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(*E*)-11,13-TETRADECADIENAL: MAJOR SEX PHEROMONE COMPONENT OF THE EASTERN BLACKHEADED BUDWORM, *Acleris variana* (Fern.) (LEPIDOPTERA: TORTRICIDAE)

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Abstract—(*E*)-11,13-Tetradecadienal (*E*11,13-14:Ald) is the major sex pheromone component of the eastern blackheaded budworm (EBB), *Acleris variana* (Fern.). The compound was identified in female pheromone gland extracts by coupled gas chromatographic–electroantennographic detection (GC-EAD), coupled GC–mass spectrometry in selected ion monitoring mode, and retention index calculations of candidate pheromone components. *E*11,13-14:Ald alone as trap bait was very attractive to male EBB. Addition of the corresponding diene alcohol or acetate or both did not enhance attraction. (*Z*)-11,13-Tetradecadienal in binary combination with (*E*)-11,13-14:Ald neither enhanced nor reduced trap catches. Increasing the amounts of pheromone from 0.01 to 10 μg increased trap catches, but increase of pheromone quantity above 100 μg proportionately reduced attraction. Stabilization of slowly polymerizing *E*11,13-14:Ald and development of a sustained, adequate release rate is required for pheromone-based monitoring of EBB populations.

Key Words—Lepidoptera, Tortricidae, *Acleris variana*, sex pheromone, (*E*)-11,13-tetradecadienal.

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INTRODUCTION

The eastern blackheaded budworm (EBB), *Acleris variana* (Fern.), is a microlepidopterous defoliator of 20 different coniferous trees, particularly balsam fir, *Abies balsamea* (L.) Mill; white spruce, *Picea glauca* (Moench) Voss; black spruce, *P. mariana* (Mill.); and western hemlock, *Tsuga heterophylla* (Raf.) Sarg. (Rose and Lindqvist, 1977). After egg hatching in late May or early June, larvae begin feeding on developing shoots. Pupation occurs in late July to early August and lasts about two weeks. Moths fly in August and September and lay eggs on the lower surface of needles.

A series of outbreaks occurred in Newfoundland, the maritime provinces, and Quebec from 1945 to 1950. The most recent EBB infestation has been reported in Newfoundland (Clarke and Carew, 1988; Clarke et al., 1989, 1990). This EBB outbreak caused extensive defoliation of 35,000 ha of mature balsam fir and was associated with feeding by the eastern hemlock looper, *Lambdina fiscellaria fiscellaria* (Guen.). In 1990, defoliation of 89,000 ha was predicted, and the biological insecticide *Bacillus thuringiensis* was evaluated as a control measure to reduce larval numbers and to protect foliage (West and Carter, 1992).

Methods of estimating EBB density are required to predict damage and measure the effectiveness of control programs. Egg counts, as described for the western blackheaded budworm (WBB), *Acleris gloverana* (Walsingham) (Shepherd and Gray, 1990), are conducted, but pheromone-based monitoring with nonsaturating traps would be a more efficient and sensitive method of monitoring EBB populations. We report the identification of the major sex pheromone component of EBB.

METHODS AND MATERIALS

Laboratory Analysis. EBB pupae were field-collected near St. John's, Newfoundland, and reared to adults at 20°C, 70% relative humidity, and a photoperiod of 14:10 hr light–dark. Male and female pupae were kept separately in Petri dishes to avoid mating of emergent moths. Maximal attraction of male WBB to female-baited traps 4 hr after sunset (Shepherd, 1979) suggested that pheromone production by female WBB peaked 4–5 hr into the scotophase. Assuming a similar timing of pheromone production in female EBB, abdominal tips of 2- to 3-day-old virgin females were removed 4–5 hr into the scotophase and extracted for 5 min in hexane. Aliquots of one female equivalent (FE) of pheromone extract were subjected to gas chromatographic–electroantennographic analysis (GC-EAD) (Arn et al., 1975), employing a Hewlett Packard 5890A gas chromatograph equipped with a DB-210 coated, fused silica column (30 m × 0.25 mm ID) (J&W Scientific, Folsom, California 95630). Coupled

lett Packard 5985 B equipped with the same column as above) in full scan and selected ion monitoring mode (SIM) was conducted to confirm the presence of candidate pheromone components in gland extracts. For GC-MS-CI-SIM, full scan electron impact spectra of synthetic (*E*)-11,13-tetradecadien-1-ol (*E*11,13-14:OH), (*E*)-11,13-tetradecadienal (*E*11,13-14:Ald), and (*E*)-11,13-tetradecadienyl acetate (*E*11,13-14:OAc) at 5 ng each were obtained to select diagnostic ions. In sequence, 200 pg of synthetic compounds, hexane, and an aliquot of 25 female equivalents of pheromone gland extract were analyzed, each time scanning for the diagnostic ions.

Synthesis of E11,13-14:Ald and Z-11,13-14:Ald. Synthesis of *E*11,13-14:Ald and *Z*11,13-14:Ald were conducted according to methods previously described (Nesbitt et al., 1973; Yamada et al., 1986).

All field-tested compounds were greater than 99% chemically and geometrically pure. None of the chemical impurities elicited antennal responses in GC-EAD recordings.

Field-Trapping Experiments. Field experiments in 1991 were conducted at Cochran Pond, 3 km west of St. John's, Newfoundland. Experiments were set up in randomized complete blocks with traps and blocks at least 20 m apart. Sticky traps (Sandia Die and Cartridge, Albuquerque, New Mexico) were suspended 1–2 m above ground from balsam fir trees and baited with rubber septa (Sigma Chemical Co., St. Louis, Missouri 63178) impregnated with candidate pheromone components in 10–50 μl of hexane (HPLC grade).

The first two-treatment, 10-replicate experiment (August 16–September 26) tested *E*11,13-14:Ald at 100 μg versus unbaited control traps. The second four-treatment, five-replicate experiment (September 27–October 8) tested *E*11,13-14:Ald (100 μg) alone, in binary combination with either *E*11,13-14:OH or *E*11,13-14:OAc at a 100:1 ratio each, and in ternary combination with *E*11,13-14:OH and *E*11,13-14:OAc at a 100:1:1 ratio. The third five-treatment, five-replicate experiment (October 1–10) tested *E*11,13-14:Ald (100 μg) alone and in binary combination with *Z*11,13-14:Ald at respective ratios of 100:1, 100:5, 100:10, and 100:100. The fourth six-treatment, four-replicate experiment (October 10–22) tested *E*11,13-14:Ald (100 μg) alone and in binary combination with *E*11,13-14:OH at respective ratios of 100:0.01, 100:0.1, 100:1, 100:10, and 100:100. A final eight-treatment, four-replicate experiment in 1991 (October 18–30) tested *E*11,13-14:Ald at the following doses: 0.01, 0.1, 1, 10, 100, 1000, and 10,000 μg.

In 1992, a two-treatment, 10-replicate experiment tested 10 μg of *E*11,13-14:Ald versus virgin female EBB. Experimental insects were reared in the laboratory (20°C, 65% relative humidity, 14:10 hr light–dark). Emergent females were individually placed in perforated plastic cups that were attached to the roof of Multipher traps (Biocontrol Services, Ste-Foy, Quebec).

RESULTS

GC-EAD analysis of female pheromone gland extracts revealed four compounds that elicited antennal responses by male EBB antennae (Figure 1). Based on retention index (RI) calculations on a DB-210 column, EAD-active compounds 1, 2, and 4 were hypothesized to be corresponding alcohol (RI: 1959), aldehyde (RI: 2038), and acetate (RI: 2165). Each compound eluted too late to be a C_{14}-monoene, but too early to be a conjugated, internal C_{14}-diene, unless the conjugated double-bond position was terminal, resulting in a lowering of the retention index. We therefore hypothesized that compounds 1, 2 and 4 were *E*11,13-14:OH, *E*11,13-14:Ald and *E*11,13-14:OAc. These synthetic dienes coincided with antennal responses to gland extract on DB-210 and DB-1 columns. Synthetic diene alcohol and acetate (50 pg) and synthetic diene aldehyde (50 pg) elicited good and very good antennal responses, respectively. GC-MS-CI-SIM of 25 FE of pheromone extract, monitoring *m/z* 211 (M + 1) and *m/z* 193 (M + 1 − H_2O) for *E*11,13-14:OH, *m/z* 209 (M + 1) and *m/z* 191 (M + 1 − H_2O) for *E*11,13-14:Ald, and *m/z* 253 (M + 1) and *m/z* 193 (M + 1 − HOAc) for *E*11,13-14:OAc, resulted in exact retention time and good ion ratio matches of synthetic and female-produced compounds, except for the diene alcohol, which was not detected by GC-MS-SIM in gland extracts.

Traps baited with *E*11,13-14:Ald were significantly more attractive than unbaited control traps (Figure 2). Addition of either *E*11,13-14:OH or *E*11,13-14:OAc or both to *E*11,13-14:Ald did not enhance attraction. Addition of *Z*11,13-14:Ald neither enhanced nor reduced attraction to *E*11,13-14:Ald.

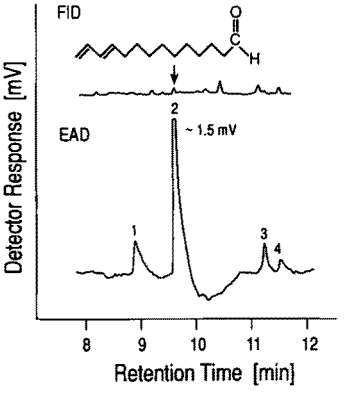

FIG. 1. GC-EAD of female EBB pheromone gland extract. The antennal recording was carried out with a male EBB antenna. (*E*)-11,13-tetradecadienal was present at about 50 pg per female equivalent of pheromone gland extract. DB-210 column (30 m × 0.25 mm ID): 1 min at 100°C, 20°C/min to 180°C, 1°C/min at 220°C.

Addition of increasing amounts of *E*11,13–14:OH to *E*11,13–14:Ald consistently reduced trap catches (Figure 3), although trap catch reduction was significant only at a 1:1 ratio of aldehyde–alcohol. In the dose–response experiment, increasing the amount of pheromone from 0.01 μg to 10 μg increased trap catches, but further increase of pheromone quantity (100–10,000 μg) proportionately reduced attraction (Figure 4).

DISCUSSION

In field-trapping experiments, several compounds tested alone or in binary combination at various ratios have been reported to attract *Acleris* moths; these compounds include: (*E*)-11-tetradecenal, (*Z*)-11-tetradecenal, (*E*)-11-tetradecenyl acetate, (*Z*)-11-tetradecenyl acetate, and (*E*)-11,13-tetradecadienal

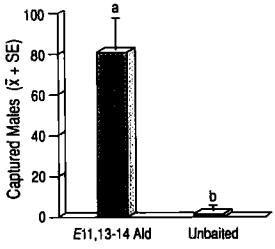

FIG. 2. Captures of EBB males in traps baited with 100 μg of *E*11,13–14:Ald, Cochran Pond, Newfoundland, August 16–September 26, 1991; $N = 10$. Bars superscripted by the same letter are not statistically different. ANOVA followed by Duncan's multiple range test on data transformed by $\log_{10}(x + 1)$, $P < 0.05$.

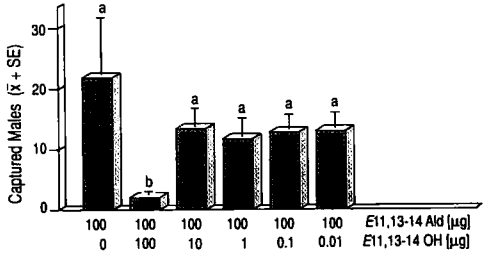

FIG. 3. Captures of EBB males in traps baited with *E*11,13–14:Ald (100 μg) alone and in binary combination with *E*11,13–14:OH at various ratios. Cochran Pond, Newfoundland, October 10–22, 1991; $N = 4$. Bars superscripted by the same letter are not significantly different. ANOVA followed by Duncan's multiple range test on data transformed by $\log_{10}(x + 1)$, $P < 0.05$.

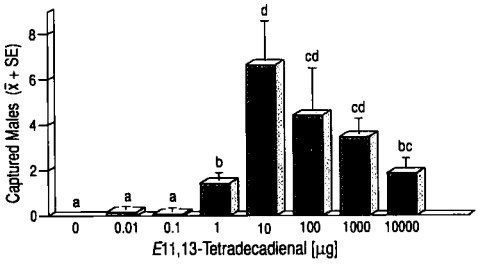

FIG. 4. Captures of male EBB in traps baited with increasing amounts of *E*11,13-14:Ald. Cochran Pond, Newfoundland, October 18–30, 1991; $N = 4$. Bars superscripted by the same letter are not significantly different. ANOVA followed by Duncan's multiple range test on data transformed by $\log_{10}(x + 1)$, $P < 0.05$.

(*E*11,13-14:Ald) (Mayer and McLaughlin, 1991). In laboratory studies of the sex pheromone of the yellowheaded fireworm, *A. minuta* (Robinson), Schwarz et al. (1983) extracted female ovipositors in heptane and analyzed extracts by GC-MS. Of eight compounds identified in ovipositor extracts, *E*11,13-14:Ald was the only compound to attract male *A. minuta* in the field. The same diene aldehyde was identified by GC-MS-CI-SIM in EBB pheromone gland extracts and constitutes the major sex pheromone component in EBB.

Picogram quantities of *E*11,13-14:Ald elicited strong antennal responses by male EBB antennae, and small amounts of synthetic diene aldehyde (100 µg) were exceedingly attractive in field experiments (Figure 2). The corresponding diene acetate was detected in gland extracts by GC-MS-CI-SIM and the corresponding diene alcohol was tentatively identified by retention index calculations of antennal responses. However, neither compound enhanced attraction to *E*11,13-14:Ald in field experiments. *E*11,13-14:OH at a 1:1 ratio with *E*11,13-14:Ald even suppressed trap catches (Figure 3). In contrast to other findings (Baker and Cardé, 1979; Sanders and Weatherston, 1976), unnatural, disproportionate ratios of *E* and *Z* isomers of the major sex pheromone component neither enhanced nor inhibited attraction of male EBB.

Further experiments were carried out in 1992 to compare attraction of virgin female EBB with that of the most effective synthetic bait, *E*11,13-14:Ald at 10 µg. Because females died within the first very cold night of testing, it remains unknown whether female EBB use a single component sex pheromone as reported for several geometrids and lymantrids (Roelofs et al., 1982; Bestmann et al., 1982; Underhill et al., 1987; Millar et al., 1987; Bierl et al., 1970, 1975). Other as yet unidentified compounds may synergize attraction to *E*11,13-14:Ald. Unknown compound 3 (Figure 1), for example, may be behaviorally active, and additional synergistic pheromone components in gland extracts may have occurred in quantities too small to elicit antennal responses in GC-EAD record-

ings. However, *E*11,13–14:Ald alone was as attractive as ovipositor extracts of female *A. minuta*, which contained eight identified components including *E*11,13–14:Ald (Schwarz et al., 1983). Female *A. minuta* and possibly also female *A. variana* may indeed use a single component pheromone.

In the dose–response experiment, increasing amounts of pheromone increased trap catches, but pheromone quantities above 100 μg proportionately reduced attraction. Use of *E*11,13–14:Ald for monitoring EBB populations requires determination of a pheromone load optimally attractive throughout the extended flight period of EBB. In addition, a method to stabilize slowly polymerizing *E*11,13–14:Ald (Wimalaratne and Slessor, unpublished) needs to be developed.

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ATTRACTION OF FEMALE MEDITERRANEAN FRUIT FLIES TO THE FIVE MAJOR COMPONENTS OF MALE-PRODUCED PHEROMONE IN A LABORATORY FLIGHT TUNNEL

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Abstract—Attraction and pheromonal activity of five major identified components of the male-produced sex pheromone of the Mediterranean fruit fly *Ceratitis capitata* to virgin laboratory-reared females was assessed in a laboratory flight tunnel. Dual-choice competitive assays were run to establish a baseline response of virgin females to live male pheromone, individual components, and an ensemble of all five compounds alone (air control) and competitively against one another. Approximately 50% of the females released in the tunnel were captured on leaf models emitting pheromonal odors from five live males. Over 37% of released females responded to an ensemble of five major identified components presented in individual capillaries. Response of females to individual components was less than 10%. Competitive assays showed the live male-produced pheromone to be more attractive than either the five major component ensemble (FMCE) or individual components. Further research is likely to identify other male-produced compounds with pheromonal activity that could improve development of a pheromone-based trap for monitoring Mediterranean fruit fly populations.

Key Words—*Ceratitis capitata*, Mediterranean fruit fly, Diptera, Tephritidae, pheromone, 1-pyrroline, attractant, flight tunnel.

INTRODUCTION

The Mediterranean fruit fly (Medfly), *Ceratitis capitata* (Wiedemann) is an economically important tephritid pest whose host range extends to over 250

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different types of fruits and vegetables. Due to its wide host range, this insect represents a significant threat to agriculture in states such as California, Florida, and Texas where much of the U.S. commercial fruits and vegetables are grown. Presently, the Mediterranean fruit fly is not known to be established in these areas; however, each year the threat of its introduction increases.

One important method used in detecting foreign introductions of Medflies into the continental United States is the use of olfactory-based detection traps placed around likely introduction points such as borders, airports, and seaports. The use of chemical attractants in detection, control, and eradication of fruit fly infestations has had a long history, primarily through the use of protein-based food baits (McPhail, 1939; Steiner, 1952; Gow, 1954) and empirically identified synthetic attractants such as trimedlure (Gertler et al., 1958; Beroza et al., 1961). Interestingly, for this species, the use of pheromone-based attractants has not generated much interest, possibly due to the difficulty in identification and formulation of the pheromonal components of the male-produced odor and/or the relatively poor performance of identified pheromonal components compared to proteinaceous or synthetic attractants.

The presence of a male-emitted pheromone attractive to virgin Medfly females was first reported by Feron (1959, 1962). Subsequently, several researchers reported on identification or attractancy of the pheromonal components from calling male Medflies (Jacobson et al., 1973; Ohinata et al., 1977; Baker et al., 1985, 1990; Jang et al., 1989; Heath et al., 1991); however, detailed information on the biological activity of individual components or blends of the pheromonal components is sparse or lacking in most cases. Baker et al. (1985) identified nine components of male Medfly odor, of which 1-pyrroline was reported as the most biologically active singly, although no data were provided. Jang et al. (1989) identified 54 components from male-produced pheromonal odors including eight of the nine reported by Baker et al. (1985) and tested activity to five individual components and a synthetic blend using a close-range cage bioassay. Baker et al. (1990) conducted field tests in Mexico of various combinations of two identified male-odor components (geranyl acetate and linalool) and five additional volatiles (three pyrazines, ammonia, and trimedlure), but they made no attempt to mimic or reproduce the male odor in their study.

Heath et al. (1991) reported on the identification, natural release rates, and formulation of three of the five major male-odor components previously identified by Jang et al. (1989), and they found limited attraction of Medfly females to three components in the field. Recently, Landolt et al. (1992) reported on flight behavior of females in a wind tunnel to the three major components of the male-odor tested by Heath et al. (1991), as well as to the odor of calling males. Flath et al. (1993) investigated the effects of fly age and time of day on the composition and complexity of volatile pheromonal emissions of Hawaiian

Medfly males and reconfirmed the identity of over 30 compounds, five of which constitute the major quantitative emissions. These chemical analyses indicate a need for more detailed research on the attractiveness of identified compounds to virgin female Medflies.

The purpose of this study was to assess the attraction of virgin female Medflies to the five major male-produced odor components identified by the present authors (Jang et al., 1989; Flath et al., 1993). The specific objectives of this study were to establish and compare the inherent attraction of virgin female Medflies in a laboratory flight tunnel to: (1) the natural male-emitted pheromone, (2) each of the five major individual components and a synthetic five-component ensemble, (3) each of the five major individual components vs. the five-component ensemble, and (4) the five-component ensemble or individual major components vs. the natural male-emitted pheromone. These competitive choice tests measured the preference in attraction (of groups) of virgin female Medflies to pairings of individual major components of the male odor, an ensemble of the five major components, or the natural-male-emitted pheromone.

METHODS AND MATERIALS

Insects. Laboratory-reared Mediterranean fruit fly pupae were obtained from the USDA-ARS, Tropical Fruit and Vegetable Research Laboratory in Honolulu, Hawaii. Males and females were segregated by sex at the late pupal stage (Cunningham et al., 1966) and placed separately by sex into groups of 50 in plastic containers (11.5 cm diameter × 7.5 cm deep) with nylon mesh covers. The flies were supplied with water, sugar, and hydrolyzed protein and tested at five to seven days postemergence. Females were held in separate rooms from males under common environmental conditions (12 : 12 hr light–dark at 23°C and 60% relative humidity) prior to testing.

Chemicals. The five identified major compounds tested were ethyl acetate, geranyl acetate, ethyl (*E*)-3-octenoate, (*E*, *E*)-α-farnesene, and 1-pyrroline. Ethyl acetate and geranyl acetate were obtained from Aldrich Chemical Co. (Milwaukee, Wisconsin). Ethyl (*E*)-3-octenoate was synthesized in the following manner: (*E*)-3-octenoic acid was synthesized from a mixture of hexanal, triethanolamine, and malonic acid by the method of Linstead et al. (1933). The acid was esterified with ethanol and the ester distilled to ca. 97% purity. 1-Pyrroline was generated by combining 5 μl of a concentrated aqueous solution of *N*-chloropyrrolidine hydrochloride and 5 μl of 4 N KOH solution and 500 μl of distilled water. Fifty microliters of the resulting solution was placed in a glass tube measuring 2.4 × 14 mm for presentation. (*E*, *E*)-α-Farnesene (97.2%) was isolated from ylang-ylang oil by fractional distillation, followed by liquid chromatography on 15% silver nitrate–silica.

All compounds except 1-pyrroline were formulated individually by placing each neat compound in individual glass capillary tubings of different sizes (1- and 5-μl microcaps, Drummond Scientific, Broomall, Pennsylvania) and different headspace levels (i.e., empty tubing space above the filling level), which allowed for different release rates for each of the five compounds (Weatherston et al., 1985; Heath et al., 1991). Individual compounds were tested by taping its capillary tube to the inside wall of the emission container. All five of the individual tubes containing compounds were taped to the sides of the emission container to produce, through simultaneous evaporation, the five major component ensemble (FMCE). Estimates of release rates for all compounds except 1-pyrroline were calculated from measurements of evaporation (drop in meniscus) in the capillaries over time in the flight tunnel. The capillary tube formulations had evaporation rates correlated to previous quantitative studies on the natural emission rates and ratios of the five major components released from calling males (Flath et al., 1993). The release rate of 1-pyrroline was estimated after adding a concentrated HCl solution to the tube containing residual 1-pyrroline at the completion of the test in order to re-form the stable *N*-chloropyrrolidine hydrochloride, which was subsequently converted to 2,3-trimethylene-4-quinazolone for quantification (Sakamoto and Samejima, 1979). The five major compounds, their purity, formulations, estimated release rates, and relationship to quantitative analysis of the five major components present in the male odor are listed in Table 1.

TABLE 1. FIVE MAJOR IDENTIFIED COMPONENTS FROM MALE ODOR OF MEDITERRANEAN FRUIT FLIES: PURITY, EVAPORATIVE RELEASE RATES, FORMULATION, AND PERCENTAGE RATIOS OF COMPONENTS USED IN FLIGHT-TUNNEL STUDIES

Compound	Purity (%)	Estimated release rate (ng/hr)	Formulation	% of total measured release rate	% of total from natural male odor[a]
(*E*,*E*)-α-Farnesene	97.2	1660–2300	2 1-μl caps, 8 mm HS[b]	10.6	6.9
Geranyl acetate	98	1472	1 1-μl cap, plugged 8 mm HS	8	12.5
Ethyl (*E*)-3-octenoate	97	5144	1 5-μl cap, 8 mm HS	27	20.4
Ethyl acetate	99.9	10,000–12,000	1 1-μl cap plugged 24 mm HS	53	46.4
1-Pyrroline	100	168	1 tube (14 × 2.5 mm)	0.9	13.6

[a]Calculated from Table 1 of Flath et al. (1993).
[b]HS = headspace, capillaries were open on both ends unless indicated as plugged at one end.

The five major components of the pheromonal odor (Jang et al., 1989; Flath et al., 1993), which together constitute greater than 90% of the total male odor, were tested individually and together as a FMCE for their attractiveness to virgin sexually mature females in a series of competitive dual-choice bioassays. Initial flight tunnel assays compared the inherent attractancy of each of the five compounds individually, the FMCE, or the odor of five sexually mature males against a clean air control. A second series of assays compared the individual compounds or the FMCE against the odor of five males. A third series of tests compared the individual components to the FMCE. Additionally, we tested the effect of doubling the emission rate (i.e., adding additional capillaries) of each individual component in the FMCE (keeping the remaining four the same) against air.

Flight-Tunnel Assay. Laboratory flight-tunnel bioassays were conducted in a rectangular glass flight tunnel as described by Jang and Light (1991). The tunnel measured 0.9 m × 0.9 m × 2.8 m and contained a measured airflow of 0.15 m/sec. Lighting inside the tunnel was maintained at ca. 2000 lux using 60-W fluorescent lights. Experiments were carried out during the morning hours (0800–1200 hr).

For each dual-choice assay, two artificial "leaf models" were placed inside the flight tunnel at a height of 40 cm above the floor of the tunnel and equidistant from each other and the sides of the tunnel. An emission container was constructed using a 250-ml plastic cylindrical bottle (Nalgene), which had the bottom third (4.5 cm) removed. A 9-mm hole was drilled into the cap of each resulting cylinder and fitted to tygon tubing. Two emission containers were connected via a T fitting to a cylinder of breathing-quality compressed air. Air was passed through each tubing at a flow rate of 50 ml/min into the plastic cylinder, which mixed and flushed odors from either calling males or synthetic components into the laminar-flow flight tunnel. The open end of the cylinder was covered with dark nylon mesh to prevent flies from landing on the emission tubes or viewing enclosed live males. Two artificial leaves (15 × 23 cm), made from dark green construction paper and covered on one side with Tanglefoot sticky glue (Tanglefoot Co., Grand Rapids, Michigan) were vertically aligned and taped above and below the emission container to form the artificial leaf model with the sticky side facing down wind. Emission tubes were taped to the inner wall of the emission container. For tests using live males, five males were placed inside the emission container portion of a similarly constructed leaf model. Clean air controls emitted only air through the leaf models.

For each assay, 50 females were released at the downwind end of the tunnel from a platform situated at midheight of the tunnel (40.5 cm). The released females were allowed to fly within the tunnel for 30 min, during which time they were observed for upwind flight and capture on the artificial leaves. At the end of the 30-min assay, the flies captured on the sticky surface of the leaf

models were counted and removed from both leaf models. After each assay, all noncaptured flies were removed (by vacuum suction) from the tunnel. New flies were then introduced at the start of subsequent tests. The tunnel's glass walls were cleaned with ethanol and allowed to dry completely whenever a change in the dual-choice tests were made. All tests were replicated at least four times using new flies for each replication. Location of the leaf models relative to each other in the flight tunnel were rotated from one side of the tunnel to the other (left or right) to avoid directional bias. Analysis for significant differences in fly capture on each of the two leaf models were compared using PROC TTEST (two-sample test) (SAS Institute, 1988).

RESULTS

Inherent Attractancy Tests. Females released into the flight tunnel in the absence of live male odor, FMCE, or individual components did not show any directed upwind anemotaxis toward the leaf model. Without odor stimuli, females congregated in the release or downwind end of the tunnel, and most did not pass the halfway point towards the upwind area of the tunnel during the course of the 30-min assay. Resulting capture of flies on the air (control) leaf model was always low (overall mean of 0.4 flies).

When live males were placed in the emission container, females exhibited a directed upwind anemotaxis to the male odor and significantly chose the male odors (pheromone) over the air control in all assays performed. Flights consisted of either a general movement upwind towards the odor source or a directed, upwind flight. Oriented upwind flights were either straight-line or a side-to-side zigzag movement characteristic of the counterturning motion of insect flight in flight tunnels to pheromonal odors. In general movement upwind, females would often take flight from the platform, alight on the sides of the glass tunnel and exhibit cleaning of antennae and legs prior to resuming their upwind flight. A mean of 24.3 $\pm$ 2.0 (48.6%) of released flies (50/test) were captured on leaf models emitting male pheromone compared to a mean of 1.0 $\pm$ 0.6 captured on the leaf model emitting air (control). This represents nearly a 50% behavioral response from which we designed further experiments.

Individual pheromonal components [except for ethyl (*E*)-3 octenoate] were significantly more attractive to virgin females than air alone (Table 2). However, the response to the individual components was less than the females' response to the male-emitted pheromone. Responses of virgin females to the FMCE approached that of live males when either was paired to air alone. A mean of 18.8 females (37.6% of released flies) were captured on the artificial leaves emitting the FMCE compared to a mean of 0.2 females captured on the leaf models emitting air alone. The response was significantly greater to both the

TABLE 2. INHERENT ATTRACTION OF VIRGIN FEMALE MEDITERRANEAN FRUIT FLIES TO NATURAL LIVE MALE PHEROMONE, FIVE MAJOR COMPONENT ENSEMBLE (FMCE), OR INDIVIDUAL COMPONENTS VERSUS AIR-ALONE CONTROL IN LABORATORY FLIGHT TUNNEL[a]

Test components	N	Mean No. of females captured ± SEM	
		Treatment	Air control
Live males	4	24.3 ± 2.0	1.0 ± 0.6**
Five components (FMCE)	24	18.8 ± 0.8	0.2 ± 0.1**
(*E*,*E*)-α-Farnesene	4	4.0 ± 1.6	0.0 ± 0.0*
1-Pyrroline	4	4.5 ± 0.9	0.0 ± 0.0**
Geranyl acetate	4	1.5 ± 0.3	0.0 ± 0.0**
Ethyl acetate	4	4.8 ± 1.8	0.0 ± 0.0*
Ethyl (*E*)-3-octenoate	4	4.0 ± 1.1	2.0 ± 0.9

[a]Five major component ensemble and individual pheromone components presented as in text at release rates specified in Table 1. Mean number of females captured were significantly different at the indicated (**$P \leq 0.01$, *$P \leq 0.05$) level of significance based on paired t tests of the means (SAS). Each replicate contained 50 females.

live male odor and the FMCE than to air. The responses to live male odor or FMCE were greater than those to the major individual components tested against air.

Competitive Attractancy Tests. Response of virgin females to individual pheromonal components compared to live male pheromone was low (Table 3). Mean responses to individual major components ranged from 0 to 2.2 females captured compared to a mean range of 16.5 to 25.8 females captured on leaf models emitting male pheromone. When evaluating the competitive efficacy relative to natural pheromone (or percentage of catch) for each of the five individual major components, ethyl acetate > 1-pyrroline > (*E*, *E*)-α-farnesene as single component attractants of females. Females showed a significantly greater preference for natural male pheromone ($\bar{X} = 16.7$) over the FMCE ($\bar{X} = 5.7$) when tested competitively. However, the FMCE did show some competitive effect in preferentially capturing 34% of the total number of females captured by the natural pheromone. This competitive activity reduced the predicted number of females expected to be captured on the leaf model emitting natural pheromone ($X = 24.3$) based on previous tests using live males.

Female responses to individual components relative to the FMCE are compared in Table 4. The FMCE caught significantly more females than any of the individual major male-odor components. Mean captures ranged from 11.8 to 19.5 to the FMCE compared to 0.3–2.3 for the individual components. In these

TABLE 3. ATTRACTION OF VIRGIN FEMALE MEDITERRANEAN FRUIT FLIES TO FIVE MAJOR COMPONENT ENSEMBLE (FMCE) OR INDIVIDUAL COMPONENTS VERSUS LIVE MALE-EMITTED PHEROMONE IN LABORATORY FLIGHT TUNNEL[a]

Test component(s)	N	Mean No. of females captured ± SEM: Treatment	Mean No. of females captured ± SEM: Live male standard	Treatment capture as a % of live male standard
Air[b]	4	1.0 ± 0.7	24.3 ± 2.0**	4.1
Five components (FMCE)	11	5.7 ± 0.7	16.7 ± 1.3**	34.1
(*E*,*E*)-α-Farnesene	4	1.0 ± 0.0	16.5 ± 2.6**	6.1
1-Pyrroline	4	1.8 ± 0.6	24.5 ± 3.4**	7.4
Geranyl acetate	4	0.0 ± 0.0	25.8 ± 1.9**	
Ethyl acetate	4	2.2 ± 1.0	21.3 ± 2.9**	10.3
Ethyl (*E*)-3-octenoate	4	0.3 ± 0.3	18.3 ± 2.2**	1.6

[a]Five major component ensemble and individual pheromone components presented as in text at release rates specified in Table 1. Mean number of females captured were significantly different at the indicated (**$P \leq 0.01$) level of significance based on a paired *t* test of the means (SAS).
[b]Data taken from Table 2.

TABLE 4. ATTRACTION OF VIRGIN FEMALE MEDITERRANEAN FRUIT FLIES TO INDIVIDUAL PHEROMONE COMPONENTS VERSUS FIVE MAJOR COMPONENT ENSEMBLE (FMCE) IN LABORATORY FLIGHT TUNNEL[a]

Test component	N	Mean No. of females captured ± SEM: Treatment	Mean No. of females captured ± SEM: Five component ensemble	Treatment capture as a % of five component ensemble
Five males[b]	11	16.7 ± 1.3	5.7 ± 0.7**	293.0
(*E*,*E*)-α-Farnesene	4	0.8 ± 0.5	16.0 ± 1.5**	5.0
1-Pyrroline	4	0.3 ± 0.3	19.5 ± 2.5**	1.5
Geranyl acetate	4	0.5 ± 0.3	11.8 ± 1.4**	4.2
Ethyl acetate	4	2.0 ± 1.2	15.3 ± 1.8**	13.1
Ethyl (*E*)-3-octenoate	4	2.3 ± 0.6	14.3 ± 2.1**	16.1
Air[c]	4	0.2 ± 0.1	18.8 ± 0.79*	1.0

[a]Five major component ensemble and individual pheromone components presented as in text at release rates specified in Table 1. Mean number of females captured were significantly different at the indicated (*$P \leq 0.05$, **$P \leq 0.01$) level of significance based on a paired *t* test of the means (SAS).
[b]Data comparing five males with the five components are from Table 3.
[c]Data on air control versus five components are from Table 2.

tests, ethyl (*E*)-3-octenoate and ethyl acetate showed the best competitive activity (16.1% and 13.1% relative capture) followed by (*E*, *E*)-α-farnesene (5.0%) and geranyl acetate (4.2%), while 1-pyrroline showed little competitive activity.

Doubling the emission rate of individual components in the FMCE one at a time resulted in mean captures ranging from 11.8 flies [ethyl (*E*)-3-octenoate] to 21.0 flies [(*E*, *E*)-α-farnesene)] (Table 5). Total capture by the augmented FMCE was, however, still significantly greater than to air alone. These data were similar to responses obtained for FMCE in earlier tests (Tables 2 and 4).

DISCUSSION

Female Medflies orient, fly upwind, and discriminate multicomponent male-produced pheromone odors from individual odors in a flight tunnel. While long-range attraction of females to calling males in the field has been reported previously (Ohinata et al., 1977; Nakagawa et al., 1981), the development of a five-component synthetic pheromonal blend (FMCE) attractive to females that approaches the attractancy of live calling males has not been previously reported. Ohinata et al. (1973), using a cage bioassay, reported that up to 50% of 100 released females responded to odor from filter paper that had been exposed to 2000 males or to a methylene chloride extract of a cold-trapped condensate from air passed over caged males. Compounds identified from that study were subsequently found to be unattractive to females. More recent attempts to formulate a synthetic pheromonal lure have centered around either a minimalist approach using only three components (Heath et al., 1991; Landolt et al., 1992) or an empirically derived combination of three identified (Howse, unpublished) components of male odor (Baker et al., 1990), which had little in common with the

TABLE 5. ATTRACTION OF VIRGIN FEMALE MEDITERRANEAN FRUIT FLIES TO FIVE MAJOR COMPONENT ENSEMBLE (FMCE) CONTAINING TWICE THE EMISSION OF ONE COMPONENT VERSUS AIR CONTROL IN LABORATORY FLIGHT TUNNEL[a]

FMCE plus additional	*N*	Treatment	Air control
Ethyl acetate	4	14.2 ± 2.2	0.5 ± 0.5**
(*E*,*E*)-α-Farnesene	4	21.0 ± 2.2	0.0**
1-Pyrroline	4	13.5 ± 1.9	0.0**
Ethyl (*E*)-3-octenoate	4	11.8 ± 1.6	0.2 ± 0.2**
Geranyl acetate	4	13.3 ± 2.5	0.3 ± 0.3**

[a] Five major component ensemble and individual components presented as in text at release rates specified in Table 1. Mean number of females captured were significantly different at the indicated (**$P \leq 0.01$) level of significance based on paired *t* tests of the means (SAS).

qualitative or quantitative complexities of the natural pheromonal odor. Neither the Heath et al. (1991) nor the Baker et al. (1990) studies directly compared the responses of their blends/formulations with a source of natural pheromone. The mean response of virgin females to the five-component ensemble in this study (37.6%) was far greater than that obtained by Landolt et al. (1992), who reported that only 3.3% of the individually released females contacted the source emitting a three-component blend, whereas 43.3% of individually released females responded to the odor of 15 caged males.

In almost all cases, responses of virgin females to individual pheromone components were low (overall $X = 0.4$ flies), while the male pheromone or the FMCE caught significantly more females, suggesting that qualitative differences or complexity in the number of pheromone components are key factors in the elicitation and degree of female attraction. Increasing the emission rate of individual components over the levels reported in this study did not significantly increase female capture (data not shown).

We believe that much of the observed differences in response between the FMCE and the natural male pheromone reported in this study are due more to qualitative differences (or complexity in odor composition) than quantitative differences in emission rates of the constituents tested. Increasing numbers of calling males (from 5 to 10) confined in the emission container of the leaf model did not increase attraction of females in our studies (data not shown). Ohinata et al. (1973) reported that there was little difference in attraction of females to 10, 25, or 50 live males in laboratory bioassays. Recent results of the present authors (Light et al., unpublished), showing that intermediate and minor male odor components can enhance the attraction of virgin females to natural male pheromone, suggests that further improvements in the synthetic five-component blend are possible.

Baker et al. (1985) reported that 1-pyrroline was the most active individual component for female attraction, an observation we confirmed in earlier close-range cage tests (Jang et al., 1989). However, in the flight tunnel assay, delta-1-pyrroline alone did not attract more females that other individual components. Apparently, its power lies in its ability to synergize with other components to produce the active pheromone (Jang et al., unpublished).

The identification of biologically active pheromone components attractive to female Medflies will be important additions to our knowledge of fruit fly semiochemicals and may lead to further studies on mating behaviors critical to development of sound control strategies.

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PHYTOTOXIC SUBSTANCES IN ROOT EXUDATES OF CUCUMBER (*Cucumis sativus* L.)

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Abstract—The addition of activated charcoal to a nutrient solution for the hydroponic culture of cucumber resulted in significant increases in the dry weight of the plant and fruit yield. Hydrophobic root exudates were collected at different growth stages with Amberlite XAD-4 resin and bioassayed with lettuce seedlings. The exudates at the reproductive stage were more phytotoxic than those at the vegetative stage. The exudates contained organic acids such as benzoic, *p*-hydroxybenzoic, 2,5-dihydroxybenzoic, 3-phenylpropionic, cinnamic, *p*-hydroxycinnamic, myristic, palmitic, and stearic acids, as well as *p*-thiocyanatophenol and 2-hydroxybenzothiazole, all of which, except 2-hydroxybenzothiazole, were toxic to the growth of lettuce.

Key Words—Activated charcoal, allelopathy, autotoxicity, *Cucumis sativus* L., hydroponics, *Lactuca sativa* L., nutrient solution, organic acid, phytotoxicity, root exudates.

INTRODUCTION

Allelopathy and autotoxicity due to root exudates of plants are important in agricultural and ecological problems such as the replant failure of horticultural crops, the selection of companion crops in mixed cropping, crop rotation, and growth reduction in some fruit vegetables during fruit enlargement (Putnam, 1986; Rizvi and Rizvi, 1992; Sarobol and Anderson, 1992; Tucker, 1981; Young, 1984). We recently reported that the growth of tomato in hydroponic culture was inhibited by organic substances that arose from the root exudates and were removed from the nutrient solution by adsorption on activated charcoal (Yu et al., 1993). The present study deals with the phytotoxicity of root exudates

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of cucumber plants and the identification of the phytotoxic substances. Cucumber is a vegetable cultivated throughout the world. However, poor growth due to successive cropping has frequently been observed. The phenomenon has been attributed to the buildup of pests, nutritional disorder, or other unknown factors (Takahashi, 1984). It has also been suggested that cucumber has allelopathic potential and some accessions of cucumber severely inhibit the growth of cucumber and weeds (Gaidamak, 1971; Lockerman and Putnam, 1979, 1981a,b; Putnam and Duke, 1974; Putnam, 1986).

METHODS AND MATERIALS

Reagents. All the reagents and solvents used were commercially available and used without further purification except for pyridine, which was dried over CaH_2 and distilled before use. *p*-Thiocyanatophenol was prepared according to the directions of Bordwell and Boutan (1956): ^{1}H NMR ($CDCl_3$) δ = 6.88 (2H, d, J = 8.8 Hz) and 7.45 (2H, d, J = 8.8 Hz); MS (70 eV) m/z (relative intensity) 151 (100), 123 (23), 96 (49), 65 (28), 39 (40), and 27 (21).

Growth Tests. Cucumber plants (*Cucumis sativus* L., cv. Tokiwa, Sakata Seed Co.) were cultivated by means of hydroponic systems as described previously (Yu et al., 1993). Each system was mainly composed of a culture bed (94 × 94 × 5 cm), a nutrient solution tank (150 dm^3), a pump with a time switch, and an inlet with an air-siphon. The nutrient solution was periodically recycled by the pump. Six cucumber seedlings at the two-leaf stage were transplanted to each bed and cultivated for about two months. The initial nutrient solution contained (in mmol/dm^3) $Ca(NO_3)_2$, 3.0; KNO_3, 6.0; $NH_4H_2PO_4$, 1.0; $MgSO_4$, 1.5 and (in mg/dm^3) $FeCl_3$, 15; H_3BO_3, 3; $MnCl_2$, 2; $ZnSO_4$, 0.22; $Cu(NO_3)_2$, 0.05; Na_2MoO_4, 0.02. The pH and electrical conductivity (EC) at 25°C of the solution were 5.5 and 1.9 dS/m, respectively. The nutrient solution was not renewed during cultivation. Nutrients and water consumed by the plants were periodically compensated on the basis of chemical analysis (Yu et al., 1993) of the nutrient solution collected weekly or biweekly. The hydroponic culture was carried out twice, from June 26 to August 22 and from September 6 to November 13, 1992, with and without the addition of 600 g of granular activated charcoal (Takeda Chemical Industry Co., Shirasagi W_2C, 4–8 mesh) to the nutrient solutions (150 dm^3) in the tanks. Each treatment was triplicated in the first cropping and duplicated in the second cropping. At the end of cultivation, the plants were divided into root, shoot, and fruit, dried at 80°C, and weighed.

Collection of Root Exudates. The residual nutrient solutions (10 dm^3) of the hydroponic cultures were passed through columns packed with 15 cm^3 of Amberlite XAD-4 resin (Sigma Chemical Co.) as an adsorbent of hydrophobic

substances. Prior to use, the resin was cleaned by treatment with hot water, followed by Soxhlet extraction with methanol, acetone, and diethyl ether, each for 24 hr (Tang and Young, 1982). The columns, after use, were washed with 200 cm^3 of water and treated with 100 cm^3 of methanol as an eluent. The eluates were concentrated to 10 cm^3 by evaporation in vacuo at 40°C.

In another experiment, the root exudates of cucumber were collected at different growth stages with a continuous trapping system similar to that devised by Tang and Young (1982). A column of the system was packed with 15 cm^3 of the precleaned XAD-4 resin. Three cucumber seedlings at the two-leaf stage were transplanted in a pot (12 dm^3) filled with 10 dm^3 of the nutrient solution with the same composition as described above. The nutrient solution was aerated and continuously recycled at the rate of 1 dm^3/hr by means of an air pump. The pot was maintained at 28°C by day and at 20°C by night in a growth chamber under natural light. The cultivation was carried out from June 12 to September 2, 1992, and the column of the system was replaced by a new column every 10 days during cultivation. The detached columns were washed with 200 cm^3 of distilled water and then treated with 200 cm^3 of methanol. The methanol solutions were concentrated to 3 cm^3 by evaporation in vacuo at 40°C. Concurrently, an additional trapping system was employed as a control, in which no cucumber plant was transplanted.

Phytotoxicity Bioassay. The assay was carried out according to the method of Tang and Young (1982). Thus, an aliquot of a test solution was applied with a micropipet to a 3.5-cm^2 disk of filter paper in a 5.5-cm-diameter Petri dish. After evaporation of the solvent, the disk was wetted with 0.2 cm^3 of distilled water. Eight lettuce seeds (*Lactuca sativa* L., cv. Shisuko; Takii Seed Co.) were placed on the disk. The seeds were incubated in a moisture-saturated box at 24°C for 50–60 hr. The mean root length of the resulting seedlings was selected as a growth index. Bioassay with cucumber seeds was also carried out in a similar manner, except that the disk area, the amount of water added, incubation time, and temperature were 19.6 cm^2, 3 cm^3, 120 hr, and 28°C, respectively.

Identification of Phytotoxic Substances. The methanol eluate of the pot experiment, after evaporation to dryness, was dissolved in 20 cm^3 of water. The resulting solution was adjusted to pH 8.0 with 0.1 mol/dm^3 NaOH and extracted three times with 30 cm^3 of ethyl acetate. The aqueous layer was then adjusted to pH 2.0 with 1.0 mol/dm^3 HCl and extracted three times with 30 cm^3 of ethyl acetate. The extracts from aqueous solutions at pH 8 and pH 2 were separately dried over anhydrous $CaSO_4$ and evaporated in vacuo to give 10 cm^3 of the concentrates, which we hereafter call neutral (NF) and acidic (AF) fractions, respectively. The residual aqueous layer was neutralized and passed through a column filled with 15 cm^3 of the XAD-4 resin. The column was eluted with 50

cm^3 of methanol, and the eluate was evaporated in vacuo to give 10 cm^3 of the concentrate, which we hereafter call the water-soluble (WF) fraction.

Aliquots (1 cm^3) of the NF, AF, and WF were evaporated in vacuo to dryness, and to the residues were added 0.2 cm^3 of pyridine and 0.2 cm^3 of *N*, *O*-bis(trimethylsilyl)trifluoroacetamide containing 1% of trimethylchlorosilane. The resulting and original solutions were analyzed with a Shimadzu GC-14A gas chromatograph and a Hitachi M-80 gas chromatograph–mass spectrometer (GC-MS). A TC-5 capillary column (60 m, GL Science) and a OV-17 column (2% Uniport HP 80/100, 2 m) were used for the gas chromatograph with He as carrier gas. The column temperature was elevated from 100°C to 270°C at the rate of 5°C/min. The ionization voltage in the electron impact mode of the mass spectrometer was 70 eV.

RESULTS AND DISCUSSION

Effect of Activated Charcoal on Growth of Cucumber. Table 1 shows the dry weights of organs and the fruit yields at the end of cultivation in the absence and presence of activated charcoal. The addition of the charcoal resulted in significant increases in dry matter production and fruit yield. During the experiments, the concentrations of nutrients were periodically regulated to be as constant as possible. Figure 1 shows changes in pH, EC, and the concentrations of some major nutrient elements during cultivation. The changes were virtually independent of the addition of the charcoal. Similar results were also obtained for Mg^{2+} and SO_4^{2-}. Hence, the effect of the charcoal on the growth of cucumber plants was not attributable to changes in the composition of inorganic nutrients but to phytotoxic organic substances, which were adsorbed on the charcoal, although cucumber plants grown without charcoal exhibited no apparent symptoms of autotoxicity except their leaves were relatively small.

TABLE 1. EFFECTS OF ACTIVATED CHARCOAL ON GROWTH OF CUCUMBER PLANT[a]

Cropping	Treatment	Dry weight (g/plant)				Fruit yield (kg/plant)
		Root	Shoot	Fruit	Total	
1	Control	6.4	95.2	50.7	152.4	1.18
	Charcoal	11.2**	123.5**	73.1**	207.8***	1.80***
2	Control	5.8	94.5	19.0	119.3	0.43
	Charcoal	8.4	131.7*	48.8*	189.4**	1.11*

[a] *, **, and *** refer to significant differences compared with the control at 0.05, 0.01, and 0.001 levels by Student's *t* test, respectively.

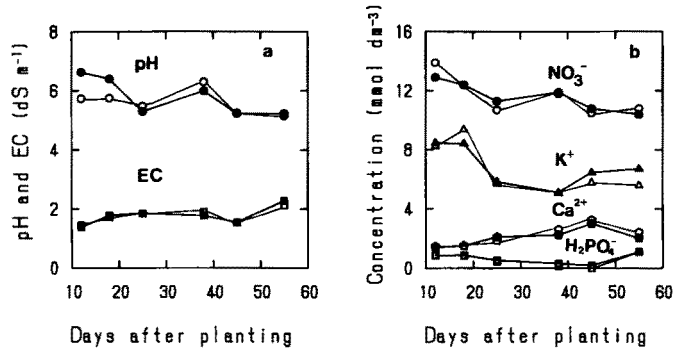

FIG. 1. Changes in pH, EC, and the concentrations of a few major ions in nutrient solutins during the first cropping of cucumber plants in the absence (open circles) and presence (solid circles) of activated charcoal.

Hydrophobic substances in the residual nutrient solutions after cultivation were collected by passing 10 dm^3 of the solutions through Amberlite XAD-4 resin columns and eluted by methanol. The eluates were concentrated to 10 cm^3, and the concentrates were bioassayed with cucumber and lettuce. Figure 2 shows the effects of the volume (V) of aliquots of the concentrates on the mean root length (MRL) of the seedlings after incubation. The MRL values for the solution treated with charcoal were significantly larger than those for the untreated solution. Furthermore, the MRL value for the latter decreased with increasing V value. These results support our presumption that cucumber plants exude phytotoxic substances that are adsorbed on activated charcoal. Figure 2 also shows

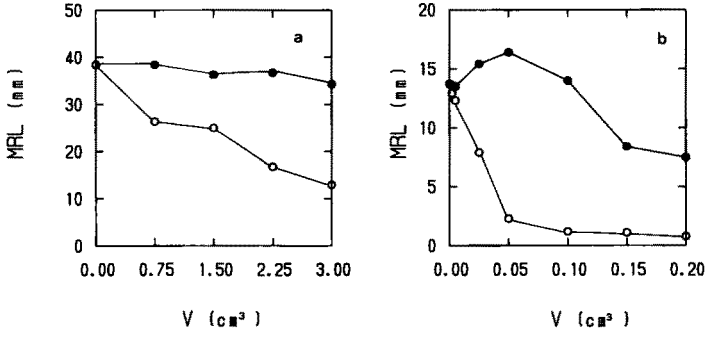

FIG. 2. Plots of the mean root lengths (MRL) vs. the volume (V) of concentrated eluates assayed with cucumber (a) and lettuce (b) for residual nutrient solutions with (solid circles) and without (open circles) charcoal.

that the bioassay with lettuce is much more sensitive than that with cucumber, so that the former was used for the subsequent experiments.

Phytotoxicity at Different Growth Stages. Pot experiments with root exudate trapping systems provided several XAD-4 resin columns through which nutrient solutions were passed for about 10 days. We treated the columns with methanol and concentrated the eluates to 3 cm^3 to give solutions containing root exudates at difference growth stages. Figure 3 shows changes in the MRL values of lettuce with sampling time. The MRL values were virtually constant for the initial periods of 40 days (vegetative growth stage), then decreased rapidly, and approached a very small constant value at the reproductive stage. The solutions from the control pot gave a virtually constant MRL value throughout the experiment. The results suggest that phytotoxic substances are mainly exuded at the reproductive stage. Similar phenomena have been reported on yellow fieldcress (Yamane et al., 1992) and tomato (Yu et al., 1993).

Identification of Phytotoxic Substances. The eluate from the XAD-4 resin in the pot experiment was evaporated to dryness, and the residue was fractionated into three fractions: NF, AF, and WF. Table 2 shows the results of bioassay with lettuce for these fractions at various volume values. The ratio value in the table refers to the ratio of the MRL value for the root exudates to that for the control. Growth inhibition was observed for NF and AF but not for WF. Thus, NF and AF were used for the GC and GC-MS analyses to identify compounds involved.

The NF gave a number of GC peaks (Figure 4), among which the main

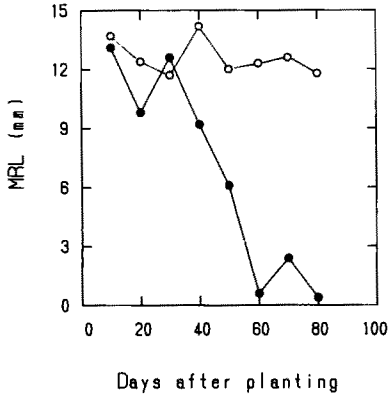

FIG. 3. Mean root lengths (MRL) of lettuce seedlings assayed for root exudates at different growth stages of cucumber plants. Open circles: control; solid circles: root exudates. Three replications with eight seeds for each were carried out at 20 mm^3 of test solutions.

TABLE 2. RATIOS OF MEAN ROOT LENGTHS FOR NEUTRAL (NF), ACIDIC (AF), AND WATER-SOLUBLE (WF) FRACTIONS FROM ROOT EXUDATES TO THOSE FROM CONTROL AT VARIOUS VOLUMES OF TEST SOLUTIONS

Volume (mm^3/disk)	Ratio		
	NF	AF	WF
5	0.96*	0.83*	0.94
10	0.90*	0.82*	0.93
20	0.66*	0.63**	0.97
40	0.37**	0.34***	0.91
80	0.19***	0.09***	1.03

"*, **, and *** refer to significant differences compared with the control at 0.05, 0.01, and 0.001 levels by Student's t test, respectively.

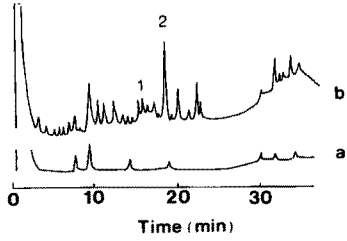

FIG. 4. Gas chromatogram of the neutral fraction (NF): (a) control; (b) root exudates. Analytical conditions: column, OV-17 (2 m); oven temperature was raised from 100°C to 270°C at 5°C/min; detector and injector temperatures, 300°C; the flow rate of carrier gas (He): 30 cm^3/min. Peak 1, p-thiocyanatophenol; peak 2, 2-hydroxybenzothiazole.

peak (peak 2) was attributed to 2-hydroxybenzothiazole by the comparison of the mass spectrum [m/z (rel. intensity) 151 (100), 123 (49), and 96 (66)] and the retention time (18.1 min) with those of the authentic sample. Similarly, the peak 1 was attributed to p-thiocyanatophenol. The contents of p-thiocyanatophenol and 2-hydroxybenzothiazole in NF were 0.7 and 8.0 mg/g root exudate, respectively. The other peaks in Figure 4 have not been identified at present. 2-Hydroxybenzothiazole has been separated from the rhizosphere of coffee tree (Waller et al., 1986), although there is no report showing that cucumber exudes the compound. There is also no report that p-thiocyanatophenol is separated from the rhizosphere of plants, including cucumber.

The GC analysis of the AF, after trimethylsilylation, gave a number of peaks (Figure 5), among which nine were assigned to trimethylsilylates of organic acids such as benzoic, 3-phenylpropionic, cinnamic, p-hydroxybenzoic, 2,5-

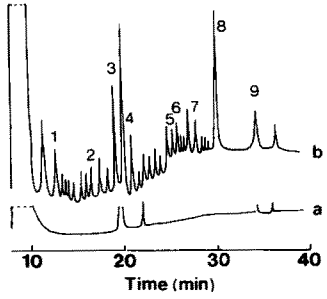

FIG. 5. Gas chromatogram of the acidic fraction (AF) after trimethylsilylation: (a) control; (b) root exudates. Analytical conditions: column, GL Sciences TC-50 (60 m); oven temperature, 100°C for 2 min and then raised to 270°C at 5°C/min; detector and injector temperatures, 300°C; the flow rate of carrier gas (He), 1 cm^3/min. Peak 1, benzoic acid; peak 2, 3-phenylpropionic acid; peak 3, cinnamic acid; peak 4, *p*-hydroxybenzoic acid; peak 5, 2,5-dihydroxybenzoic acid; peak 6, myristic acid; peak 7, *p*-hydroxycinnamic acid; peak 8, palmitic acid; peak 9, stearic acid.

dihydroxybenzoic, myristic, *p*-hydroxycinnamic, palmitic, and stearic acids, based on the comparison of their GC-MS data with those of authentic samples. These compounds were not detected in the AF of a control, indicating that they were the constituents of root exudates. They have frequently been separated from the root residue, root exudates, and rhizosphere of plants (AlSaadawi et al., 1983; Pérez and Ormeño-Nuñez, 1991b; Rice, 1984; Tang and Young, 1982; Yamane et al., 1992), although not from the root exudates of cucumber. The mean rates of release for these compounds at the vegetative and reproductive stages were calculated on the basis of the amounts of the compounds detected and the days needed for the collection (Table 3). Significant amounts of the fatty acids, as well as benzoic acid, were released at both stages, whereas the other aromatic carboxylic acids were mainly released at the reproductive stage. The calculated rates of release for the aromatic carboxylic acids were similar to those for allelochemicals observed for other plants, ranging from less than 1 μg/day to several micrograms per day (Pérez and Ormeño-Nuñez, 1991a,b; Tang and Takenaka, 1983; Yamane et al., 1992).

Table 4 shows the phytotoxicity of the identified compounds at various concentrations. Most of the compounds exhibited significant phytotoxicity at concentrations higher than 0.1 mmol/dm^3. Among them, *p*-thiocyanatophenol was the most phytotoxic. 2-Hydroxybenzothiazole had no inhibitory effect on the growth of lettuce. The inhibitory effect of phenolic acids such as ferulic and *p*-hydroxycinnamic acids on the growth of cucumber plants and their nutrient uptake has been reported (Blum et al., 1985; Blum and Dalton, 1985; Holappa and Blum, 1991; Lyu et al., 1990).

TABLE 3. IDENTIFIED ORGANIC ACIDS IN ACIDIC FRACTION (AF) AND MEAN RATES OF RELEASE FOR THEM AT DIFFERENT GROWTH STAGES

Peak	m/z	Organic acid	Release rate (μg/day/plant)	
			Vegetative	Reproductive
1	194	Benzoic	1.5	1.5
2	222	3-Phenylpropionic	ND[a]	0.7
3	220	Cinnamic	ND	1.6
4	282	*p*-Hydroxybenzoic	ND	1.5
5	370	2,5-Dihydroxybenzoic	0.1	0.4
6	300	Myristic	1.4	1.5
7	308	*p*-Hydroxycinnamic	ND	0.9
8	328	Palmitic	2.6	5.0
9	356	Stearic	0.5	1.1

[a]ND: not detected.

TABLE 4. EFFECT OF IDENTIFIED COMPOUNDS AT VARIOUS CONCENTRATIONS ON ROOT LENGTH (mm) OF LETTUCE

Compounds	Concentration (mmol/dm^3)				
	0	0.01	0.05	0.10	0.50
Benzoic acid	11.2a[a]	11.2a	10.1a	6.3b	3.4c
p-Hydroxybenzoic acid	11.2a	9.6a	9.6a	6.3bc	4.7c
2,5-Dihydroxybenzoic acid	11.2a	11.3a	11.1a	9.2ab	8.2b
3-Phenylpropionic acid	11.2a	10.5a	9.2a	6.9b	2.4c
Cinnamic acid	11.2a	10.2a	9.6a	6.4bc	5.4c
p-Hydroxycinnamic acid	11.2a	11.0a	9.9a	9.6a	7.8b
Myristic acid	11.2a	10.4a	9.2ab	8.3ab	8.4b
Palmitic acid	11.2a	11.0a	10.9ab	10.3ab	6.9b
Stearic acid	11.2a	10.9a	9.4ab	9.3ab	8.1b
p-Thiocyanatophenol	11.2a	9.7a	9.4b	6.8c	0.8d
Mixture[b]	11.2a	10.3a	8.2ab	7.7.b	6.9b

[a]Numbers with different letters within a row refer to significant difference at 0.05 level according to Duncan's multiple-range test.
[b]The mixture of the above 10 compounds, each concentration being one tenth of those cited.

The above results clearly indicate that autotoxic and allelopathic compounds were accumulated in the nutrient solutions for cucumber, especially at the reproductive stage of growth. However, as shown in Figures 4 and 5, we identified only some of compounds involved in NF and AF, and most of them

remained to be identified. The identified compounds were less than 5% by weight of the collected exudates. Furthermore, the fractions might contain nonvolatile compounds that could not be detected by gas chromatography. Thus, it is reasonable to consider that not only the identified but also unidentified compounds are responsible for the phytotoxicity of the fractions. It is also possible that the effect of the phytotoxic compounds is additive, synergistic, or antagonistic (Leather and Einhellig, 1986; Rice, 1984). It is important to examine the effect of mixed compounds on the growth of cucumber plants. These problems remain to be solved.

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CONTROL OF PLANT-PARASITIC NEMATODES BY A NEMATICIDAL STRAIN OF *Aspergillus niger*

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Abstract—An isolate of *Aspergillus niger* (designated PD-42) was evaluated in laboratory, greenhouse, and field trials for efficacy in controlling plant-parasitic nematodes. In greenhouse experiments, PD-42 drenches containing spores of PD-42 on oatmeal significantly reduced galling on tomato due to root-knot nematode as compared to untreated controls. In a one-half acre field experiment, PD-42 incorporated in seed coats was associated with significantly increased yield and decreased root-knot galling on pepper. In a second one-half acre field experiment, PD-42 drenches significantly reduced tomato and pepper root galling due to *Meloidogyne incognita*, and nonsignificant yield increases occurred. In each field experiment, treatment with PD-42 reduced *Rotylenchulus reniformis* populations. The nematicidal components of the *A. niger* culture filtrates include citric acid, oxalic acid, and undetermined molecules larger than 8000 MW.

Key Words—*Aspergillus niger*, biological control, plant-parasitic nematodes, *Meloidogyne incognita*, nematicidal activity, *Caenorhabditis elegans*, *Rotylenchulus reniformis*.

INTRODUCTION

An early observation of toxicity of *Aspergillus* to nematodes was made by Haven Metcalf (1903). In this paper, which, to our knowledge, also records the first successful attempt to produce an axenic culture of nematodes, Metcalf describes the lethal effect of a black-spored *Aspergillus* on *Rhabditis brevispina* (Claus) Butschli. He wrote "No investigation was made of the by-products of this

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Aspergillus, which are probably poison. Might not the line of investigation here suggested be fruitful if followed out with reference to certain pathogenic nematodes?'' Later, other references to the nematicidal affects of *Aspergillus* and its filtrates appeared, among them reports by Murad (1966), Mankau (1969), Alam et al. (1973), and Khan et al. (1981).

The chinampa agricultural soils of Mexico contained organisms that exert a suppressive effect on plant-pathogenic fungi (Lumsden et al., 1987) and plant-parasitic nematodes (Zuckerman et al., 1989). The predominant nematicidal fungi from these soils were species of *Aspergillus* which, in agar culture, produced green, yellow, black, or brown spores. Of these, the most toxic isolate was identified by M. Bigelow, University of Massachusetts, as a strain of *Aspergillus niger* van Tieghem (coded PD-42). The current paper reports on laboratory, greenhouse, and field studies to examine the nematicidal properties of this *A. niger* isolate on plant-parasitic nematodes.

METHODS AND MATERIALS

Organisms. The *A. niger* strain in these experiments produces black spores and is referred to by its coded designation PD-42. PD-42 was the most abundant fungus encountered from the chinampa agricultural soils (Zuckerman et al., 1989). Nematicidal *Aspergillus* with green, brown, or yellow spores were also frequently isolated, but PD-42 showed the highest activity against plant nematodes in greenhouse tests and was selected for further study. PD-42 was maintained in cryopreservation buffer at −80°C and under liquid nitrogen. When required for experiments, it was grown on potato dextrose agar (PDA) or in potato dextrose broth (PDB).

Meloidogyne incognita (Kofoid and White) race 3 was obtained from Dr. M. McClure, University of Arizona, Tucson, and was maintained on tomato (*Lycopersicon esculentum* L.) cv. Rutgers in the greenhouse. Inoculum for greenhouse experiments consisted of eggs obtained by the method of Hussey and Barker (1973).

The free-living nematode *Caenorhabditis elegans* Dougherty and Calhoun for in vitro studies of nematicidal activity were cultured axenically at 22°C on heme medium (McClure and Zuckerman, 1982). Tomato cv. Floradade and pepper (*Capsicum frutescens* L.) cv. Cubanelle were used in the field trials.

Laboratory Assays for Nematicidal Activity. The nematicidal activity of PD-42 was first observed in an assay in which *C. elegans* was placed in proximate contact with the fungus in an agar plate (Zuckerman et al., 1989). This procedure was followed by a similar in vitro assays of the effect on second stage larvae of *M. incognita*. The criterion for high nematicidal activity was death of the worms within 24 hr. Culture filtrates of *A. niger*, or the acid components

of these filtrates, in Falcon multiwell tissue culture plates (24 wells) were also used to measure toxicity against *C. elegans*.

Greenhouse Trials. PD-42 was applied to pots in greenhouse trials on infested oatmeal, as a drench of a liquid shake culture, as a spore suspension, or as a seed coat (Townshend et al., 1989). Six experiments were run utilizing the several methods of inoculum preparation. Since all gave approximately the same results, only two representative trials are reported (Tables 1 and 2). The drench application was prepared as follows: each culture was initiated with a 5-mm plug taken from a culture of PD-42 grown on PDA at 25°C, then grown in 50 ml PDB in a 250-ml Erlenmeyer flask on a rotary shaker at ambient room temperature for seven days. Applications were at a concentration of $1 \times 10^{5-6}$ colony forming units (CFU/ml water/50 ml/plant. The first trial contained PDB medium alone as a control. The medium control showed no significant effect on plant growth or gall formation due to *M. incognita* on tomato. Therefore this control was not included in succeeding experiments.

Oatmeal cultures were prepared by autoclaving 25 g oatmeal in a 250-ml Erlenmeyer flask for 20 min at 120°C. The cultures were allowed to cool and then inoculated with 15 ml sterile, distilled water containing a 5-mm plug of PD-42 from a PDA culture. The flasks were inoculated at 25°C for seven days and shaken by hand daily to ensure even colonization of the oatmeal. For the greenhouse trial, each pot received 25 g of oatmeal–fungus culture or oatmeal alone, as appropriate (Table 2).

Tomato seedlings, *L. esculentum* cv. Rutgers, were grown in a mixture of one part washed sand to one part sterilized soil. A 2 to 3-week-old tomato seedling was transplanted into each pot and allowed to grow one week before the PD-42 drench was applied. After seven days, 5000 *M. incognita* eggs were added to a 1/2-in.-deep hole made in the soil of each pot. There were five replicates per treatment. Pots were arranged on a greenhouse bench as a ran-

TABLE 1. GREENHOUSE TRIAL EVALUATING EFFECTS OF *Aspergillus nige* DRENCH ON GALL FORMATION CAUSED BY *Meloidogyne incognita* (MI) AND GROWTH OF RUTGERS TOMATO[a]

Treatment	Fresh shoot wt (g)	Fresh root wt (g)	Galls/plant	Control (%)
MI-infested control	29.9 ± 4.5a	7.1 ± 1.4a	384 ± 34a	
MI + *A. niger* drench	40.3 ± 7.4b	16.8 ± 1.8b	158 ± 80b	60

[a]Each treatment replicated five times. Numbers followed by different letters differ at $P = 0.05$. Means separated by *t* tests (Minitab).

TABLE 2. GREENHOUSE TRIAL EVALUATION OF EFFECTS OF *Aspergillus niger* GROWN ON OATMEAL ON GALL FORMATION CAUSED BY *Meloidogyne incognita* (MI) AND GROWTH OF RUTGERS TOMATO[a]

Treatment	Fresh shoot wt (g)	Fresh root wt (g)	Galls/plant	Control (5)
Oatmeal control (25 g)	168.5 ± 13.2a	36.8 ± 5.8a		
Oatmeal + *A. niger* (25 g)	196.3 ± 21.0b	49.1 ± 7.9b		
MI-infested control	132.5 ± 18.6c	37.6 ± 3.5a	122.6 ± 17.5a	0
MI + *A. niger* on oatmeal (25 g)	169.9 ± 7.7a	37.3 ± 14.8a	55.8 ± 24.4b	55

[a]Data followed by different letters differ from each other significantly at $P = 0.05$.

domized Latin square and fertilized weekly with a 20–20–20 (N-P-K) fertilizer (Table 1).

After six weeks, plants were harvested, their roots washed, and data taken on the fresh weight of plant tops and roots, the dry weight of plant parts, and the number of galls per root system. Only the fresh weight data are reported. Data were analyzed by analysis of variance supplemented by paired *t* tests (Minitab, Pennsylvania State University).

Field Trials. Field trials were performed at the University of Puerto Rico, Isabela Experiment Station, Puerto Rico, where high soil populations of *M. incognita* and *Rhotylenchulus reniformis* Linford and Oliviera occurred.

The first experiment was on pepper with PD-42 applied in a seed coat, on rice, or as a drench. Each treatment was replicated four times, with each replicate consisting of four rows of 20 ft each, for a total of 80 plants. Plants were spaced 1 ft apart, giving 180 ft^2 as the size of each plot. The treatments reported here were part of a larger experiment containing 11 treatments and 44 plots and testing other organisms as biocontrol agents. Seedlings from seeds coated with PD-42 were transplanted directly to the field. Where rice was the carrier, 50 g of rice containing the biocontrol agent was placed in each hole at the time of planting.

One method of application was by seed coat (Table 3). Methyl cellulose seed coats were prepared by the methods of Townshend et al. (1989). Three coated pepper (*Capsicum annuum* cv. Cubanelle) seeds were planted in a 2-in. pot containing heat-sterilized coto clay (72.5% clay, 21.1% sand, and 6.4% silt) taken from the field plot site, thinned to one plant after germination, and transplanted to the field at three to four weeks.

PD-42 was also applied on a rice carrier. Long grain rice (unhulled, organically grown) was boiled for 3 min, drained, and 200 g transferred to quart-size

TABLE 3. FIELD EVALUATION OF *Aspergillus niger* AND *Bacillus thuringiensis* IN CONTROLLING ROOT-KNOT OF PEPPER, UNIVERSITY OF PUERTO RICO, ISABELA EXPERIMENT STATION[a]

Treatment[b]	Yield/20 plant plot (Kg)	Root-knot gall index
A. niger seed coat + drench	16.1 ± 4.2b	0.5c
A. niger on rice	6.8 ± 3.3c	1.1b
Rice alone (control)	7.8 ± 1.5b	0.7bc
A. niger + CR-371 (drench)	15.3 ± 0.9b	1.0bc
Untreated control	11.4 ± 2.0c	2.1a
Fenamiphos	19.3 ± 2.6a	0.4c

[a]Each treatment contained four replicate plots. CR-371 = nematicidal strain of *Bacillus thuringiensis*. Numbers followed by different letters differ at $P = 0.05$.
[b]All PD-42 treated plots received a second application of PD-42 drench four weeks later.

Mason jars and autoclaved for 30 min at 121°C. Each jar was inoculated with PD-42 from a PDA culture and incubated at room temperature in the dark with vigorous agitation by hand for 5 min daily for seven days. The contents of the Mason jars were then spread on aluminum foil, covered with newspaper, and allowed to dry for 48 hr. Dried inoculum was weighed into plastic bags and stored at 4°C until use.

A combination of two biocontrol organisms was also applied as a drench, specifically PD-42 and CR-371 (Table 3), the latter being a nematicidal *Bacillus thuringiensis*, which was previously described by Zuckerman et al. (1993).

The drench application in this trial varied from that in the second trial (Table 3) in that each plant received 50 ml of the organism at a concentration of $1 \times 10^{5-6}$ CFU/ml in PDB culture medium applied directly in the hole made at the time of planting. Plants in the control plots each received 50 ml PDB. A PD-42 drench was applied twice at 30-day intervals to all plots that received either PD-42 or a combination of PD-42 and CR-371. The nematicide plots were treated once with fenamiphos (15 A.I.) at the rate of 7.92 g/row of 20 plants. Peppers were harvested five times. Nematode population estimates were obtained using a combined sieving and Baermann funnel extraction (Barker and Niblack, 1990). *R. reniformis* populations were counted in all soil samples. Nematode soil populations were sampled three times: at planting, 30 days later, and at 60 days. Damage from root-knot nematode was evaluated by a gall index as follows: 0 = no galls or egg masses, 1 = 1–25 galls or egg masses per plant, 2 = 26–50, 3 = 51–75, 4 = 76–99, 5 = 100 plus. In each trial, 20 plants/replicate were pulled after harvest and assigned a gall index. Twenty-five soil samples also were analyzed for the presence of black-spored aspergilli at

the termination of each experiment using the same procedures described for soil sampling by Zuckerman et al. (1989), except that the only media employed were 3% potato dextrose agar or 2% Bacto-agar each with 0.3% streptomycin sulfate added.

The same protocol was followed in trial 2 (Table 4) with several exceptions. Host plants in this experiment were pepper cv. Cubanelle and tomato cv. Floradade. Liquid cultures for drench application were initiated by growing a 5-mm plug of PD-42 in 1 liter PDB for five days, adding this to 10 liters PDB held in a 14-liter Chemap Fermenter and fermenting for 24 hr to a final concentration of $1 \times 10^{5-6}$ CFU/ml. Prior to shipping, the inoculum was concentrated by centrifugation and the supernatant PDB medium removed. Fermentations were performed in Massachusetts. The concentrated inoculum was packed in 500-ml containers and shipped on ice to Puerto Rico by overnight carrier. The inoculum was brought up to concentration in the field by the addition of water, and applied at 50 ml PD-42 drench per plant. Controls were untreated and fenamiphos treated plots. During the first period of the 12-week experiment, PD-42 was applied four times at two-week intervals. In this trial, there were 40 tomato plants and 40 pepper plants in each plot, with a total of 160 tomato plants and 160 pepper plants on four plots for each treatment. The total yield from three weekly tomato harvests and five weekly pepper harvests were combined for analysis (Table 4). Nematode soil populations were sampled when the experiment was initiated, three weeks later, six weeks later, and at 53 days (during harvest).

Characterization of Nematicidal Exometabolites. In vitro nematicidal activity of PD-42 exometabolites was evaluated in two ways. For the first, PD-42 was grown four days in PDB at room temperature on a rotary shaker at ambient room temperature (23°–25°C). Culture extracts were then centrifuged at 10,000*g*

TABLE 4. EFFECTS OF *A. niger* ON YIELD AND GALL FORMATION CAUSED BY *Meloidogyne incognita* ON TOMATO AND PEPPER IN FIELD TRIAL, UNIVERSITY OF PUERTO RICO, ISABELA EXPERIMENT STATION[a]

	Tomato			Pepper		
Treatment	Yield (kg)	Yield over control (%)	Gall index	Yield (kg)	Yield over control (%)	Gall index
Untreated control	114.8 ± 10.6a		4.3 ± 3.6a	18.3 ± 1.4a		0.92 ± 0.6a
Fenamiphos	133.2 ± 9.3a	16	4.45 ± 2.2a	19.4 ± 4.3a	6	0.75 ± 0.6a
A. niger drench	125.2 ± 6.7a	9	0.5 ± 1.1b	21.9 ± 2.3a	19	0.63 ± 0.6b

[a]Each treatment replicated four times. Numbers followed by different letters differ at $P = 0.05$.

for 1 min, the supernatant removed, and passed through a sterile 0.22-μm filter. Thermostability of the extract was tested first by boiling the culture filtrate (CF) for 5 min and then bioassaying against *C. elegans* to evaluate loss or retention of nematicidal activity. Second, CF was dialyzed against Spectrophor membrane tubing (6000–8000 molecular weight cutoff), and the fraction remaining within the tubing evaluated for nematicidal activity as described below.

The *C. elegans* test for nematicidal activity proceeded as follows: 2.5 ml of CF were placed in a well in a sterile, multiwell plate at a concentration of one part filtrate to one part liver extract medium (HLE) prepared as described by Sayre et al. (1963). Further concentrations of the extract tested were two parts CF to one part HLE, four parts CF to one part HLE, and eight parts CF to one part HLE (Table 5). Since HLE was diluted by CF as increasing concentrations of filtrate were tested and this factor had an effect on *C. elegans* growth and mobility, a series with HLE dilutions without PD-42 filtrate were run as a control. For the toxicity assay, one drop of a 7-day-old mixed popu-

TABLE 5. EFFECT OF *Aspergillus niger* CULTURE FILTRATE ON *Caenorhabditis elegans*[a]

Treatment[b]	Liver extract dilution	Time			
		15 min	1 hr	3 hr	24 hr
Potato-dextrose broth (PDB)	1:1	+	+	+	+
	1:2	+	+	+	+
	1:4	+	+	+	+
	1:8	+	+	+	+
4-day-old culture in 50 ml PDB,	1:1	+	+	+	+
centrifuge 1 min, test	1:2	+	+	+/−	−/+
supernatant	1:4	+	−/+	−	−
	1:8	+	−/+	−	−
4-day-old culture, centrifuge	1:1	+	+	+	+
1 min, sterile filter	1:2	+	+	+/−	+/−
	1:4	+	−/+	−/+	−/+
	1:8	+	−/+	−/+	−/+
4-day-old culture, filter Whatman	1:1	+	+	+	+/−
No. 1, test	1:2	+	+	+/−	−
	1:4	+	+/−	−/+	−
	1:8	+	+/−	−	−
4-day-old culture, filter Whatman	1:1	+/−	+/−	+/−	+/−
No. 1, boiled 5 min	1:2	+/−	+/−	−	−
	1:4	−	−	−	−
	1:8	−	−	−	−

[a]Key: + = vigorous movement; +/− = 50% dead; −/+ = 75% dead; − = 100% dead.
[b]Each dilution was replicated four times.

lation, axenic *C. elegans* was added to each well (50–100 worms). Observations on toxicity were taken at 15 min, 1 hr, 3 hr, and 24 hr. The rating scale was: + = vigorous movement; +/− = 50% immobile; −/+ = 75% immobile; and − = 100% immobile. Observations over longer time periods showed that immobile nematodes were dead.

High-Pressure Liquid Chromatographic (HPLC) Analysis. Sterile CF from 2-, 4-, 6-, and 8-day-cultures, were prepared as previously described and analyzed by HPLC on a Hewlett Packard 1090 analyzer. A Bio-Rad HPX-87H cation exchange column with a 5-cm guard column containing the same resin was used. The eluent was 0.005 N sulfuric acid at a flow rate of 0.7 ml/min. The UV detector setting was 210 nm. The system was run at ambient room temperature.

The HPLC analysis revealed large amounts of citric and oxalic acids in the CF; therefore, a second series was run on the nematicidal effects of pH and these two organic acids on *C. elegans*. One-milliliter samples of several concentrations of oxalic and/or citric acid were applied to water agar plates and 0.1 ml of a 7-day-old axenic *C. elegans* culture added. Bioassays were conducted at room temperature and plates analyzed at three days for *C. elegans* viability. Controls were sterile distilled water, PDB, and filter sterilized PD-42 extracts from 2-, 4-, and 6-day-old cultures (Table 6).

RESULTS

Greenhouse Trials. PD-42 drench applied to tomato in the greenhouse gave 60% control of galling caused by *M. incognita*. A PDB control was not included in this trial since previous tests showed that the PDB medium did not effect growth of tomato. In this trial, PD-42 treatment was associated with a significant enhancement of tomato growth and a significant reduction in root galling (Table 1).

PD-42 applied on an oatmeal carrier gave 55% control of root-knot galling (Table 2). The oatmeal significantly ($P = 0.05$) enhanced tomato shoot growth (Table 2).

Field Trials. In the first field trial at the Isabela Experiment Station (Table 3), significant control of root-knot galling by *M. incognita* ($P = 0.05$) was a significant increase in yield ($P = 0.05$) occurred in the combinations where PD-42 was applied as a drench and seed coat, but not when applied with rice as a carrier. The rice carrier was phytotoxic at the rate applied, as indicated by the lesser growth in the rice control. Rice also depressed galling; this probably was associated with the high concentration of nitrogen that the carrier introduced into the rhizosphere.

A significant reduction in tomato root galling due to *M. incognito* ($P =$

TABLE 6. EFFECTS OF *Aspergillus niger* CULTURE FILTRATE (CF) AND/OR CITRIC AND OXALIC ACID ON VIABILITY OF *C. elegans*[a]

Treatment	Viability[b]
Sterile distilled water	+
Potato–dextrose broth	+
CF	
2-day-old culture	+
4-day-old culture	−
6-day-old culture	−
Oxalic acid	
8 g/liter	+
10 g/liter	−
12 g/liter	−
16 g/liter	−
Citric acid	
8 g/liter	+
10 g/liter	+
12 g/liter	+
16 g/liter	+
Oxalic acid	
2 g/liter + citric acid 2 g/liter	+
4 g/liter + citric acid 4 g/liter	−
8 g/liter + citric acid 8 g/liter	−

[a] Readings taken 72 hr after initiation of assay.
[b] + = mostly live − = all dead; four replicates for each treatment.

0.05) occurred as compared to untreated control plants in the second field trial (Table 4). The failure of fenamiphos to control root-knot was unexplained. There were no significant differences in yield, although both the biological and chemical nematicide showed mathematical yield increases. The incidence of root galls was very low on pepper. However, PD-42-treated plants showed significant reductions in galling ($P = 0.05$) as compared to untreated plants. Yield results were similar to those on tomato: slight increases but no significant differences. At the last soil sampling of the second field trial, populations of *R. reniformis* were 93% lower in both the PD-42 and fenamiphos plots as compared to the untreated controls. At the last soil sampling of the first experiment, reductions in *R. reniformis* as compared to untreated controls were 30% in fenamiphos-treated plots, 20% for PD-42 seed coat + CR-371 drench, and 57% for PD-42 alone. The differences were not statistically significant due to variations between replicate plots.

Sixty days after treatment, a black-spored *A. niger* was recovered from

PD-42-treated plots, but not from untreated plots. It was not determined if this isolate was the same strain as that inoculated.

Culture Filtrate Studies. Dialysis studies indicated that molecules in CF larger than 8000 MW killed *C. elegans* within 10 min and *M. incognita* second-stage larvae in 60 min. CF retained nematicidal activity against both species after boiling for 5 min.

Low concentrations of CF were generally not toxic to *C. elegans*, but at higher concentrations 75–100% mortality occurred within 3 hr (Table 5).

Combining bioassay and HPLC analysis, 8-day-old CF showed nematicidal activity at mean oxalic acid residues of 6.1 g/liter and citric acid of 0.9 g/liter. When pH of the medium was adjusted, no effect on nematode viability occurred in the range pH 2–9. The nematicidal principal in CF appeared after four days in culture. Exposure of *C. elegans* to prepared concentrations of oxalic and citric acids showed no nematicidal effect of oxalic acid at 12 g/liter, but toxicity at 16 g/liter *C. elegans* tolerated citric acid concentrations of 16 g/liter. However, concentrations of 4 g oxalic acid and 4 g citric acid combined were nematicidal (Table 6), demonstrating a synergistic action between the two compounds.

These results indicate that the PD-42 culture filtrate contains a number of nematicidal molecules, of size both smaller and larger than 8000 MW.

DISCUSSION

These studies indicate that *A. niger* strain PD-42 has potential as a commercial biological control agent for plant-parasitic nematodes. While recognizing that aspergilli are reported as pathogenic to humans (Wyllie and Morehouse, 1978), options exist for safe delivery to the host rhizosphere. Several reports on the toxicity of microbial exometabolites to nematodes are reviewed by Sayre and Starr (1988). Oxalic acid was previously reported as being an exometabolite of a nematicidal *A. niger* (Mankau, 1969). Mankau (1969) also noted the thermostability of the *A. niger* toxic principal and its nematicidal action both in vitro and in the soil against *Aphelenchus avenae* Bastian.

A novel finding in the current study is that both oxalic and citric acids are partial determinants of the nematicidal activity and, more importantly, that synergism between these two acids is an important factor in explaining the mode of action of PD-42 nematoxicity. This was shown in studies in which neither citric nor oxalic acid killed *C. elegans* at concentrations of these acids produced in shake culture, but that combinations of lower concentrations of the acids were lethal. The current report also is the first on quantifying oxalic and citric acid levels in *A. niger* CF. The dialysis study indicated that nematicidal components greater than 8000 MW are also present in the *A. niger* CF.

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CHANGES IN PHEROMONE TITER OF OBLIQUE-BANDED LEAFROLLER, *Choristoneura rosaceana*, VIRGIN FEMALES AS A FUNCTION OF TIME OF DAY, AGE, AND TEMPERATURE

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Abstract—Under a 16:8 hr light–dark photoperiod and 20°C constant temperature, the titers of (*Z*)-11-tetradecenyl acetate (*Z*11–14:Ac), (*E*)-11-tetradecenyl acetate (*E*11-14:Ac) and (*Z*)-11-tetradecenol (*Z*11–14:OH) produced by different-aged *Choristoneura rosaceana* virgin females varied significantly during the scotophase, with the maximum titer occurring before the onset of calling in day-0 and day-3 females, while in day-5 females the titer remained constant throughout the calling period. There was a significant decrease in the titer of all pheromone components with age, explaining the lesser attractiveness of day-5 females relative to day-0 and day-3 females observed in the field. Under a cold thermocycle simulating condition during the second flight period in the fall, the titers of all pheromone components did not vary with time of day. There was a significant decrease in the amount of *Z*11–14:Ac with age but no changes occurred in the minor components. Furthermore, for any given age tested, the amount of each component produced during the period of maximal calling activity remained relatively similar at the two temperature regimes. However, as with the expression of calling behavior, pheromone production was initiated earlier at cooler than at warmer temperatures. At both temperature regimes, female age and time of day influenced the ratio of each pheromone component. These results are discussed in relation to the hypothesis that by calling earlier, less attractive older females may increase their probability of mating.

Key Words—Pheromone titer, (*Z*)- and (*E*)-11-tetradecenyl acetate, (*Z*)-11-tetradecenol, age, time of day, constant and fluctuating temperature,

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Choristoneura rosaceana, oblique-banded leafroller, Lepidoptera, Tortricidae.

INTRODUCTION

It has been suggested that the advance in the onset time of calling as females age is an adaptation that permits older individuals in several species of Lepidoptera to increase their probability of attracting mates before younger females start calling (Swier et al., 1977; Turgeon and McNeil, 1982; Webster and Cardé, 1982). Similarly, the earlier onset of calling in response to decreasing ambient temperatures has been interpreted as an adaptation permitting females of all ages to attract mates before temperatures fall below a level that inhibits male flight activity (Cardé et al., 1975; Webster and Cardé, 1982; Turgeon and McNeil, 1983; Delisle and McNeil, 1987a, b).

Delisle (1992a) reported such age- and temperature-related changes in the onset time of calling of the oblique-banded leafroller (OBL), *Choristoneura rosaceana* (Harris), a bivoltine species in eastern Canada (Delisle, 1992b). However, whether or not these behavioral changes afford any reproductive advantage to older OBL females was further examined by comparing the relative attractiveness of different-aged (0-, 3-, and 5-day-old) females during both flight periods (Delisle, 1992a). Day-5 females attracted significantly fewer males than day-0 and day-3 individuals throughout the season, suggesting that older females may release less pheromone than younger ones. However, while the relative attractiveness of day-0 to day-3 females remained the same in both flights, day-5 females were comparatively more attractive in the fall than in the summer. Delisle (1992a) suggested that these results may support the hypothesis that by calling earlier, older females avoid competition with younger females and are particularly advantaged in the fall, when low temperatures limit male flight. However, another explanation for the lower degree of competitiveness of younger females in the fall is that temperature affected their pheromone production more than that of older individuals.

In order to verify if pheromone titer in OBL was influenced by age, and if there was a differential effect of both time and temperature on pheromone synthesis by different-aged females, we undertook a series of experiments to determine the periodicity of pheromone production of 0-, 3-, and 5-day-old females at 20°C constant temperature under a 16:8 hr light–dark photoperiod. The experiment was also repeated under a cold thermocycle that simulated temperature conditions prevailing during the fall flight.

METHODS AND MATERIALS

Moths. All females used in these experiments were obtained from an annually restocked laboratory colony, established using field-collected pupae at Quebec City, and maintained at 20 ± 0.5°C, 65± 1% relative humidity, under

a 16:8 hr light–dark photoperiod. Larvae were reared on a pinto bean artificial diet (Shorey and Hale, 1965). Upon pupation, individuals were sexed and only females retained. At emergence, females were kept individually in 150 cm^3 plastic vials with an 8% sucrose solution and held under standard colony conditions until needed.

Gland Extraction and Pheromone Quantification. The titers of the major component, (*Z*)-11-tetradecenyl acetate (*Z*11–14:Ac), as well as two minor components, (*E*)-11-tetradecenyl acetate (*E*11–14:Ac) and (*Z*)-11-tetradecenol (*Z*11–14:OH) (Hill and Roelofs, 1979) were determined. Quebec populations, like those of New York (Hill and Roelofs, 1979), do not produce (*Z*)-11-tetradecenal (*Z*11–14:Ald), a component isolated from OBL females in the Okanagan Valley, British Columbia (Vakenti et al., 1988). Prior to detailed quantification, the efficiency of pheromone extraction was determined by comparing the amount of *Z*11–14:Ac extracted from glands excised 2 hr after the onset of scotophase from day-1 females. Individual glands were soaked in 20 μl of hexane, containing 1.5 ng of dodecanoic acid methyl ester ($C_{13}H_{26}O_2$) as an internal standard, for 0.5, 1, 5, 10, 15, 20, 25, and 30 min. The titer of *Z*11–14:Ac did not vary significantly when extraction times exceeded 20 min. However, when glands were soaked >20 min, extraneous compounds were observed, so 20 min was chosen as the period of extraction.

To assess the effect of age, time of day, and temperature on the OBL pheromone titer, individual glands of 0-, 3-, and 5-day-old virgin females, held at 20°C constant temperature, were excised at −1, 0, 1, 2, and 3 hr, with 0 hr being the onset of the scotophase. As previously reported by Delisle (1992a), the mean onset time of calling (MOTC) of 0-, 3-, and 5-day-old females at 20°C constant temperature occurred 2.1, 0.9, and 0.7 hr after the onset of scotophase, respectively. The pheromone titer of similar-aged females was also quantified under a thermocycle (17°–9°C; $\bar{X}$ = 14.9°C) reflecting conditions during a cool summer or a fall night. Females were kept at 20°C constant temperature and at the appropriate age were transferred to the thermocycle 5 hr before lights-off on the day the pheromone titer was determined. At the time of transfer, the temperature was 17.5°C and decreased to 17.0, 16.5, 16.0, 14.5, and 13.0°C during the next 5 hr, respectively. Pheromone glands were excised at −2, −1, and 0 hr of the scotophase, coinciding with the period during which calling is initiated by the different-aged females under this thermocycle (Delisle, 1992a). The pheromone titer was not determined after the onset of scotophase as, based on the reduced male flight activity and the low incidence of matings observed at these temperatures in the field (Delisle, personal observations), it was unlikely that OBL females would attract mates at temperatures below 13°C, even though calling can be expressed under such conditions (Delisle, 1992a). As previous temperature conditions may affect some aspects of calling ecology (Baker and Cardé, 1979; Delisle and McNeil, 1987a), all females were precon-

ditioned under the same photoperiod and temperature prior to testing to allow direct comparisons of the effect of different ambient temperatures on pheromone production.

For each pheromone gland extract, 5 μl were injected onto a fused silica capillary column, SP 2340 (30 m $\times$ 0.32 mm ID), in a 5890 HP gas chromatograph, equipped with a flame ionization detector (FID) and an HP-3393A integrator. The column temperature program was: 110°C for 30 sec, increased to 160°C at 6°C/min, held at 160°C for 15 sec, increased to 230°C at 15°C/min, and then held for 15 sec. The detector was at 300°C while the splitless injector was at 205°C. Hydrogen was used as the carrier gas, at a flow rate of 1 ml/min. The retention times of the internal standard and the three pheromone components, *E*11–14:Ac, *Z*11–14:Ac and *Z*11–14:OH were 2.57, 6.08, 7.15, and 8.36 min, respectively. For each age category, under both temperature regimes, a minimum of 20 pheromone glands were excised per time point.

Statistical Analyses. Each value of *Z*11–14:Ac and *E*11–14:Ac (expressed in nanograms) was raised to the power 0.4 while the naperian logarithm of each value of *Z*11–14:OH, ln (*Z*11–14:OH + 0.01) was used to normalize the data and stabilize the variance (homoscedasticity). Similarly, each value of the relative proportion of *Z*11–14:Ac, *E*11–14:Ac, and *Z*11–14:OH was transformed using: (1) ln (1.01 − proportion of *Z*11–14:Ac), (2) ln (proportion of *E*11–14:Ac + 0.01), and (3) ln (proportion of *Z*11–14:OH + 0.001), respectively. Transformed data were analyzed separately for each temperature regime, using an analysis of variance. In addition, orthogonal contrasts (Snedecor and Cochran, 1967) were performed to test for differences in the mean level of pheromone (least square means: adjusted means) as a function of age (three levels: 0, 3, and 5 days), time of day (five levels at 20°C: −1, 0, 1, 2, 3 hr and three levels under the cold thermocycle: −2, −1, 0 hr) and the interaction between the two factors. Additional contrasts were also carried out to assess the trend (linear, quadratic, cubic, or quartic) of each source of variation. Polynomial equations that best described the relationship between pheromone titer, female age, and time of day were determined and used to generate the response surfaces of each component as well as their relative proportion. In the calculation of these equations, the time values −1, 0, 1, 2, 3 and −2, −1, 0 were replaced by 1400, 1500, 1600, 1700, 1800, and 1300, 1400, 1500, respectively, which coincided with eastern standard time. Lights-off occurred at 1500 hr. All statistical analyses were performed using the GLM procedure of SAS/Stat (SAS Institute, 1990).

RESULTS

At 20°C, the titers of *Z*11–14:Ac and *E*11–14:Ac were generally low prior to lights-off but increased and reached a peak in the first or second hour of the scotophase (Table 1). These temporal variations were highly significant

TABLE 1. MEAN TITER (NANOGRAMS) (LEAST SQUARE MEAN OF BACK-TRANSFORMED VALUES) OF *Z*- AND *E*11–14:AC AND Z11–14:OH PRODUCED BY DIFFERENT-AGED (0-, 3-, AND 5-DAY-OLD) *Choristoneura rosaceana* VIRGIN FEMALES AT DIFFERENT HOURS OF 16:8 HR LIGHT–DARK PHOTOPERIOD AT 20°C CONSTANT TEMPERATURE OR COOL THERMOCYCLE (0 HR REPRESENTS ONSET OF SCOTOPHASE)[a]

	Z11–14:Ac at day				E11–14:Ac at day				Z11–14:OH at day			
Time	0	3	5	$\bar{X}$ (time)	0	3	5	$\bar{X}$ (time)	0	3	5	$\bar{X}$ (time)
20°C constant temperature												
−1	23.57	29.46	24.24	25.73	0.33	0.27	0.22	0.27	0.03	0.04	0.01	0.03
0	11.07	38.79	14.69	29.93	0.78	0.77	0.10	0.46	0.03	0.06	0.01	0.03
1	66.51	43.67	28.15	44.23	1.49	0.80	0.27	0.74	0.09	0.09	0.02	0.06
2	61.06	44.15	20.54	39.77	1.40	0.69	0.19	0.65	0.26	0.17	0.03	0.11
3	39.69	30.50	21.94	30.25	0.74	0.39	0.26	0.44	0.35	0.10	0.06	0.12
$\bar{X}$ (age)	44.65	37.01	21.63		0.88	0.55	0.20		0.09	0.08	0.02	
Cool thermocycle												
−2	37.68	43.91	19.63	32.60	0.67	1.15	0.85	0.88	0.07	0.11	0.08	0.09
−1	49.95	33.83	24.93	35.48	0.92	0.78	0.98	0.89	0.13	0.04	0.03	0.06
0	44.89	37.89	20.38	33.41	0.75	0.87	0.90	0.84	0.13	0.06	0.08	0.09
$\bar{X}$ (age)	43.91	38.56	21.63		0.78	0.93	0.91		0.11	0.06	0.06	

[a]The transformed LS mean values of the standard errors of Z11–14:Ac and E11–14:Ac were 0.09 and 0.25 at 20°C constant temperature and 0.08 and 0.24 under the cool thermocycle respectively, regardless of age and time. For the Z11–14:OH, the LS mean values varied with age and time between (0.37–0.40) at 20°C constant temperature and between (0.48–0.74) under the cool thermocycle.

TABLE 2. MEAN SQUARE (MS) VALUES AND LEVEL OF SIGNIFICANCE (P) ASSOCIATED WITH EACH ORTHOGONAL CONTRAST USED TO TEST FOR DIFFERENCES IN TITER OF Z- AND E11-14:Ac AND Z11-14:OH PRODUCED BY *Choristoneura rosaceana* VIRGIN FEMALES UNDER 16:8 HR LIGHT-DARK PHOTOPERIOD AT 20°C CONSTANT TEMPERATURE OR UNDER COOL THERMOCYCLE, IN RESPONSE TO AGE (A), TIME DURING PHOTOPERIOD (T), AND INTERACTION BETWEEN THE TWO (AT)

		Z11-14:Ac		E11-14:Ac		Z11-14:OH	
Source of variation	*df*	MS	*P*	MS	*P*	MS	*P*
20°C constant temperature							
Time	4	14.447	0.0001	1.431	0.0006	32.565	0.0001
Linear (T)	1	9.653	0.0419	1.287	0.0342	121.950	0.0001
Quadratic (T^2)	1	37.473	0.0001	4.129	0.0002	0.007	0.9616
Cubic (T^3)	1	5.036	0.1412	0.085	0.5850	7.500	0.1090
Quartic (T^4)	1	5.625	0.1200	0.224	0.3754	0.542	0.6658
Age	2	61.283	0.0001	8.030	0.0001	63.208	0.0001
Linear (A)	1	107.880	0.0001	15.000	0.0001	91.517	0.0001
Quadratic (A^2)	1	14.686	0.0122	1.073	0.0530	36.119	0.0005
Age * time	8	5.726	0.0124	0.594	0.0359	3.781	0.2416
AT	1	9.763	0.0407	0.621	0.1409	9.887	0.0659
AT^2	1	26.807	0.0007	2.594	0.0027	0.296	0.7497
AT^3	1	0.081	0.8519	0.014	0.8264	4.123	0.2342
AT^4	1	1.580	0.4095	0.081	0.5955	0.549	0.6637
A^2T	1	1.178	0.4765	0.161	0.4530	7.894	0.1001
A^2T^2	1	0.677	0.5892	0.587	0.1521	5.137	0.1843
A^2T^3	1	0.489	0.6463	0.338	0.2770	0.242	0.7729
A^2T^4	1	5.234	0.1336	0.358	0.2634	2.775	0.3289
Error IV	510	2.319		0.285		2.901	
Cool thermocycle							
Time	2	0.417	0.7848	0.012	0.9383	1.754	0.5929
Linear (T)	1	0.051	0.8640	0.011	0.8084	0.001	0.9851
Quadratic (T^2)	1	0.785	0.4999	0.013	0.7945	3.496	0.3086
Age	2	32.567	0.0001	0.119	0.5397	2.993	0.4112
Linear (A)	1	53.471	0.0001	0.176	0.3395	5.467	0.2036
Quadratic (A^2)	1	11.731	0.0095	0.061	0.5726	0.979	0.5893
Age * time	4	2.099	0.3024	0.152	0.5316	2.611	0.5391
AT	1	0.740	0.5125	0.007	0.8489	1.043	0.5773
AT^2	1	0.203	0.7313	0.052	0.6025	5.294	0.2109
A^2T	1	1.850	0.3003	0.211	0.2960	2.914	0.3524
A^2T^2	1	5.557	0.0734	0.336	0.1874	0.381	0.7363
Error IV	269	1.720		0.192		3.335	

for both acetate components (Table 2). Furthermore, the titers of both acetates varied with female age, particularly during the period of maximal pheromone production (Table 1), with younger females producing significantly more *Z*11–14:Ac and *E*11–14:Ac than older ones (Table 2). However, there was a significant interaction between age and time for both components (Table 2), indicating that the temporal pattern described above was not typical of all OBL females. The titers of both acetates produced by day-5 females remained relatively constant throughout the 5-hr period compared with those produced by day-0 and day-3 females (Table 1), which could explain the significant interaction.

Temporal changes in the titer of *Z*11–14:OH were also noted (Table 1), with the production increasing linearly as the night progressed (Table 2). Furthermore, as observed with respect to the two acetate components, female age had a significant effect on the titer of *Z*11–14:OH (Table 2), with younger females producing more alcohol than older ones. The best model that described the relationship between titer in nanograms ($\hat{Y}$), time of day (T), and female age (A) for each acetate, as well as the alcohol component, was expressed by the following polynomial equations

$$\hat{Y}_{Z11\text{-}14:Ac} = (-84.0304 + 10.9383\,T - 0.3349\,T^2 + 17.5113\,A - 0.0595\,A^2 - 2.1499\,AT + 0.0657\,AT^2)^{2.5}$$

$$\hat{Y}_{E11\text{-}14:Ac} = (-26.9178 + 3.4754\,T - 0.1075\,T^2 + 5.1883\,A - 0.0161\,A^2 - 0.6542\,AT + 0.0204\,AT^2)^{2.5}$$

$$\hat{Y}_{Z11\text{-}14:OH} = \exp(-9.7615 + 0.4623\,T + 0.3461\,A - 0.127\,A^2) - 0.01$$

and served to generate the response surfaces shown in Figure 1A–C, respectively.

Given these differences, one would expect the relative proportion of each pheromone component to vary with time and age. As seen in Tables 3 and 4, there was a significant linear decline in the proportion of present *Z*11–14:Ac with time during the scotophase, and older females produced relatively more *Z*11–14:Ac (~0.99) than younger individuals (~0.96) at any given hour. There was no significant interaction between the two factors. The proportion of *E*11–14:Ac remained constant during the 5-hr period but, as expected, it decreased significantly from 0.02 to 0.003 with age. In contrast, the relative proportion of *Z*11–14:OH increased linearly with time and younger females produced more *Z*11–14:OH (~0.003) than older ones (~0.001). However, there was significant interaction between age and time with respect to the proportion of *Z*11–14:OH in the pheromone blend (Table 4). The best relationship between age and time on the relative proportion of each OBL pheromone component is described by the following polynomial equations,

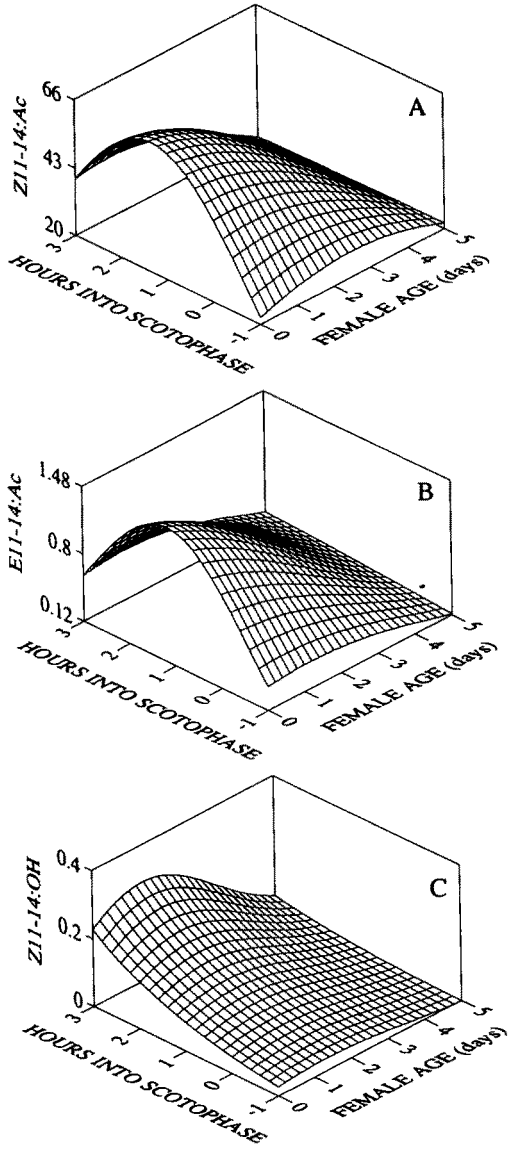

FIG. 1. Predicted values (ng) of Z11-14:Ac (A), *E*11-14:Ac (B), and Z11-14:OH (C) produced by different-aged *Choristoneura rosaceana* virgin females at different hours of the 16:8 hr light-dark photoperiod at 20°C constant temperature. 0 hr represents the onset of the scotophase.

TABLE 3. MEAN PROPORTIONS (LEAST SQUARE MEAN OF BACK-TRANSFORMED VALUES) OF *Z* AND *E*11-14:Ac AND *Z*11-14:OH PRODUCED BY DIFFERENT-AGED (0-, 3-, AND 5-DAY-OLD) *Choristoneura rosaceana* VIRGIN FEMALES AT DIFFERENT HOURS OF 16:8 HR LIGHT-DARK PHOTOPERIOD AT EITHER 20°C CONSTANT TEMPERATURE OR COOL THERMOCYCLE (0 HR REPRESENTS ONSET OF SCOTOPHASE)[a]

	*Z*11-14:Ac at day				*E*11-14:Ac at day				*Z*11-14:OH at day			
Time	0	3	5	$\bar{X}$ (time)	0	3	5	$\bar{X}$ (time)	0	3	5	$\bar{X}$ (time)
20°C constant temperature												
−1	0.971	0.983	0.994	0.985	0.016	0.007	0.003	0.007	0.002	0.002	0.000	0.001
0	0.974	0.979	0.995	0.984	0.017	0.013	0.003	0.009	0.001	0.002	0.000	0.001
1	0.971	0.980	0.992	0.982	0.023	0.010	0.004	0.100	0.002	0.003	0.001	0.002
2	0.957	0.978	0.992	0.979	0.028	0.011	0.003	0.100	0.006	0.004	0.001	0.003
3	0.963	0.983	0.983	0.978	0.019	0.007	0.006	0.100	0.010	0.003	0.002	0.005
$\bar{X}$ (age)	0.968	0.981	0.991		0.020	0.009	0.003		0.003	0.003	0.001	
Cool thermocycle												
−2	0.977	0.956	0.969	0.968	0.019	0.038	0.023	0.026	0.002	0.003	0.003	0.002
−1	0.972	0.955	0.951	0.960	0.024	0.038	0.044	0.034	0.003	0.002	0.001	0.002
0	0.957	0.966	0.939	0.955	0.029	0.030	0.050	0.035	0.005	0.002	0.004	0.003
$\bar{X}$ (age)	0.969	0.959	0.954		0.024	0.035	0.037		0.003	0.002	0.002	

[a]The transformed LS mean values of the standard errors of the proportions of *Z*11-14:Ac, *E*11-14:Ac and *Z*11-14:OH varied with age and time between 0.15–0.16, 0.29–0.31 and 0.25–0.27 at 20°C constant and 0.14–0.16, 0.16–0.21, and 0.30–0.41 under the cool thermocycle, respectively.

TABLE 4. MEAN SQUARE (MS) VALUES AND LEVEL OF SIGNIFICANCE (P) ASSOCIATED WITH EACH ORTHOGONAL CONTRAST USED TO TEST FOR DIFFERENCES IN PROPORTIONS OF Z AND E11–14:Ac AND Z11–14:OH PRODUCED BY *Choristoneura rosaceana* VIRGIN FEMALES UNDER 16:8 HR LIGHT–DARK PHOTOPERIOD AT 20°C CONSTANT TEMPERATURE OR UNDER COOL THERMOCYCLE IN RESPONSE TO AGE (A), TIME DURING PHOTOPERIOD (T) AND INTERACTION BETWEEN THE TWO (AT)

		Z11–14:Ac		E11–14:Ac		Z11–14:OH	
Source of variation	*df*	MS	*P*	MS	*P*	MS	*P*
20°C constant temperature							
Time	4	0.747	0.1702	0.910	0.7244	14.154	0.0001
Linear (T)	1	2.834	0.0139	2.418	0.2429	50.088	0.0001
Quadratic (T^2)	1	0.008	0.8924	1.060	0.4391	2.247	0.1945
Cubic (T^3)	1	0.127	0.6006	0.014	0.9301	3.906	0.0875
Quartic (T^4)	1	0.019	0.8398	0.030	0.8965	0.878	0.4169
Age	2	16.512	0.0001	59.868	0.0001	21.043	0.0001
Linear (A)	1	32.456	0.0001	117.017	0.0001	32.322	0.0001
Quadratic (A^2)	1	0.665	0.2313	3.115	0.1853	10.147	0.0061
Age * time	8	0.399	0.5487	1.488	0.5853	1.748	0.2353
AT	1	0.073	0.6914	0.163	0.7618	2.843	0.1447
AT^2	1	0.157	0.5607	2.286	0.2562	1.784	0.2475
AT^3	1	0.528	0.2862	1.933	0.2963	2.058	0.2143
AT^4	1	0.417	0.3430	0.850	0.4884	0.388	0.5892
A^2T	1	0.742	0.2062	1.542	0.3509	5.369	0.0454
A^2T^2	1	1.200	0.1083	2.600	0.2263	1.562	0.2792
A^2T^3	1	0.027	0.8105	0.122	0.7925	0.011	0.9293
A^2T^4	1	0.192	0.5196	2.628	0.2235	0.115	0.7684
Error IV	293	0.462		1.766		1.329	
Cool thermocycle							
Time	2	0.592	0.0950	0.950	0.0968	1.095	0.4880
Linear (T)	1	1.109	0.0360	1.492	0.0555	0.549	0.5487
Quadratic (T^2)	1	0.037	0.6976	0.298	0.3878	1.776	0.2818
Age	2	0.754	0.0509	1.642	0.0190	0.661	0.6477
Linear (A)	1	1.507	0.0150	3.152	0.0059	1.086	0.3993
Quadratic (A^2)	1	0.028	0.7349	0.322	0.3700	0.366	0.6244
Age * time	4	0.518	0.0852	0.770	0.1102	1.400	0.4539
AT	1	0.000	0.9968	0.105	0.6084	0.766	0.4789
AT^2	1	0.139	0.4534	0.221	0.4576	1.043	0.4089
A^2T	1	1.975	0.0056	2.690	0.0107	2.093	0.2428
A^2T^2	1	0.046	0.6661	0.008	0.8860	1.311	0.3546
Error IV	100	0.245		0.397		1.515	

$$\hat{Y}_{(\text{ratio of } Z11\text{-}14:\text{Ac})} = 1.01 - \exp(-4.2492 + 0.0699\,T - 0.1621\,A)$$

$$\hat{Y}_{(\text{ratio of } E11\text{-}14:\text{Ac})} = \exp(-3.7933 - 0.3078\,A) - 0.001$$

$$\hat{Y}_{(\text{ratio of } Z11\text{-}14:\text{OH})} = \exp(-11.0921 + 0.3484\,T + 2.7968\,A - 0.6105\,A^2 - 0.1648\,AT + 0.0340\,A^2T) - 0.001$$

and were used to produce the response surfaces shown in Figure 2A–C, respectively.

Under cold thermoperiodic conditions, females of the same age produced a similar titer of *Z*11–14:Ac over the 3-hr period (Table 1) but, overall, older individuals produced significantly less than younger ones (Table 2). This was similar to the trend observed at 20°C constant temperature. No significant interaction was found between age and time (Table 2), and the best model

$$\hat{Y}_{Z11\text{-}14:\text{Ac}} = (4.5498 + 0.1387\,A - 0.0729\,A^2)^{2.5}$$

was used to simulate the response surface (Figure 3). It is worth noting that, for any given age tested, the titers of *Z*11–14:Ac were quite comparable under both temperature regimes (Table 1, Figures 1A and 3).

The production of *E*11–14:Ac, as well as *Z*11–14:OH, under the cold thermocycle did not vary significantly with either age or time during the scotophase (Tables 1 and 2). The mean titer of *E*11–14:Ac was ~1 ng compared with 0.1 ng for the alcohol component.

There was considerable variation in the relative proportion of both acetate components produced by the different-aged OBL females over time due to significant interactions between factors (Tables 3 and 4) under the cool thermocycle. While there was no clear pattern for *Z*11–14:Ac, older females produced relatively more *E*11–14:Ac than younger ones. The best relationship between time and age on the ratios of both acetate components was described using the following polynomial equations

$$\hat{Y}_{(\text{ratio of } Z11\text{-}14:\text{Ac})} = 1.01 - \exp(-6.3025 + 0.2221\,T + 4.0339\,A - 0.8198\,A^2 - 0.2835\,AT + 0.0586\,A^2T)$$

$$\hat{Y}_{(\text{ratio of } E11\text{-}14:\text{Ac})} = \exp(-5.1545 + 0.1040\,T + 4.5671\,A - 0.9229\,A^2 - 0.3189\,AT + 0.0658\,A^2T) - 0.001$$

which served to generate the response surfaces of Figure 4.

However, contrary to the situation observed at 20°C, the ratio of *Z*11–14:OH did not vary with female age or time of day (Tables 3 and 4).

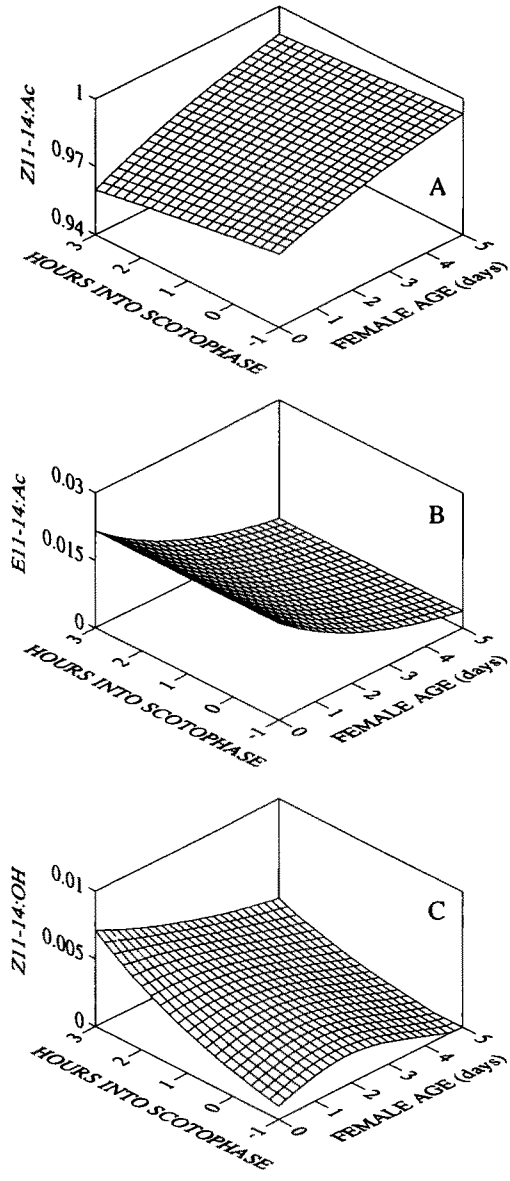

FIG. 2. Predicted values of the relative proportions of Z11-14:Ac (A), E11-14:Ac (B), and Z11-14:OH (C) of different-aged *Choristoneura rosaceana* virgin females at different hours of the 16:8 hr light-dark photoperiod at 20°C constant temperature. 0 hr represents the onset of the scotophase.

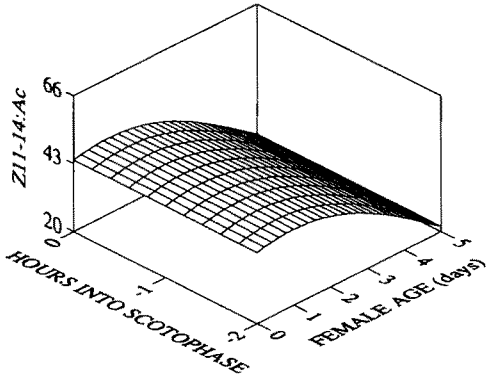

FIG. 3. Predicted values (ng) of Z11-14:Ac produced by different-aged *Choristoneura rosaceana* virgin females at different hours of the 16:8 hr light–dark photoperiod under a cool thermocycle. 0 hr represents the onset of the scotophase.

DISCUSSION

The present study demonstrates that younger OBL females produced more pheromone than older ones, both at warm and cold temperatures, thereby supporting the hypothesis that the greater attractiveness of day-0 and day-3 females compared with that of day-5 individuals during both flight periods (Delisle, 1992a) was probably associated with their higher pheromone content, if the amounts released are proportional to the amounts produced. This interpretation is further supported by the fact that the capture of OBL males in traps baited with the three-component pheromone blend (Z11-14:Ac, E11-14:Ac, Z11-14:OH) increased significantly with concentration, irrespective of the flight period (Delisle, 1992b). The decline in pheromone titer as females age has been linked with senescence associated with increased oviposition in *Helicoverpa zea* (Giebultowicz et al., 1990) and *Lymantria dispar* (Teal and Tumlinson, cited in Giebultowicz et al., 1990). However, in this study, senescence could not account for the decline observed in pheromone titer with age, as all ovipositing females, irrespective of age, were excluded from the experiment.

The relative composition of the OBL pheromone blend varied both in time and with age, although variation in the ratios of Z- and E11-14:Ac isomers was relatively small (less than 10%). These results fit within the generalization made by Ono et al. (1990) that species using geometrical isomers, such as *Argyrotaenia velutinana* (Miller and Roelofs, 1980) and *Pectinophora gossypiella* (Collins and Cardé, 1985), exhibit a narrow range of ratios compared with species such as *Phthorimaea operculella* (Ono et al., 1990), *Agrotis segetum* (Löfstedt et al., 1985), and *Ephestia cautella* (Barrer et al., 1987) that

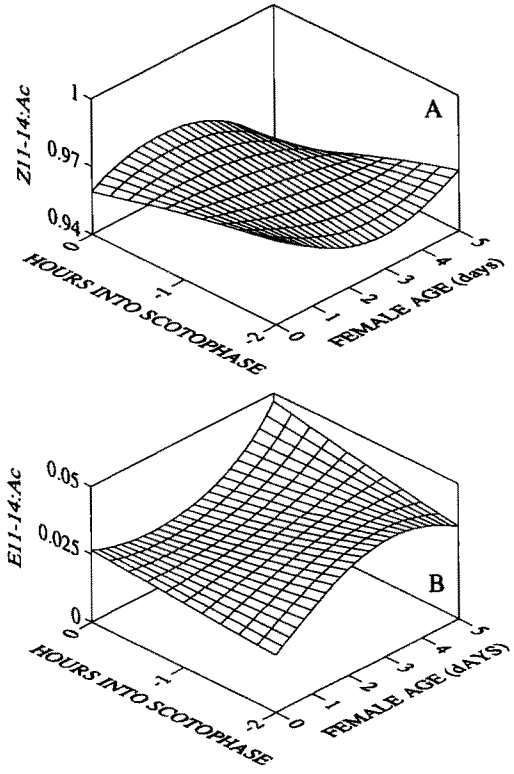

FIG. 4. Predicted values of the relative proportions of $Z11$–14 : Ac (A) and $E11$–14 : Ac (B) of different-aged *Choristoneura rosaceana* virgin females at different hours of the 16 : 8 hr light–dark photoperiod under a cool thermocycle. 0 hr represents the onset of the scotophase.

produce blends with very different compounds. The variation observed in the pheromone blend produced by OBL females fell within the range of variation that gave maximum male response in the field (Hill and Roelofs, 1979; Vakenti et al., 1988; Delisle, 1992b). This is not surprising as, in the field, it has been shown that males generally respond to a wider range of isomeric ratios (Flint et al., 1977; Baker et al., 1988) than those produced by females, and this may be accentuated by seasonal changes in temperature (Linn and Roelofs, 1988). However, the presence or absence of a pheromone component generally has a stronger influence on male attraction (Linn and Roelofs, 1983) than variation in blend ratios. In the case of OBL, it is clear that males responded preferentially to blends containing three pheromone components rather than the acetates only

(Delisle, 1992b). In this context, the lower amounts of Z11–14:OH in the pheromone blend of older females at 20°C, combined with the lower total pheromone titer, could reasonably explain their lower ability to attract males during the summer flight. However, under a cool thermocycle there were no differences in the amount or proportion of Z11–14:OH and thus does not support the idea that seasonal changes in the relative attractiveness of older females (Delisle, 1992a) could be attributed to the effects of age and temperature on pheromone production. Therefore, we believe our data support the original hypothesis that the relative increase in attractiveness of older females over younger ones observed in the fall (Delisle, 1992a) is due to the significant advance in the onset time of calling with age (Delisle, 1992a): by doing so, older females not only avoid competition with the more attractive younger females but also would be less affected by the limiting effect of low temperatures on male flight activity. On the other hand, when the mating periodicity of different-aged OBL females was examined under field conditions (Delisle, unpublished data), the onset of calling of older females coincided with peak foraging activity of a presently unidentified spider species that preyed on tethered females (Delisle, personal observation). This suggests there may be a cost associated with calling early and could explain why younger females do so later than older individuals, irrespective of temperature conditions (Delisle, 1992a).

This model probably holds true for other Lepidoptera, such as the omnivorous leafroller, *Platynota stultana*. All *P. stultana* virgin females call on the day following emergence and the onset time of calling occurs earlier on successive nights (Webster and Cardé, 1982). Furthermore, the progressive decline in the pheromone titer with age (Webster and Cardé, 1982) would explain the lower attractiveness of older females (day-5 and -6) compared with younger ones (day-2) under field conditions (AliNiazee and Stafford, 1971). However, whether the earlier onset of calling of less attractive older females enhances their degree of competitiveness, particularly in response to cool nights, remains to be tested. This seems likely as under cooler conditions calling is initiated earlier, especially in older females (Webster and Cardé, 1982). On the other hand, this scenario does not necessarily hold for all species where it has been shown that pheromone titer varies with age (Table 5). For example, in *Plusia chalcites*, even though older females initiate calling earlier than younger ones, pheromone production also increases with age (Snir et al., 1986).

The literature available on the diel patterns of pheromone production and calling in Lepidoptera (Table 6) indicates that most species (15 species of 25) show a close synchrony between the timing of calling and maximal pheromone production. Two different asynchronous patterns have been reported in the others: in six species, the peak of pheromone production occurs before the peak of calling, while in two noctuids, *Pseudaletia unipuncta* (Delisle and McNeil, 1987a) and *P. chalcites*, (Snir et al., 1986) the peak of pheromone production

TABLE 5. LEPIDOPTERAN SPECIES IN WHICH PHEROMONE TITER, DETERMINED BY GC ANALYSIS OR MALE RESPONSE, VARIED WITH FEMALE AGE[a]

Family	Age(s) of maximal production	Species	Reference
Arctiidae	4	*Holomelina lamae*	Schal et al. (1987)
Gelechiidae	1	*Phthorimaea operculella*	Ono et al. (1990)
Geometridae	3–4[b]	*Rheumaptera hastata*	Werner (1977)
Lymantriidae	4	*Lymantria dispar*	Giebultowicz et al. (1992),
	2–3		Tang et al. (1992)
	6–8[b]	*Orgyia pseudotsugata*	Swaby et al. (1987)
Noctuidae	7–20[b]	*Euxoa ochrogaster*	Struble and Jacobson (1970)
	5–8	*Heliothis peltigera*	Dunkelblum and Kehat (1992)
	3	*H. virescens*	Mbata and Ramaswamy (1990)
	2–3	*Helicoverpa zea*	Raina et al. (1986)
	7–9[b]	*Mamestra configurata*	Struble et al. (1975)
	4	*Plusia chalcites*	Snir et al. (1986)
	2	*Spodoptera littoralis*	Dunkelblum et al. (1987),
	2		Martinez and Camps (1988)
	2[b]	*S. frugiperda*	Sekul and Cox (1967)
	6	*Trichoplusia ni*	Bjostad et al. 1980
Pyralidae	3[b]	*Anagasta kühniella*	Traynier (1970)
	1–5	*Diaphania nitidalis*	Valles et al. 1992
	1–4	*Plodia interpunctella*	Sower and Fish (1975)
	2	*Sesamia nanogrioides*	Babilis and Mazomenos (1992)
Tortricidae	4–5	*Argyrotaenia velutinana*	Miller and Roelofs (1977)
	3–4	*Choristoneura fumiferana*	Sanders and Lucuick (1972),
	1–3		Morse et al. (1982),
	1–4		Grant et al. (1982)
	5[b]	*Epiphyas postvittana*	Lawrence and Bartell (1972)
	2	*Platynota stultana*	Webster and Cardé (1982)

[a]The age at which the maximal pheromone production occurred is also given.
[b]In these species, the age of maximal production was determined using male response.

occurs after the peak of calling. In a third noctuid, *H. zea*, the relationship is far from clear as conflicting data have been presented in two papers (Raina et al., 1986, 1991).

If one assumes that calling and pheromone biosynthesis are both circadian, as demonstrated experimentally for *P. unipuncta* (Delisle and McNeil, 1987a), then species that showed synchrony probably used the same *zeitgeber* for the two processes. However, in most species that show asynchrony (Table 6), calling is expressed relatively early in the scotophase, while the accumulation of either pheromone or the precursors occurs in the photophase. Thus, one could

TABLE 6. RELATIONSHIP BETWEEN TIMING OF CALLING AND PHEROMONE PRODUCTION DETERMINED BY GLAND EXTRACT (G) OR AIRBORNE COLLECTION (A) IN LEPIDOPTERA

Family	Pheromone quantification	Species	Period of calling	Reference
Peak of pheromone coincides with peak of calling				
Lymantriidae	A	*Lymantria dispar*	early scotophase	Charlton and Cardé (1982),
	G			Tang et al. (1992),
	G			Giebultowicz et al. (1992)
Noctuidae	G	*Heliothis peltigera*	late scotophase	Dunkelblum and Kehat (1992)
	G, A	*H. subflexa*	middle scotophase	Heath et al. (1991)
	G	*H. virescens*	middle-late scotophase	Heath et al. (1991),
	G, A[a]			Mistrot Pope et al. (1982),
	G			Mbata and Ramaswamy (1990, 1991)
	A	*Mamestra brassicae*	late scotophase	Bestmann et al. (1988),
				Subchev (1983)
	A	*Spodoptera sunea*	late scotophase	Bestmann et al. (1988)
	G	*S. frugiperda*	throughout scotophase	Ramaswamy et al. (1988)
Pterophoridae	A[a]	*Platyptilia carduidactyla*	middle scotophase	Haynes et al. (1983)
Pyralidae	G, A	*Diaphania nitidalis*	middle scotophase	Valles et al. (1992)
	G	*Ephestia cautella*	throughout scotophase	Coffelt et al. (1978),
	A			Barrer et al. (1987)
	G	*Plodia interpunctella*	early scotophase	Coffelt et al. (1978),
	A			Sower and Fish (1975)
	G	*Sesamia nanogrioides*	late scotophase	Babilis and Mazomenos (1992)
Tortricidae	A	*Cacoecimorpha pronubana*	throughout photophase	Witzgall and Frérot (1989)
	A	*Choristoneura funiferana*	early scotophase	Ramaswamy and Cardé (1984),
	G, A			Morse et al. (1982)
	A	*Chryptophlebia leucotreta*	late photophase	Bestmann et al. (1988)

TABLE 6. CONTINUED

Family	Pheromone quantification	Species	Period of calling	Reference
Peak of pheromone occurs before peak of calling				
Arctiidae	A, G	*Holomelina lamae*	late photophase	Schal et al. (1987)
Gelechiidae	G	*Phthorimaea operculella*	early scotophase	Ono et al. (1990)
Noctuidae	G	*Spodoptera littoralis*	early scotophase	Dunkelblum et al. (1987),
	G			Martinez and Camps (1988)
	G,A[a]	*Trichoplusia ni*	early scotophase	Hunt and Haynes (1990)
Pyralidae	G	*Conogethes punctiferalis*	middle scotophase	Konno (1986)
Tortricidae	G	*Adoxophyes* sp.	middle scotophase	Kou (1992)
	G	*Platynota stultana*	early scotophase	Webster and Cardé (1982)
Peak of pheromone occurs after peak of calling				
Noctuidae	G	*Plusia chalcites*	early scotophase	Snir et al. (1986)
	G	*Pseudaletia unipuncta*	late scotophase	Delisle and McNeil (1987a)
Conflicting results				
Noctuidae	G	*Helicoverpa zea*	early/middle scotophase	Raina et al. (1986, 1991)

[a]The release rate was determined from forcibly extruded glands.

hypothesize that the lack of synchrony is due to different cues being used for the two processes: lights-on for pheromone biosynthesis and lights-off for calling.

However, care must be taken when making generalizations based on available literature due to differences in the experimental approaches used to determine the pattern of pheromone production. For example, while the onset time of calling differs with age in most species examined (McNeil, 1991), the pheromone titer of different-aged females was usually measured at one specific hour of the scotophase and/or photophase within the calling window. Similarly, the diel changes in the pheromone titer are generally examined at a specific chronological age and only at constant temperatures. A comparison of the pattern of calling and pheromone production by different-aged OBL females at 20°C and under the cool thermocycle (Figure 5) demonstrates that these approaches may lead to somewhat biased estimates and thus to potentially erroneous conclusions. If the pheromone titers were determined at the time of peak calling activity at 20°C, one would conclude that day-0 and day-3 females produce similar amounts of pheromone (~45 ng), with day-5 females producing significantly less (~20 ng) than younger females. However, if the pheromone titers were determined at the mean onset time of calling at 20°C, then the conclusion would be that day-0 females (61 ng) produce significantly more pheromone than day-3 females (43.7 ng), with day-5 females still producing less than 30 ng. In

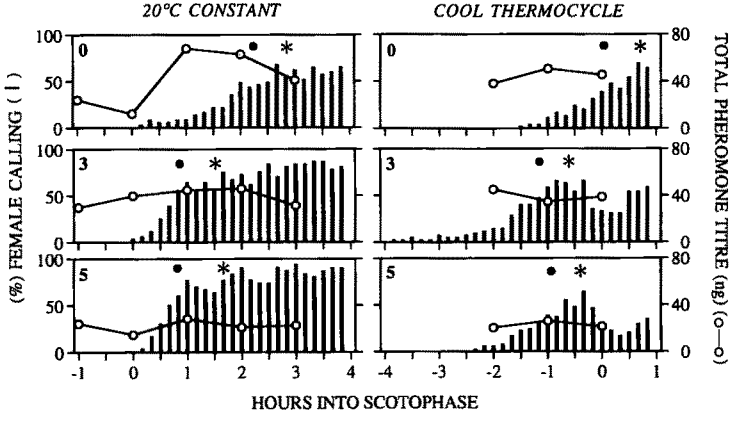

FIG. 5. The calling pattern (bars), and the mean total pheromone titer (circles) produced by 0-, 3-, and 5-day-old *Choristoneura rosaceana* virgin females at different hours of the 16:8 hr light–dark photoperiod at either 20°C constant temperature or a cool thermocycle. 0 hr represents the onset of the scotophase. The mean onset time of calling (solid circle) as well as the peak of call (*) are indicated for each age category and temperature regime. The proportion of females calling were obtained from Delisle (1992a).

contrast, under the cool thermocycle, estimates of the amount of pheromone produced by either 0- or 3-day-old females would be similar whether analyses were carried out at the mean onset time of calling or peak period of calling. As before, estimates of day-5 females would be less than those of younger ones at both times.

Furthermore, the age at which the patterns of calling and pheromone production is estimated could influence the conclusions drawn. At 20°C, the pheromone titer of day-0 OBL females reached its peak 1 hr prior to the onset of calling and dropped as soon as calling began, suggesting that the events are asynchronous, with the peak of pheromone preceding the peak of calling. In contrast, the pheromone patterns of day-3 and day-5 females showed that the peak of both calling and pheromone occurred at the same time, therefore leading to the conclusion that the two events are synchronous. The temperature regime at which the analyses are carried out could also affect the results, for as seen under the cold thermocycle, pheromone titers did not fluctuate considerably with age, regardless of whether analyses were made at the onset time of calling or at the peak calling time.

Using chronological rather than calling age (Turgeon and McNeil, 1982) is an additional factor that may result in errors when evaluating age and diel pheromone titers of species where females do not all initiate calling on the night following emergence. For example, in species such as the true armyworm (Turgeon and McNeil, 1982; Delisle and McNeil, 1986), the cotton bollworm (Kou and Chow, 1987), or the oriental armyworm (Han and Gatehouse, 1991), a population of 5-day-old females will have some individuals that have not yet started calling, while others are on their first, second, or third night of calling. Thus, any diel and/or age patterns obtained could be confounded if, as seen in this study of the OBL, there are changes in titer on successive days of calling. To make the point, let us assume that the OBL, rather than initiating calling on the night following emergence, did so at different ages. If we sampled a population of 5-day-old individuals that had equal numbers of females in their first, fourth and sixth night of calling (the first scotophase being day 0) then, using the data from Figure 5, we would obtain the pattern seen in Figure 6. While this would be an accurate population pattern, it would not give a clear diel pattern of females of a specific calling age. In fact, under both temperature regimes, the periodicity of calling, as well as pheromone production of the population (Figure 6), would be very close to the pattern expressed by females in their fourth night of calling (3-day-old) (Figure 5), but not for those in their first (0-day-old) or sixth night of calling (5-day-old) (Figure 5).

We would, therefore, recommend that future studies examining the synchrony of pheromone biosynthesis and calling take into account the importance of determining the temporal pattern of calling and pheromone biosynthesis as a function of calling age rather than chronological age, thus avoiding possibly

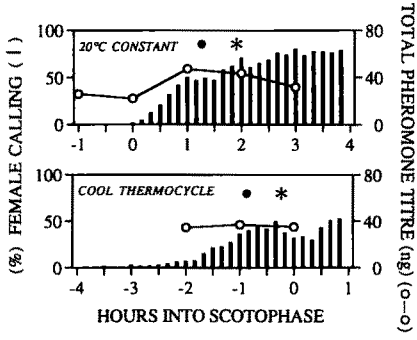

FIG. 6. The calling pattern (bars), and the mean total pheromone titer (circles) of a 5-day-old population of *Choristoneura rosaceana* virgin females that contained females in their first, fourth, and sixth night of calling under a 16:8 hr light–dark photoperiod at either 20°C constant temperature or a cool thermocycle. 0 hr represents the onset of the scotophase. The mean onset time of calling (solid circle) as well as the peak of calling (*) are indicated for each population under both temperature regimes. The proportion of females calling were obtained from Delisle (1992a).

incorrect estimates. This, we believe, is essential for valid interspecies comparisons, as well as for experiments examining the external cues governing the diel periodicity of calling and pheromone synthesis.

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FLIGHT-MEDIATED ATTRACTION OF *Biprorulus bibax* BREDDIN (HEMIPTERA: PENTATOMIDAE) TO NATURAL AND SYNTHETIC AGGREGATION PHEROMONE

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Abstract—The attraction of female spined citrus bugs, *Biprorulus bibax*, to natural and synthetic aggregation pheromone was studied using an olfactometer and a large flight cage. No locomotory response by postdiapause, prereproductive females to heptane extracts of male dorsal abdominal glands (DAGs) (site of pheromone production) was recorded in the olfactometer study. However, postdiapause, prereproductive females showed significant attraction to sites baited with DAG extracts in the flight cage (1.9–3.0 times that of unbaited sites). Prereproductive and reproductive females showed greatest attraction to sites baited with a synthetic blend of pheromone [(3*R*,4*S*,1′*E*)-3,4-bis(1′-butenyl)tetrahydro-2-furanol, linalool, farnesol, nerolidol] (2.3–4.7 times the attraction of unbaited sites). Females also responded significantly to sites baited with the hemiacetal major component alone (1.7–2.2×). Diapausing females collected from fall populations did not respond to natural or synthetic pheromone baits. Potential applications of the synthetic aggregation pheromone are discussed with respect to *B. bibax* management.

Key Words—Aggregation pheromone, *B. bibax*, Citrus, Hemiptera, Pentatomidae, dorsal abdominal gland, hemiacetal.

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INTRODUCTION

The spined citrus bug, *Biprorulus bibax* Breddin (Hemiptera: Pentatomidae), is a recent and increasing pest of commercial citrus in inland southeastern Australia (James, 1989). Damage to citrus crops, particularly lemons and mandarins, can be considerable and results in internal staining, gumming, and fruit drop (Hely et al., 1982). Recent research has focused on understanding the biology and ecology of this native species (e.g., James, 1990a–c, 1991; James and Warren, 1989; James et al., 1990) as a prelude to development of an integrated management strategy (James, 1992b).

James and Warren (1989) reported the presence of sexual dimorphism in the dorsal abdominal glands (DAGs) of *B. bibax* (males have enlarged glands while those of females are very small). This was considered analogous to the situation reported for certain predaceous pentatomids where similar dimorphism was related to pheromone production by glands in males (Aldrich et al., 1984). Aldrich and Oliver (unpublished) determined the chemical constituents of the DAG of male *B. bibax* to consist of a novel hemiacetal, linalool, two isomers of farnesol, and nerolidol. The hemiacetal and linalool are the major components and comprise 85–90% of male DAG extracts. DAGs from female *B. bibax* contain only linalool. Oliver et al. (1992) isolated the 12-carbon unsaturated hemiacetal and identified it as *cis*-3,4-bis[(*E*)-1′-butenyl]tetrahydro-2-furanol. Subsequently, Mori et al. (1992) established the absolute configuration of the compound, (3*S*,4*R*,1′*E*)-3,4-bis(1′-butenyl)tetrahydro-2-furanol and presented a synthesis. Absolute configuration of the compound was later revised to 3*R*,4*S*,1′*E* (Mori et al., 1993).

Pheromone produced in the enlarged male DAG of the predaceous pentatomid, *Podisus maculiventris* (Say) serves as a long-range attractant for conspecifics (Aldrich et al., 1984), and a synthetic version is now available commercially. The pheromone produced by male *B. bibax* may have a similar function, and a synthetic version could have potential as a monitoring or management tool for this pest.

This study attempts to evaluate attraction of *B. bibax* to both natural and synthetic pheromone, in a pedestrian olfactometer and in a flight cage, under various physiological conditions.

METHODS AND MATERIALS

Olfactometer Study. A plastic Y-tube olfactometer was used to determine the response of female *B. bibax* to male DAG/solvent extracts. Bugs could only walk in the olfactometer (10 mm diam., arms 22 cm long) and each arm ended in a rectangular plastic container (17 × 12 × 5 cm). Clean air (from outside the testing room) was passed through the containers via plastic tubing from an

air pump (85–100 ml/min). The holding chamber for bugs was also a plastic container with a muslin-covered rear hole through which the air current exited the system. A smoke test demonstrated a laminar airflow in both arms and in the base tube. Bugs walked upwind in the base tube, chose an arm, and ended up in the sample or control chamber. Tests were conducted in a constant environment room (27.5 ± 0.5°C, 50 ± 10% relative humidity, 13:11 hr light–dark). Lighting was provided by two 40-W fluorescent tubes located in front of the two arms to encourage bug movement. Each test was conducted over a period of five days and 8–15 female *B. bibax* were used per test. Male DAG extracts were prepared by excising the paired glands from five bugs under a stereomicroscope and placing them in a glass vial containing 1 ml of heptane. The glands were lightly crushed with a glass rod and plastic film pierced with a pin was used to cover the vial. A vial with heptane only was used in the control chamber. Two immature lemon fruit were placed in each of the three chambers as food. Data on bug movement to the chambers were recorded at approximately hourly intervals between 0800 and 1700 hr daily. At each recording period any bugs in choice chambers were removed and replaced in the holding chamber. Twelve tests were conducted over a 10-week period with different females used in each test. To compensate for possible minor asymmetry in the olfactometer or experimental conditions, DAG and control samples were alternated between arms between each test. After each test, the olfactometer was washed thoroughly in acetone and hot water. Initial tests using heptane only in both choice chambers demonstrated neutrality of the system to female *B. bibax*. All females used were postdiapause, prereproductive individuals collected from overwintering aggregations (James, 1989, 1990a, 1991) and held in the laboratory for 8–12 weeks under "winter" conditions (15 ± 0.5°C, 10:14 hr light–dark). The male-produced pheromone of *B. bibax* is thought to be a long-range attractant aimed primarily at bringing sexually immature individuals together for mating (James and Warren, 1989).

Flight Cage Studies. A large (17 × 6 × 3.6 m) shadecloth-covered, concrete floored structure was used as a flight cage for female *B. bibax*. Bugs made flight-mediated choices between sites baited with host fruit only or host fruit and pheromone. A bait site was located on each of nine or 12 evenly distributed tables (two rows of six) and consisted of a seed tray filled with immature lemon fruit and a jar of fresh lemon foliage (replaced every other day). A maximum of three pheromone treatments, each replicated three times, were conducted in each experiment. At the beginning of each experiment 50–80 female *B. bibax* were released in the center of the flight cage. Some mortality occurred but numbers of bugs were kept above 40 for the duration of each experiment by introducing additional bugs when necessary. Counts of bugs at each bait site were conducted three times daily (0800, 1200, 1600 hr), and treatments were

randomly reassigned to bait sites at the end of each day. Bugs at bait sites were removed and placed in the center of the flight cage.

One natural and two synthetic pheromone formulations were used in four experiments. Male DAG/heptane extracts were prepared as described previously although a shortage of male bugs meant that two pairs of DAGs per vial were used instead of five. The racemate of (3*R*,4*S*,1′*E*)-3,4-bis(1′-butenyl)tetrahydro-2-furanol was synthesized in Japan (Mori et al., 1992) and shipped to Australia. The other components of male *B. bibax* DAG secretion [linalool, farnesol (mixed isomers) and nerolidol] were obtained from Sigma-Aldrich Pty Ltd (Castle Hill, New South Wales). The synthetic blend was formulated (by volume) to approximate the composition of male DAG extracts and extracts of airborne volatiles from live males (hemiacetal 46%, linalool 40%, farnesol 13%, nerolidol 1%) (Aldrich, unpublished). The blend was mixed with 1 ml of heptane in a plastic film-capped glass vial. The plastic film was pierced with a pin, and vials were attached to the jars containing citrus foliage. Vials containing heptane only were used at control sites. A climate data-logger was used to record temperature and relative humidity during each experiment.

Experiment 1. This was conducted during November 19–24, 1992, using postdiapause, prereproductive females collected from overwintering aggregations and held in the laboratory for 8–12 weeks under "winter" conditions (15 ± 0.5°C, 10:14 hr light–dark). Nine tables were baited with DAGs, synthetic blend, or heptane only. The blend was a formulation of 16 mg of the racemate of (3*R*,4*S*,1′*E*)-3,4-bis(1′-butenyl)tetrahydro-2-furanol (38%), 16 mg of linalool (38%), 5 mg of farnesol (12%), and 5 mg of nerolidol (12%).

Experiment 2. This was conducted during December 5–11, 1992, using prereproductive, postdiapause females as in experiment 1. Twelve tables were baited with DAGs, synthetic blend, hemiacetal only, or heptane only. Blend formulation was as in experiment 1, 16 mg of the racemate of (3*R*,4*S*,1′*E*)-3,4-bis(1′-butenyl)tetrahydro-2-furanol and 1 ml of heptane comprised the hemiacetal treatment.

Experiment 3. This was conducted during March 4–15, 1993, using reproductive females collected from summer breeding populations in local lemon groves. Twelve tables were baited with DAGs, synthetic blend, hemiacetal only, or heptane only. A blend formulation of 29 mg of the racemate of (3*R*,4*S*,1′*E*)-3,4-bis(1′-butenyl)tetrahydro-2-furanol (46%), 25 mg of linalool (40%), 7 mg of farnesol (13%), and 1 mg of nerolidol (1%) was used, 29mg of the hemiacetal in 1 ml of heptane comprised the hemiacetal treatment.

Experiment 4. This was conducted during April 8–12, 1993, using females collected from lemon groves during the first week of April. At this time (fall) *B. bibax* enters reproductive diapause (James et al., 1990; James, 1991) and forms overwintering aggregations on nonlemon citrus (James, 1990 a,b). Dissection of a sample of 10 females showed all to have undeveloped ovaries.

Twelve tables were baited with DAGs, synthetic blend, hemiacetal only, or heptane only. The same formulations were used as in experiment 3.

Data were analyzed by chi-square, analysis of variance (ANOVA), and LSD procedures, where appropriate.

RESULTS

Olfactometer Study. No significant attraction to DAG/heptane extracts was demonstrated. During 12 tests, 501 individual bug movements were recorded, 258 (51.5%) movements were made to the chambers containing DAG/heptane extract and 243 (48.5%) to chambers containing heptane only ($\chi^2 = 0.44$, $P > 0.5$).

Flight Cage Studies. Female *B. bibax* released in the flight cage demonstrated significantly greater attraction to pheromone baited sites than unbaited sites in experiments 1–3 but not in experiment 4 (Table 1).

Experiment 1. Flight activity of prereproductive, postdiapause females released in this experiment was considerable. Bugs spent much time "patrolling" the cage, avoiding landings on the walls and roof. Flights of 1–4 min were frequently observed. More than three times as many bugs were recorded from synthetic blend-baited sites than from unbaited sites (Table 1). Almost twice as many bugs were recorded from DAG-baited sites than unbaited sites. Synthetic blend-baited sites attracted 1.6 times as many bugs as DAG-baited sites. All differences were significant ($P < 0.05$). A combined sites daily mean of 28 bugs was recorded. Temperatures during the experiment ranged from 13 to 29°C with daily mean maxima and minima of 28.5°C and 14.2°C, respectively.

Experiment 2. Flight activity of bugs was again considerable with flights of 1–4 min frequently observed. The synthetic blend sites attracted 4.7 times as many bugs as unbaited sites (Table 1). Three times as many bugs were recorded from DAG-baited sites as from unbaited sites ($P < 0.05$). Sites baited with hemiacetal only recorded a similar number of bugs to the DAG sites (NS, $P > 0.05$) and both treatments were significantly less attractive than the synthetic blend. A combined sites daily mean of 14 bugs was recorded. Temperatures during the experiment ranged from 14 to 35°C with daily mean maxima and minima of 30.3°C and 15.4°C, respectively.

Experiment 3. Reproductive females were less flight active than the prereproductive females used in experiments 1 and 2. All observed flights were of short (< 1 min) duration. Sites baited with synthetic blend or hemiacetal attracted 2.3 and 1.7 times as many bugs as unbaited sites, respectively ($P < 0.05$) (Table 1). DAG-baited sites were 1.5 times as attractive as unbaited sites, but this was weakly nonsignificant ($P > 0.05$). A combined daily mean of 26 bugs was recorded. Temperatures during the experiment ranged from 10 to 32°C with daily mean maxima and minima of 29.4°C and 14°C, respectively.

TABLE 1. NUMBERS OF FEMALE *B. bibax* ATTRACTED TO PHEROMONE-BAITED SITES IN FLIGHT CAGE EXPERIMENTS[a]

	Synthetic blend		DAG/heptane extract		Synthetic hermiacetal		Unbaited	
Experiment	Total (all sites)	Daily mean per site (± SE)	Total (all sites)	Daily mean per site ± SE	Total (all sites)	Daily mean per site ± SE	Total (all sites)	Daily Mean per site ± SE
Prereproductive, postdiapause females								
1	82	4.8 ± 0.8 a	52	2.9 ± 0.5 b	—	—	27	1.5 ± 0.4 c
2	40	1.9 ± 0.3 a	26	1.2 ± 0.3 b	20	0.9 ± 0.3 b	9	0.4 ± 0.1 c
Reproductive females								
3	110	3.0 ± 0.4 a	76	1.9 ± 0.3 bc	77	2.2 ± 0.3 b	46	1.3 ± 0.2 c
Prereproductive, diapausing females								
4	14	0.9 ± 0.2 a	11	0.7 ± 0.4 a	9	0.7 ± 0.1 a	6	0.5 ± 0.1 a

[a] Values with different letters (within experiments) are significantly different ($P < 0.05$).

Experiment 4. Flight activity of diapausing females used in this experiment was very limited with no flights of > 1 min observed. There was no significant difference in numbers of bugs recorded at blend, hemiacetal, DAG, or unbaited sites ($P > 0.05$) (Table 1). Low numbers of bugs were recorded at the sites (combined sites daily mean = 8) and most individuals remained on the roof or walls of the flight cage. Temperatures during the experiment ranged from 5 to 31°C with daily mean maxima and minima of 26.8°C and 10.2°C, respectively.

DISCUSSION

Males of other pentatomids, both predatory and phytophagous, produce long-range attractant pheromones (Aldrich, 1988; Aldrich et al., 1984, 1991). The similarity in glandular morphology of *B. bibax* to the well-researched North American predatory pentatomid, *Podisus maculiventris* (Say), led us to consider the possibility that the two species have a similar chemical communication system. Male *P. maculiventris* have enlarged DAGs from which they release an attractant pheromone consisting of a mixture of (*E*)-2-hexenal, α-terpineol, linalool, terpinen-4-ol, and benzyl alcohol. Both sexes are attracted to natural or synthetic versions of this volatile blend and at least four parasitoids use the pheromone as a kairomone (Aldrich et al., 1984). This study demonstrates that the secretion from enlarged DAGs in male *B. bibax* is also a pheromone attractant to conspecific females in flight. This is the first example of an Australian pentatomid with glands exclusively devoted to the production and storage of a pheromone. It is also the first instance of a phytophagous pentatomid using enlarged DAGs to produce attractant pheromone.

Data from the flight cage studies are convincing evidence of the attraction of postdiapause, prereproductive and reproductive *B. bibax* females to natural and synthetic versions of the pheromone; however, data from the laboratory olfactometer study did not reveal this situation. Airflow olfactometers are commonly used to measure responses of insects to infochemicals (e.g., Akinlosotu, 1978; Read et al., 1970; Rotheray, 1981; Sabelis and van de Baan, 1983; Shahjahan, 1974; Vet et al., 1983). In most published accounts, test insects demonstrate clear preferences for the chamber containing the attractive material, but olfactometers usually only allow subjects to respond by walking, which is clearly inappropriate for insects which orient to infochemicals after initiation of flight. Long-range attractant pheromones by definition, must act on flying insects, and it is therefore logical to expect *B. bibax* to respond best to such a pheromone in a flight cage situation. A similar case was reported for the braconid parasitoid *Cotesia* (= *Apanteles*) *glomerata* (L.), which responded to volatile infochemicals in a glasshouse flight chamber but showed no response in an olfactometer (Steinberg et al., 1992). There are other factors, of course, such as natural

daylight and air turbulence that enable flight cage systems to more closely simulate natural field situations and therefore provide more reliable bioassays of attraction.

Although all experiments in this study used female *B. bibax* as test subjects, it is quite likely that male *B. bibax* also respond to the pheromone, as is the case with *P. maculiventris* (Aldrich et al., 1984). Mating leks have been observed in *B. bibax* (James, 1989; James and Warren, 1989) and may be mediated by the male DAG-produced pheromone. Reproductive and postdiapause, prereproductive females responded to pheromone in the flight cage but responses appeared to be greater in the latter. Postdiapause, prereproductive females clearly displayed greater flight activity than reproductive females. Increased flight activity is often characteristic of sexually immature insects (Johnson, 1969), and it is this young adult phase that may be most sensitive to male-produced aggregation pheromone in *B. bibax*. In the ancestral habitat of *B. bibax* (western areas of New South Wales and Queensland) (James, 1992a) host plants [*Eremocitrus glauca* (Lindl. Swing)] occur in isolated patches, often separated by many kilometers. The value of effective long-range chemical communication to *B. bibax* in such a habitat is clear.

In contrast, female *B. bibax* collected from fall populations in the early stages of reproductive diapause showed no response to natural or synthetic aggregation pheromone. Overwintering populations of *B. bibax* in inland southeastern Australia are nonreproductive from April to September (James, 1990b; James et al., 1990), and DAGs in winter males are approximately 80% smaller than in reproductive males (James and Warren, 1989). The unresponsiveness of diapausing females to aggregation pheromone supports the idea that male DAG secretion in *B. bibax* primarily serves to bring the sexes together for mating. From these results, the characteristic aggregative behavior of overwintering *B. bibax* (James, 1990a) appears to be mediated by other male- and/or female-produced pheromones, or possibly by other sensory stimuli.

The synthetic blend of the natural pheromone was the most attractive bait for responsive female *B. bibax*, being 1.6–1.7 times as attractive as DAG extracts. The major component of the pheromone [the racemate of (3*R*,4*S*,1′*E*)-3,4-bis(1′-butenyl)tetrahydro-2-furanol] when used on its own was less attractive than the blend and similar in attraction to DAG extracts.

A synthetic blend of the aggregation pheromone of *B. bibax* has potential as a management or monitoring tool for this pest of Australian citrus. Pheromone-baited traps could provide a reliable and timely indication of bug populations in citrus groves. This would substantially improve integrated management of *B. bibax*, which currently relies on time-consuming and sometimes unreliable tree searches (James, 1992b). Alternatively, a trapping system using pheromones may have sufficient impact on *B. bibax* populations to be a management tool in its own right.

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PRIOR EXPOSURE TO CARBARYL ALTERS BEHAVIOR OF *Tetranychus urticae* KOCH ON ACARICIDE-TREATED LEAF SURFACES

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Abstract—Preadult exposure to carbaryl affected the subsequent behavior of two-spotted spider mites (*Tetranychus urticae* Koch) on leaf surfaces with discontinuous acaricide residues. In dicofol bioassays, preexposure to carbaryl caused a loss of avoidance behavior (eliminating the tendency of mites to feed and stand longer off treated areas). In amitraz bioassays, preexposure to carbaryl had the opposite effect. It increased the propensity of mites to feed and stand off amitraz-treated areas, resulting in increased avoidance of amitraz. Carbaryl preexposure therefore resulted in diametrically opposed behavioral changes in subsequent encounters with two acaricides. These effects provide additional evidence of the unpredictable nature of interactions between pesticides and show how a chemical, irrespective of degree of toxicity, can alter the behavior of arthropods in response to subsequent chemical encounters.

Key Words—*Tetranychus urticae*, Acarina, Tetranychidae, amitraz, dicofol, carbaryl, discontinuous residues, behavior, pesticide efficacy.

INTRODUCTION

When organic pesticides were first used on a broad scale in agriculture, researchers noted the occurrence of severe outbreaks of some pests following applications in certain crops (Ripper, 1956; van den Bosch and Stern, 1962). Outbreaks were of two distinctly different kinds: (1) those resulting from rapid resurgences of the pests originally targeted by pesticide treatments, and (2) severe build-up of

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so-called secondary or occasional pests known previously to cause crop damage only in certain years or within limited areas (Luck et al., 1977; Newsom et al., 1976; Metcalf, 1986). Much research has been done to characterize the mechanisms of chemically induced outbreaks of pests (e.g., see Metcalf, 1980; Waage et al., 1985). In many cases, the elimination of natural enemies has been implicated as the key determinant of outbreaks (Root and Skelsey, 1969; Croft and Brown, 1975; Waage et al., 1985). Yet, it was found that disruption of biological control could not account fully for the phenomenon, because chemically induced pest outbreaks were found to occur in some instances where natural enemies were not a factor. Studies of this nature extended the conceptual framework of pest–pesticide–natural enemy interactions to include an appreciation of the potential stimulatory action of pesticides on pest physiology (hormoligosis; see Luckey, 1968), sublethal effects on natural enemies (e.g., Croft and Brown, 1975) and effects of pesticides on plant physiology (e.g., Jones et al., 1986; Youngman et al., 1989).

Carbaryl is used widely in agriculture, owing to its safety to mammals and its relatively broad spectrum of insecticidal activity. There are numerous published reports of carbaryl-induced outbreaks of arthropods (e.g., Bentley et al., 1987; Flaherty and Huffaker, 1970; Inoue et al., 1986; Messing et al., 1988; Pike and Allison, 1987; Sandhu et al., 1987). Many such outbreaks have been attributed to the greater toxicity of carbaryl to beneficial organisms, relative to pests. However, additional factors have been shown to be involved in some arthropod outbreaks induced by carbaryl. For example, Flaherty and Huffaker (1970) showed that resurgences of *Tetranychus pacificus* occurred following applications of carbaryl to central California vineyards, even in the absence of the principle predator, *Metaseiulus occidentalis*. Similarly, Messing et al. (1988) concluded that natural enemy mortality was not the only factor leading to carbaryl-induced resurgences of the filbert aphid, *Myzocallis coryli* (Goeze) and showed that aphids exposed to carbaryl residues produced significantly greater numbers of offspring than did untreated aphids.

In this paper we expand the documentation of nonlethal effects of pesticides on pest biology by showing how prior exposure to carbaryl altered the behavior of mites on leaf surfaces treated with acaricides. These findings provide additional evidence of the unpredictable nature of interactions between pesticides commonly used in agriculture and illustrate, at the level of pest behavior, the point that potentially any chemical encountered by pests, irrespective of degree of toxicity, could alter significantly the response of arthropods to subsequent chemical encounters.

METHODS AND MATERIALS

Preparation of Spider Mite Cultures. Two-spotted spider mites (*Tetranychus urticae*) were raised on Cranberry Bean seedlings (*Phaseolus vulgaris* L.) at 26 ± 3°C, 70 ± 10% relative humidity, and a 16-hr photoperiod. Cultures

were isolated in cages as described by Dennehy and Granett (1984). Dicofol-susceptible mites [homozygous susceptible (SS) for the dicofol-resistance gene] were from a laboratory culture originally collected from a New York State Agricultural Experiment Station apple orchard (Orchard 12) located in Ontario County during 1985 (Rizzieri et al., 1988).

In all treatments, females were removed singly from the cultures, during the quiescent phase following the deutonymph stage. This period is characterized by immobile females that are guarded by males. The females were placed individually within a Tanglefoot ring (The Tanglefoot Company, Cedar Rapids, Michigan) on the upperside of Cranberry Bean leaves, and 48 hr later, observations of newly emerged adult females were made.

Observation leaves were treated with either dicofol (100 ppm) or amitraz (360 ppm) solutions. Dicofol solutions were prepared from Kelthane EC (18.5%, Rohm and Haas, Philadelphia, Pennsylvania). Amitraz solutions were prepared from Ovasyn (19.8%, NOR-AM Chemical Company, Wilmington, Delaware). For the behavioral bioassays described herein, concentrations of dicofol and amitraz were chosen based on prior studies of the log-concentration/probit response of *T. urticae* to dicofol and amitraz (Dennehy et al., 1988, 1993). The concentrations selected, 100 ppm dicofol and 360 ppm amitraz, caused approximately 70% mortality of susceptible mites in 72-hr residual bioassays (continuous residues).

Preparation of Discontinuous Residues. In agricultural settings, pesticide residues typically are deposited unevenly. To model the implication of this in a behavioral assay, we followed a procedure where either dicofol (100 ppm) or amitraz (360 ppm) solutions were sprayed on leaves as discontinuous checkerboards. A wire grid, having 0.7-cm squares alternately filled with wax, was clamped to the underside of an observation leaf. The leaf underside was used because *T. urticae* are more likely to walk off the top of the leaf (Kolmes et al., 1990). This assembly was then placed in a Potter Precision Laboratory spray tower (Burkhard, Rickmansworth, Herts., UK), and it was sprayed with 2.0 ml of newly prepared acaricide solution. After this treatment, the grid was removed, and the residue was allowed to dry for 24 hr. Treated squares were identified by placing a small dot of red ink in their centers.

Experimental Assay. The assay comprised 1 hr of observation of mite behavior on the 24-hr-old discontinuous residue. To construct the observation arenas, 4 × 4 square areas of leaves were cut out and situated bottom side upwards on moist cotton bedding. Female mites were placed individually, one per leaf, on these treated areas. Following an acclimation period of 5 min, behavior patterns were recorded for 1 hr for each mite.

Behavioral observations of mites were recorded, using a TRS-102 laptop computer (used as a real-time event recorder), a Volpi Interlux 5000 fiberoptic cool light source, and a Wild M-3Z dissecting microscope. Fourteen mites were

studied for each of the four experimental treatments listed below. Each treatment group required five or six days of behavioral observation. The dates of this work were spread out between June 19, 1992 and July 29, 1992, and dates were alternated between treatment groups rather than having the observations for any single treatment group occur as a block. A total of 5293 events were recorded, and their durations were measured. One observer (J.A.D.) collected all of the behavioral data.

Behavioral Observations and Analysis. Three behavior patterns—walking, standing and feeding—were recorded. Walking was defined as mite movement across the leaf-surface. Standing was defined as a period of mite immobility. Feeding was defined by the following actions: the pumping action of the mouth parts, small postural movements, and the body being held head downwards at an angle to the leaf surface. Frequencies and durations of each behavior were analyzed using chi-square goodness-of-fit tests and Wilcoxon-Mann-Whitney tests, respectively. Analysis of behavioral data using combined values for each treatment group produces a conservative set of analyses. Individual variability among mites could make it more difficult to detect treatment group differences, but differences detected between treatment groups will be highly robust. The use of nonparametric statistics is appropriate for this sort of behavioral data, which do not resemble a normal distribution (Siegel and Castellan, 1988).

Four Treatment Groups Evaluated. The four treatment groups consisted of: female mites, reared on bean plants sprayed with 600 ppm carbaryl, placed on either dicofol or amitraz; and female mites, reared on bean plants without carbaryl, placed on either dicofol or amitraz.

RESULTS

Effect of Dicofol on Mites Reared on Carbaryl-Free Plants. Walking bout frequencies on and off residue squares (Figure 1) did not differ significantly, according to a χ^2 goodness-of-fit test ($\chi^2 = 2.98$, $df = 1$, $P > 0.05$). Walking bout durations were significantly longer off residue squares than on residue squares, according to a Wilcoxon-Mann-Whitney test ($U = 844374$, $P = 0.033$) (Figure 1), although the size of the difference was small.

Feeding bout frequencies on residue squares were significantly lower than those observed off residue squares ($\chi^2 = 42.26$, $df = 1$, $P < 0.005$) (Figure 1). Feeding bout durations on and off residue squares were not significantly different ($U = 5806.5$, $P = 0.056$) (Figure 1). Feeding bout durations off residue squares tended to be longer, although this was not statistically significant at the 0.05 probability level.

Standing bout frequencies on residue squares were significantly lower than those off residue squares ($\chi^2 = 26.14$, $df = 1$, $P < 0.005$) (Figure 1). Standing

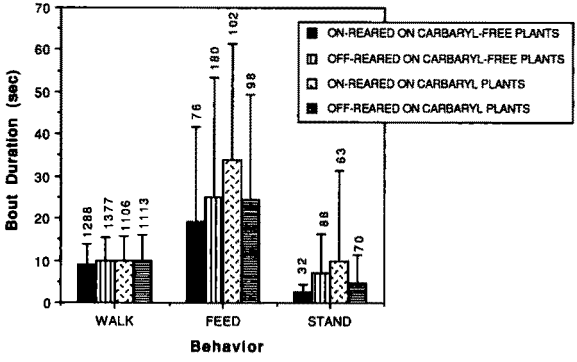

FIG. 1. Behavior on discontinuous dicofol residues of *Tetranychus urticae* reared on and off plants sprayed with carbaryl. Walking, feeding, and standing bout durations are expressed as median values. Error bars represent the semiinterquartile range of the data. Values above error bars represent the walking, feeding, and standing bout frequencies.

bout durations were significantly longer off residue squares than on residue squares ($U = 1017.5$, $P = 0.021$) (Figure 1).

Effect of Dicofol on Mites Reared on Plants Sprayed with Carbaryl. Walking bout frequencies on and off residue squares did not differ significantly, according to a χ^2 goodness-of-fit test ($\chi^2 = 0.022$, $df = 1$, $P > 0.05$) (Figure 1). Walking bout durations on and off residue squares were indistinguishable, according to a Wilcoxon-Mann-Whitney test ($U = 590081$, $P = 0.092$) (Figure 1).

Feeding bout frequencies on and off residue squares were not statistically different ($\chi^2 = 0.080$, $df = 1$, $P > 0.05$) (Figure 1). Feeding bout durations on and off residue squares also did not differ significantly ($U = 4771.5$, $P = 0.580$) (Figure 1).

Standing bout frequencies on and off residue squares were not significantly different ($\chi^2 = 0.368$, $df = 1$, $P > 0.05$) (Figure 1). Standing bout durations on and off residue squares were not significantly different ($U = 1780$, $P = 0.056$) (Figure 1). Standing bout durations on residue squares tended to be longer, although this was not statistically significant at the 0.05 probability level.

Effect of Amitraz on Mites Reared on Carbaryl-Free Plants. Walking bout frequencies on and off residue squares did not differ significantly, according to a χ^2 goodness-of-fit test ($\chi^2 = 2.16$, $df = 1$, $P > 0.05$) (Figure 2). Walking bout durations on and off residue squares also did not differ significantly, according to a Wilcoxon-Mann-Whitney test ($U = 1032837.5$, $P = 0.4529$) (Figure 2).

Feeding bout frequencies were significantly greater off residue squares than on residue squares ($\chi^2 = 22.70$, $df = 1$, $P < 0.005$) (Figure 2). Feeding bout

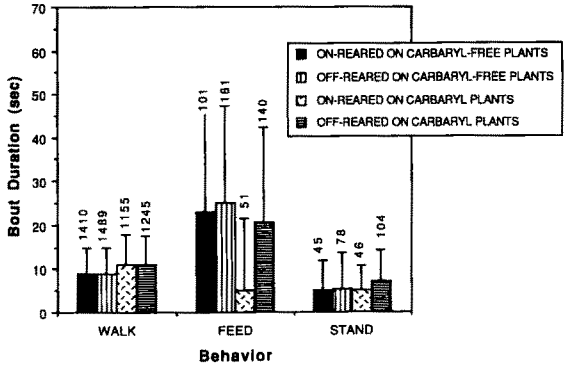

FIG. 2. Behavior on discontinuous amitraz residues of *Tetranychus urticae* reared on and off plants sprayed with carbaryl. Walking, feeding, and standing bout durations are expressed as median values. Error bars represent the semiinterquartile range of the data. Values above error bars represent the walking, feeding, and standing bout frequencies.

durations on and off residue squares were not significantly different ($U = 8714.5$, $P = 0.5165$) (Figure 2).

Standing bout frequencies were significantly greater off residue squares than those on residue squares ($\chi^2 = 8.86$, $df = 1$, $P < 0.05$) (Figure 2). Standing bout durations on and off residue squares were not significantly different ($U = 1628.5$, $P = 0.5065$) (Figure 2).

Effect of Amitraz on Mites Reared on Plants Sprayed with Carbaryl. Walking bout frequencies on and off residue squares were not significantly different, according to a χ^2 goodness-of-fit test ($\chi^2 = 3.38$, $df = 1$, $P > 0.05$) (Figure 2). Walking bout durations on and off residue squares were also statistically insignificant, according to a Wilcoxon-Mann-Whitney test ($U = 705571.5$, $P = 0.429$) (Figure 2).

Feeding bout frequencies were significantly greater off residue squares than those on residue squares ($\chi^2 = 41.48$, $df = 1$, $P < 0.005$) (Figure 2). Feeding bout durations were greater in length off residue squares than on residue squares ($U = 2590.5$, $P = 0.0038$) (Figure 2).

Standing bout frequencies were significantly lower on than off residue squares ($\chi^2 = 22.42$, $df = 1$, $P < 0.005$) (Figure 2). Standing bout durations on and off residue squares were not significantly different ($U = 2149.5$, $P = 0.323$) (Figure 2).

DISCUSSION

Our results demonstrate that preexposure to carbaryl significantly changed the behavior of spider mites on leaf surfaces treated with acaricide. The full impact of this chemically induced alteration of the spider mite–acaricide inter-

face is most readily visualized by computing from the values given in Figures 1 and 2, the products of bout frequencies and bout lengths, in order to compare for each group the total time that mites resided on versus off residue, when feeding and standing (Figures 3 and 4). Preexposure to carbaryl eliminated the tendency of mites to feed and stand longer off dicofol residues (Figure 3). In other words, carbaryl resulted in a loss of spider mite avoidance of dicofol-treated areas. However, the opposite effect of preexposure to carbaryl was observed with amitraz. Mites exposed to amitraz spent less total time feeding and standing on the residue if they were preexposed to carbaryl (Figure 4). This suggested that carbaryl somehow intensified behaviors resulting in avoidance of amitraz.

Earlier studies demonstrated that, given the opportunity, spider mites avoided dicofol residues and that the mechanism of this avoidance in susceptible *T. urticae* involved feeding deterrence caused by dicofol. This avoidance behavior was lost by mites resistant to dicofol (Kolmes et al., 1990). In the present study, we have recorded a remarkable impact of carbaryl on the amount of time mites spent feeding and standing on acaricide residues (Figures 3 and 4). Although we cannot predict the impact on field efficacy resulting from such carbaryl-mediated decreases (for dicofol) or increases (for amitraz) in avoidance of residues, studies are currently underway to correlate these behavioral observations with efficacy evaluations.

Resistant spider mites reared on dicofol-treated leaves exhibited a prefer-

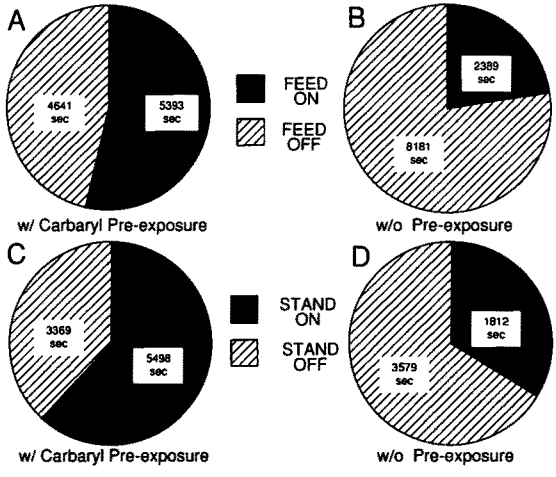

FIG. 3. Impact of preexposure to carbaryl on the total time that mites fed and stood on dicofol residues. Feeding and standing behaviors are expressed as total summed durations.

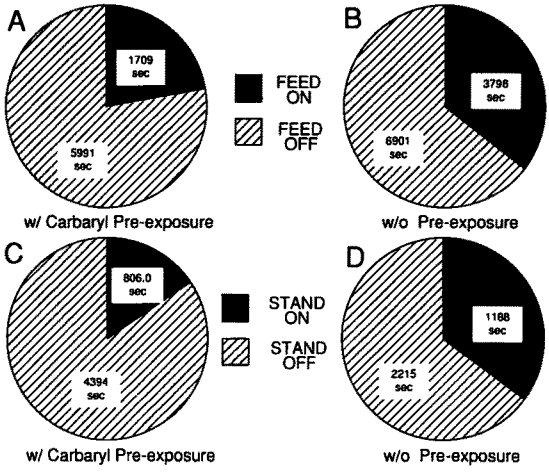

FIG. 4. Impact of preexposure to carbaryl on the total time that mites fed and stood on amitraz residues. Feeding and standing behaviors are expressed as total summed durations.

ence for feeding on dicofol-treated leaves relative to untreated leaves (Kolmes et al., 1992). Similarly, mites preexposed to carbaryl exhibited a feeding preference for dicofol-treated areas on discontinuous dicofol residues (Figure 3).

The results described herein extend our understanding of how dramatically pest behavior can change following exposure to pesticides. From our results, it is quite plausible to hypothesize that some carbaryl-induced pest outbreaks have been promoted by increased residue avoidance of pests. Our findings provide additional evidence of the unpredictable nature of interactions between pesticides commonly used in agriculture and illustrate, at the level of pest behavior, the point that potentially any chemical encountered by pests, irrespective of degree of toxicity, could alter significantly the response of arthropods to subsequent chemical encounters.

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PHEROMONE DIFFERENCES BETWEEN SIBLING TAXA *Diachrysia chrysitis* (LINNAEUS, 1758) AND *D. tutti* (KOSTROWICKI, 1961) (LEPIDOPTERA: NOCTUIDAE)

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Abstract—The noctuid sibling taxa *Diachrysia chrysitis* s. str. and *D. tutti*, of yet uncertain taxonomic status, have previously been shown to possess differences in morphology and to be attracted to different mixtures of the two presumed pheromone components (Z)-5-decenyl acetate and (Z)-7-decenyl acetate. Typical *D. tutti* males (clearly broken forewing marking) are known to respond to a 2 : 100 mixture of the two isomers, whereas *D. chrysitis* males (unbroken marking) are attracted to a 100 : 10 mixture. We investigated female pheromone production and male electroantennographic (EAG) response in *Diachrysia* families raised in the laboratory from field-collected gravid females. Extracts of individual females from typical *D. tutti* and *D. chrysitis* families were subjected to gas chromatography with simultaneous flame ionization and electroantennographic detection. All females produced mixtures of Z5– and Z7–10 : OAc, but female *D. chrysitis* produced predominantly Z5–10 : OAc

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and the antennae of their brothers responded more strongly to the Z5 peak than to the Z7-10:OAc peak, whereas the opposite was true for *D. tutti* families. The pheromone components were shown to be biosynthesized from hexadecanoic and tetradecanoic acid, respectively by Z11-desaturation followed by chain shortening, reduction, and acetylation. The EAG responses of males trapped with the typical *D. tutti* and *D. chrysitis* blends, as well as with an intermediate blend, were investigated. Males trapped with the *D. tutti* mixture almost exclusively had a clearly broken wing marking and showed strongest EAG response to Z7-10:OAc. The intermediate blend and the *D. chrysitis* mixture gave more mixed catches, but with a prevalence of males with an unbroken (or almost unbroken) wing marking and with a higher mean response to Z5-10:OAc. Some males with typical *D. tutti* EAG responses were attracted in the field to the *D. chrysitis* pheromone. In the flight tunnel some *D. chrysitis* males were attracted also to the *D. tutti* mixture. This indicates that cross attraction may take place between the two taxa under natural conditions.

Key Words—Lepidoptera, Noctuidae, *Diachrysia chrysitis*, *Diachrysia tutti*, pheromones, sibling taxa, electroantennographic responses, biosynthesis, cross-attraction.

INTRODUCTION

In his taxonomic revision of palearctic Plusiinae, Kostrowicki (1961) described *Diachrysia tutti* as a species that could be separated from *D. chrysitis* (Linnaeus, 1758) on morphological grounds. According to Kostrowicki the two taxa appear sympatrically in Europe, Asia Minor, Iran, and the Caucasus, whereas only *D. tutti* occurs east of the Urals. Kostrowicki's distinction between the two taxa was, however, not confirmed by other taxonomists, and *D. tutti* was generally considered a synonym of *D. chrysitis* (Lempke, 1965; Urbahn, 1966, 1967) until Priesner (1985) reported the attraction of two distinct *Diachrysia* populations, assignable to *D. tutti* and *D. chrysitis*, to two different mixtures of (Z)-5-decenyl acetate (Z5-10:OAc) and (Z)-7-decenyl acetate (Z7-10:OAc). Typical *D. tutti* specimens have a clearly broken forewing pattern (Wp 1; confluence grades 1 and 2 according to Rezbanyai-Reser, 1985), and such males are attracted to a 2:100 mixture of Z5-10:OAc/Z7-10:OAc, whereas *D. chrysitis* males with an unbroken wing pattern (Wp 5) are attracted to a 100:10 mixture. The *chrysitis* sex attractant rarely attracts males with Wp 1 and few males with Wp 5 are found in *tutti* traps, but insects with intermediate wing patterns (grades 2–4) are frequently found in both kinds of traps (Priesner, 1985; Rezbanyai-Reser, 1985; Tòth et al., 1988; Svensson et al., 1989).

Allozyme analysis of males trapped with the two types of sex attractants demonstrated that the samples were similar but not identical with respect to allele frequencies. However, no diagnostic loci were found, and the allozyme data were not clear in terms of cross-attraction and reproductive isolation (Svens-

son et al., 1989). The noctuid moths *D. chrysitis* and *D. tutti* provide an opportunity for the evolutionary biologist to study the role of pheromones in reproductive isolation and speciation. In the present study we investigate differences in female pheromone production and male electrophysiological response between *D. tutti* and *D. chrysitis*. Males attracted in the field were subsequently subjected to EAG analysis in the laboratory. The specificity of male attraction to synthetic pheromone was thus investigated by studying electroantennographic responses of the trapped males to synthetic pheromone components.

METHODS AND MATERIALS

Insects. Insects were classified based on their forewing pattern according to the five confluence grades suggested by Rezbanyai-Reser (1985) (see Figure 5B) below. Gravid females were collected in Germany, Hungary, and Sweden. The larvae developing from eggs were raised on a natural diet consisting of *Urtica dioica*, *Taraxacum vulgare*, *Plantago major*, or other suitable host plants. Insects for pheromone analysis were obtained from 12 families (Table 1). Hind wing scales from the male moths of family 3 were magnified 500 × and analyzed for density of ridges according to the method of Bruun (1987). *D. chrysitis* specimens used for flight-tunnel experiments and biosynthetic labeling experiments were the F1 progeny of a cross between families 6 and 7.

Pheromone Gland Extracts. Extracts for analysis of pheromone components and pheromone precursors were prepared from individual 2- to 5-day old female *Diachrysia* spp. The pheromone gland, located at the dorsal side of the intersegmental membrane between the eighth and ninth abdominal segments, was excised with a pair of forceps and extracted in 10 μl of redistilled hexane. The extract was subjected to gas chromatography (GC) analysis. For total lipid extraction, 10 μl of chloroform–methanol (2 : 1 v/v) was subsequently added to the hexane-extracted glands. This second extract was subjected to base methanolysis (Bjostad and Roelofs, 1981), and the methyl esters thus formed were analyzed by GC or coupled GC–mass spectrometry (GC-MS).

Experiments on Pheromone Biosynthesis. Deuterium-labeled fatty acids in DMSO (approximately 4 μg in 0.2 μl), mixtures or individual components, were applied topically (Bjostad and Roelofs, 1981; Löfstedt et al., 1986) to the pheromone glands of 2- to 4-day old *Diachrysia* sp. The acids were applied at the beginning of the dark period, and the pheromone glands were dissected after approximately 30 min of incubation. Most of the experiments were carried out with F1 insects from a cross between families 6 and 7 (classified as *D. chrysitis* based on female pheromone production and male electroantennographic and flight-tunnel responses). A few experiments were carried out with insects from family 3.

TABLE 1. DATA ON *Diachrysia* FAMILIES ANALYZED IN PRESENT STUDY[a]

No.	Place and year of collection	Wp		Offspring wing pattern				
				Wp 1	Wp 2	Wp 3	Wp 4	Wp 5
1	Seewiesen, Germany, 1986	5	females	0	0	0	3	5
2	Seewiesen, Germany, 1986	5	females	0	0	0	1	5
3	Seewiesen, Germany, 1989	2	females	0	0	3	0	0
			males	2	1	3	1	0
4	Budapest, Hungary, 1988	3	females	0	0	1	3	0
			males	0	0	0	3	2
5	Budapest, Hungary, 1988	3	females	0	0	6	5	5
			males	0	0	0	6	7
6	Budapest, Hungary, 1989	5	females	0	0	0	2	34
			males	0	0	1	5	46
7	Budapest, Hungary, 1989	5	females	2	3	4	6	13
			males	1	2	4	13	11
8	Delsbo, Sweden, 1988	3	females	0	1	0	0	0
9	Delsbo, Sweden, 1988	4	—	—	—	—	—	—
10	Delsbo, Sweden, 1989	3	females	0	0	1	0	0
			males	0	0	2	1	0
11	Ljusdal, Sweden, 1989	2	females	0	0	2	0	0
			males	0	2	0	0	0
12	Ljusdal, Sweden, 1989	2	females	0	2	1	0	0
			males	0	1	0	1	0

[a]In families 6, 7, 11, and 12, the wing patterns (Wp) were noted for all emerging adults. In other families Wp was generally noted for those insects used in experiments. In a few cases Wp information was lost for some individuals, but in these cases the frequencies given should still represent an unbiased subsample.

Electrophysiology. Recordings of electroantennographic responses (EAG) were performed on excised male antennae in Lund and in Budapest, using slightly different methods. In Lund, an antenna was placed with the base in a pipet electrode filled with Beadle-Ephrussi Ringer, and grounded via a Ag–AgCl wire. The distal tip of the antennae was placed in contact with a recording electrode, similar to the indifferent electrode. The tip electrode was connected to a high impedance DC amplifier with automatic baseline drift compensation. One microgram of the stimulus was applied to a 7 × 15 mm^2 piece of filter paper, which was placed inside a Pasteur pipet. The antenna was constantly flushed by a charcoal-filtered and moistened airstream. The airstream passed through a glass tube (ID 8 mm) at a velocity of 0.5 m/sec. The glass tube ended 8 mm before the preparation. The stimulus was injected as a short puff (50 msec) by a stimulation device (Syntech, Hilversum, The Netherlands) into the constant airstream. As only two stimuli were tested on each antenna, no normalization was needed.

In Budapest, the connection between the platinum electrodes and the insect tissue was maintained by an electrically conducting gel (Valleylab, Boulder, Colorado). Responses were amplified by a high-impedance amplifier (Rumbo, 1981). One microgram of a test compound applied to a 10 × 10 mm^2 piece of filter paper inside a Pasteur pipet was used as an odor source. Stimuli were provided by injecting 1 ml of air through the Pasteur pipet into an airstream (80 liters/hr) flushing over the antenna. The interval between stimuli was at least 1 min. Responses were normalized against a common standard that was administered before and after stimulation with the test compound. In these tests, 2- to 4-day-old laboratory-reared males or feral males captured in sticky traps were used.

EAG response profiles of *D. tutti* were generated from recordings on male antennae from typical *D. tutti* specimens trapped with the 2:100 blend (see below). *D. chrysitis* males with typical wing pattern were trapped with the 100:10 mixture or they were sampled from the F1 generation of the cross-bred families 6 and 7.

Relative EAG response of an individual male to Z5–10:OAc (expressed as percent Z5–10:OAc) was calculated as [response to Z5–10:OAc/(response to Z5–10:OAc + response to Z7–10:OAc)] × 100, using the responses in millivolts from one of its antennae.

GC-FID and GC-EAD. GC-FID was performed on a Hewlett Packard 5880 GC equipped with a 30-m × 0.25-mm-ID DB-Wax column (J&W Scientific, Folsum, California). Chromatography with simultaneous FID and electroantennographic detection (FID-EAD) (Arn et al., 1975) was performed on a Hewlett Packard 5830 GC equipped with an effluent split and a DB-Wax column. Hydrogen was the carrier gas, and the effluent split ratio was approximately 1:1. The outlet for the EAD was placed in a purified airstream flowing over the antennal

preparation at a speed of 0.5 m/sec. The electrophysiological methods were as described above for the Lund laboratory.

Mass Spectrometry and NMR Spectroscopy. GC-MS with electron impact ionization (70 eV) was performed on a Hewlett Packard model 5970B GC-MS system equipped with a 59970B computer system and interfaced with a Hewlett Packard model 5890 GC. Helium was the carrier gas and the column used was a 30-m × 0.25-mm-ID DB-Wax column. The GC-MS was operated in the selected ion monitoring mode for the detection of incorporation of labeled precursors into pheromone components and intermediates. Acquisition programs were designed to monitor diagnostic ions of native and labeled methyl esters and acetates. Selected ions were monitored in groups of two to three depending on the experiment, and the groups were changed at preset times in the course of the separation, based on the retention times of synthetic standards. This allowed selective and sensitive detection of each of the different compounds of interest. The following diagnostic ions were chosen for the detection of unlabeled specimens: decenyl acetates *m/z* 138.15, 10:Me *m/z* 186.20, monounsaturated 10:Me *m/z* 152.20, 12:Me *m/z* 214.20, monounsaturated 12:Me *m/z* 180.20, 14:Me *m/z* 242.25, monounsaturated 14:Me *m/z* 208.20, 16:Me *m/z* 270.25 and monounsaturated 16:Me *m/z* 236.25. Corresponding specimens labeled with 3, 5, and 9 deuterons were monitored at mass fragments 3, 5, and 9 Daltons higher, respectively.

EI mass spectra for documentation of the synthesis of monounsaturated labeled acids were recorded on a Finnigan 4021 mass spectrometer and high resolution mass spectra on a VG ZAB instrument.

^{1}H and ^{13}C NMR spectra were recorded on a Varian XL-300 spectrometer in $CDCl_3$ solutions with Me_4Si as internal reference. The ^{13}C signals for the deuterated carbon atoms were not assigned.

Field Tests. Trapping experiments with synthetic pheromone components were carried out in Budakeszi, Pest County, Hungary, between May 26 and June 12, 1988, and June 1 and August 30, 1989, at Gyöngyös, Heves County, Hungary, between August 20 and September 15, 1989. Sticky traps similar in shape and size to those described by Arn et al. (1979), but made from polyethylene sheets, were used. Traps were suspended from the branches of trees at a height of 1.5 m. Male moths captured were taken alive to the lab. In 1988 the males were transferred to Lund for electrophysiological studies, whereas in 1989 EAG recordings were carried out at the lab in Budapest.

Dispensers for trapping experiments were prepared from pieces of rubber tubing (Taurus, Budapest, Hungary; No. MSZ 9691/6; extracted in ethanol and dichloromethane prior to usage). The required amounts of compounds in hexane solutions were administered to the surface of the dispensers.

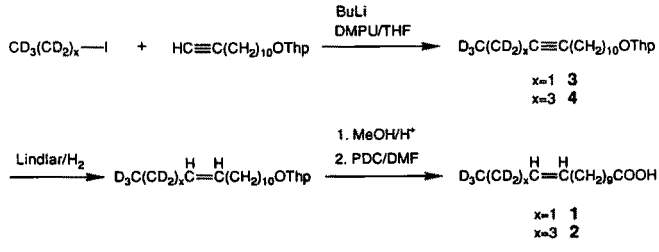
Flight-Tunnel Experiments. Experiments were carried out in an open

Plexiglas flight tunnel 0.9 m wide × 0.9 m high × 2.5 m long, 3–4 hr into the scotophase. Flight-tunnel conditions were 18–20°C, 35–45% relative humidity, 1 lux, and 0.35 m/sec wind speed. Synthetic mixtures to be tested were applied to rubber septa (Arthur H. Thomas, Co.) in 100 μl of hexane. The dispensers were placed on the top of 30-cm-tall metal rods. Males were released individually into the plume from a cylindrical screen cage with the open end facing upwind. Six behavioral steps were typically observed in the flight tunnel: wing fanning (WF), taking flight (TF), orientation (Or), upwind flight 30 cm from the release cage in the plume (30 cm), flying half the distance between the source and the release cage in the plume (HW), and source contact (SC). Three different mixtures were tested, containing 10 μg Z5–10:OAc and 1 μg Z7–10:OAc ("*chrysitis* blend"), 0.2 μg Z5–10:OAc and 10 μg Z7-10:OAc (*tutti* blend), and 5 μg of each compound (intermediary blend), respectively.

Chemicals. Z5–10:OAc and Z7–10:OAc were purchased from the Institute for Pesticide Research, Wageningen, The Netherlands. The overall chemical purity was about 98%, and the purity with respect to geometric isomers was above 98.5%. Other acetates, alcohols, and aldehydes used for EAG screening were from the laboratory collection of pheromone components at the Plant Protection Institute, Budapest, Hungary.

Deuterium-labeled saturated fatty acids were purchased from Larodan Fine Chemicals, Malmö, Sweden. The deuterium enrichment of these omega-labeled acids was 99%. The monounsaturated deuterium-labeled fatty acids were synthesized as described below (Scheme 1). The products were purified by flash chromatography (Taber, 1982) on TLC-Silica gel 60 H (Merck) and argentation liquid chromatography (Houx et al., 1974). Final products were more than 99.8% isomerically pure. 1,3-Dimethyl-2-oxo-hexahydropyrimidine (DMPU, or *N,N'*-dimethylpropyleneurea) was purchased from Fluka AB. Immediately before use, it was distilled over CaH_2 at reduced pressure and kept over 4 Å molecular sieves under an argon atmosphere.

(Z)-[13,13,14,14,14,-$^2H_5$]11-tetradecenoic acid [(D_5)-Z11–14:COOH] **(1)** was prepared from 1-(2-tetrahydropyranyloxy)-dodecyne (2.2 g, 8.2 mmol), *n*-butyllithium (6.1 ml, 1.42 M in hexane) in dry THF (8 ml) and [$^2H_5$]ethyl



SCHEME. 1.

iodide (2.2 g, 8.2 mmol) in DMPU (14 ml) according to a method previously described for similar systems (Bengtsson and Liljefors, 1988; Bengtsson 1988; Löfstedt and Bengtsson, 1988) yielding 2.1 g (86%) of the product (**3**, Scheme 1) after flash chromatography. Reduction with Lindlar catalyst (Leznoff et al., 1977; Wong et al., 1984) gave the (*Z*)-monoene, which was converted to the corresponding alcohol by treatment with *p*-toluenesulfonic acid in methanol. Oxidation with pyridinium dichromate (PDC) in dry dimethylformamide (DMF) (Corey and Smidt, 1979) gave the final product (**1**) in 60% overall yield: *m/z* 231 (M^+, 2%), 213(4), 171(3), 161(2), 151(2), 138(4), 123(6), 110(10), 97(21), 83(29), 73(42), 69(52), 60(58), 55(96), 43(100), 33(3). ^{1}H NMR (300 MHz); ∂ 1.25–1.28 (m, 12H, CH_2CH_2), 1.58–1.66 (m, 2H, $C\underline{H}_2$—C—COOH), 1.97-2.05 (m, 2H, CH_2—C=C), 2.35 (t, 2H, $C\underline{H}_2$—COOH), 5.27–5.37 (m, 2H, CH=CH). ^{13}C NMR (300 MHz); ∂ 24.69, 27.09, 29.05, 29.16, 29.23, 29.39, 29.45, 29.76, 33.84, 129.34, 131.43, 178.89. High-resolution mass spectroscopy on the corresponding methyl ester: $[M]^+_{calc}$ = 245.24031, $[M]^+_{obs}$ = 245.23801.

(Z)-[13,13,14,14,15,15,16,16,16-$^2H_9$]11-hexadecenoic acid [(D_9)-Z11-16:COOH] (**2**) was prepared as described above for (D_5)-Z11-14:COOH) (**1**) from 1-(2-tetrahydropyranyloxy)dodecyne (1.1 g, 4.1 mmol), *n*-butyllithium (3.1 ml, 1.42 M in hexane) in dry THF (4 ml) and [$^2H_9$]butyl iodide (1.3 g, 6.5 mmol) in DMPU (7 ml) affording 1.1 g (81%) of the product (**4**) (Scheme 1) after flash chromatography: *m/z* 245 (M^+ − 18, 9%), 217(1), 203(6), 177(1), 165(2), 161(6), 150(3), 137(5), 133(4), 123(9), 119(3), 110(16), 96(35), 84(46), 74(93), 69(54), 59(67), 55(100), 41(65), 34(12). ^{1}H NMR (300 MHz); ∂ 1.26–1.28 (m, 12H, CH_2CH_2), 1.58–1.68 (m, 2H, $C\underline{H}_2$—C—COOH), 1.96–2.05 (m, 2H, CH_2—C=C), 2.34 (t, 2H, $C\underline{H}_2$—COOH), 5.33-5.36 (m, 2H, CH=CH). ^{13}C NMR (300 HMz); ∂ 24.67, 27.17, 29.05, 29.91, 29.24, 29.38, 29.44, 29.74, 33.87, 129.80, 129.85, 179.09. High-resolution mass spectroscopy on the corresponding methyl ester: $[M]^+_{calc}$ = 277.29672, $[M]^+_{obs}$ = 277.27782.

RESULTS

Analysis of Female Pheromone Production. Pheromone gland extracts of individual female *Diachrysia* were subjected to GC analysis with EAG detection. Females for extraction were obtained from family rearings (the offspring of individual field collected gravid females) and antennae from their brothers, i.e., males from the same family, were used as detectors. Analysis of insects from typical *chrysitis* families (predominantly grade 5) yielded two significant EAD responses. The strongest response was obtained from a compound with the same retention time as Z5-10:OAc and a smaller response was obtained

from a compound with the same retention time as Z7–10:OAc. In analyses of insects from a typical *tutti* family, a small EAD response was obtained at the retention time of Z5–10:OAc and a larger response at the retention time of Z7–10:OAc (Figure 1). The structural assignments of the active peaks were corroborated by GC-MS. The two active peaks in both *D. chrysitis* and *D. tutti* gave similar mass spectra, containing the fragments *m/z* 138 (M−60), 109, 95, 82, 67, 61, and 43 characteristic of decenyl acetates.

Individual females from 12 different families of *Diachrysia* were analyzed by GC-FID or GC-MS for ratios of the two decenyl acetates (Figure 2A). In nine of the 12 families, the family means of percent Z5–10:OAc were above 75% (range 76–92%). These nine families were assigned *D. chrysitis* based on their pheromone production. In the other three families (3, 11, and 12), the average percent Z5–10:OAc in the pheromone was below 20% (range of family means 2–15%). These families were tentatively called *D. tutti*.

Although there was a correlation between wing pattern and pheromone ratio at the family level, it was obvious that wing pattern cannot be used as a diagnostic character for pheromone type. Among those females analyzed for pheromone production, all individuals with grade 4 or 5 were of the *D. chrysitis* type (>70% Z5–10:OAc), but females with wing pattern grade 2 and 3 were found in both the *D. chrysitis* and *D. tutti* families.

EAG Responses of Diachrysia *Families.* Eight different families were inves-

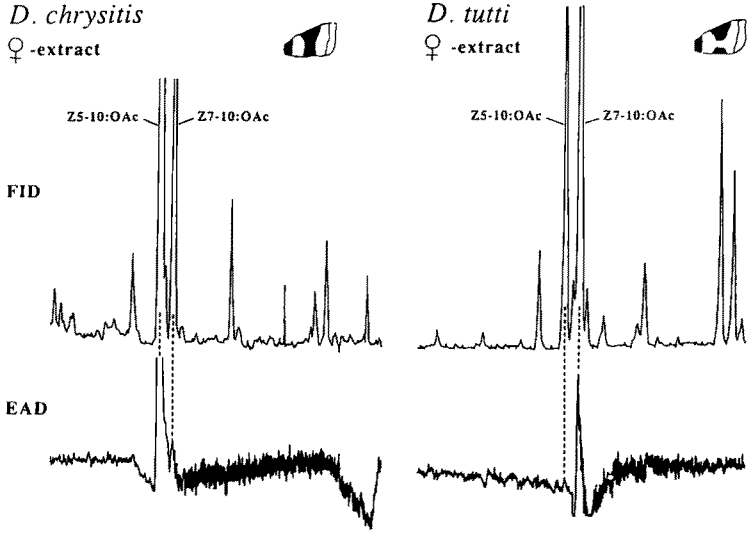


FIG. 1. Gas chromatograms of pheromone gland extracts from individual *Diachrysia* females on a DB-Wax column with simultaneous FID and EAD.

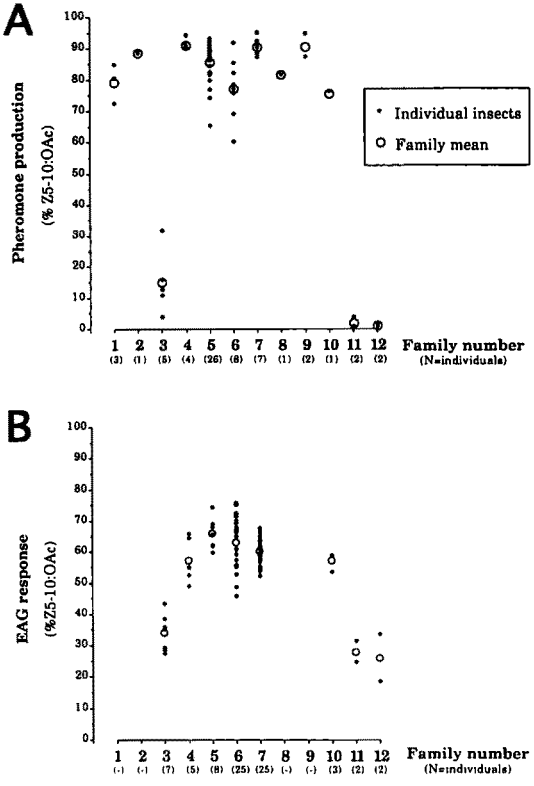


FIG. 2. Ratios of pheromone components produced by individual *Diachrysia* females from 12 families (A) and relative EAG responses of males from eight of these families (B).

tigated with respect to the relative EAG responses of individual males (Figure 2B). The families had been classified as *chrysitis* or *tutti* based on predominant wing patterns and female pheromone production. The assigned *chrysitis* families (4–10) responded more strongly to synthetic Z5–10:OAc than to Z7–10:OAc (range of family means 56–65), with only a few individuals responding more strongly to the Z7 than to the Z5 isomer. Unfortunately, only a few laboratory-reared males belonging to families of the *tutti* type were available, but all *tutti* males (families 3, 11, and 12) responded more strongly to Z7–10:OAc than to Z5–10:OAc.

Pheromone Biosynthesis. Gas chromatographic analysis of lipid extracts from glands of *D. chrysitis* and *D. tutti* revealed a number of unsaturated fatty acids that could be involved in the pheromone biosynthesis (Table 2). The

methyl esters were identified based on the correspondence of their retention times with those of synthetic standards. No unsaturated decenoates were detected, but traces of dodecenoates and relatively large amounts of tetradecenoates and hexadecenoates were identified. Based on the fatty acyl moieties identified, pathways to the two pheromone components, starting with *Z*11 desaturation of

TABLE 2. RELATIVE AMOUNTS OF FATTY ACYL MOIETIES IN EXTRACTS OF *Diachrysia tutti* AND *D. chrysitis*

No.	Methyl ester	Relative titer[a]	
		D. chrysitis	*D. tutti*
1	12:Me	0.38 ± 0.09	1.03 ± 0.48
2	Z7-12:Me	+	+
3	Z9-12:Me	+	+
4	14:Me	3.42 ± 1.03	7.82 ± 1.18
5	Z9-14:Me	8.43 ± 1.75	5.02 ± 0.09
6	*E*11-14:Me	2.54 ± 0.19	5.03 ± 0.22
7	Z11-14:Me	0.92 ± 0.09	4.92 ± 1.01
8	16:Me	100	100
9	Z7-16:Me	1.19 ± 0.79	1.86 ± 0.12
10	Z9-16:Me	10.81 ± 2.15	6.54 ± 3.00
11	Z11-16:Me	53.41 ± 7.35	82.59 ± 15.98

[a] + = detected, but below the limit of quantification.

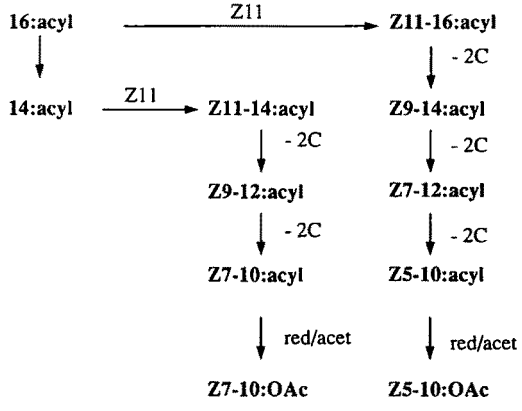


FIG. 3. Proposed biosynthetic routes to the pheromone components of *D. chrysitis* and *D. tutti*.

tetradecanoate and hexadecanoate respectively, are proposed (Figure 3). The suggested pathways were supported by the results of labeling experiments using deuterium-labeled fatty acid precursors. In *D. chrysitis* (D_3)-16:COOH was incorporated into both Z5-10:OAc and Z7-10:OAc whereas topical application of (D_3)-14:COOH resulted in detectable amounts of labeled Z7-10:OAc only (two experiments with each of the precursors). The amount of Z11-14:COO^- relative to Z11-16:COO^- differed between the *D. chrysitis* and *D. tutti* (Table 2). In *D. chrysitis*, application of (D_9)-Z11-16:COOH (presumed precursor of Z5-10:OAc) and (D_5)-Z11-14:COOH (presumed precursor of Z7-10:OAc) in a 1:1 ratio resulted in the production of 25% labeled Z5-10:OAc (N = 5), whereas the average native ratio was approximately 85% Z5-10:OAc (Figure 4). Application of the same precursors in a 100:2 ratio resulted in labeled pheromone consisting of 93% Z5-10:OAc (N = 2). The application of these precursors in a 100:10 ratio to *D. tutti* resulted in a strongly Z5-10:OAc biased pheromone ratio (>85% Z5-10:OAc, N = 3) also in this taxon.

EAG Responses of Field-Trapped Males. The EAG responses of male *Diachrysia* sp. trapped with three different pheromone blends and classified with respect to wing pattern were compared with those of the laboratory-reared individuals. There was a strong correlation between attraction to the different baits and EAG response; all of the males attracted to the 2:100 mixture of Z5-10:OAc/Z7-10:OAc showed a stronger EAG response to the Z7 than to the Z5 isomer. The majority of the males attracted to the 100:100 and 100:10 mixtures responded more strongly to Z5-10:OAc, but the relative EAG responses of these males covered a wide range from 20 to 72 (Figure 5A).

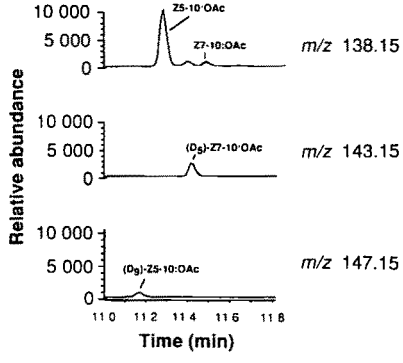


FIG. 4. GC-MS analyses of pheromone gland extracts from a female *Diachrysia chrysitis* gland treated with a 1:1 mixture of (D_9)-Z11-16:COOH and (D_5)-Z11-14:COOH. *m/z* 138.15 indicates native acetates, *m/z* 143.15 indicates D_5-labeled and *m/z* 147.15 indicates D_9-labeled acetates. Labeled specimens elute earlier than the native ones and their abundances are multiplied by a factor 2.

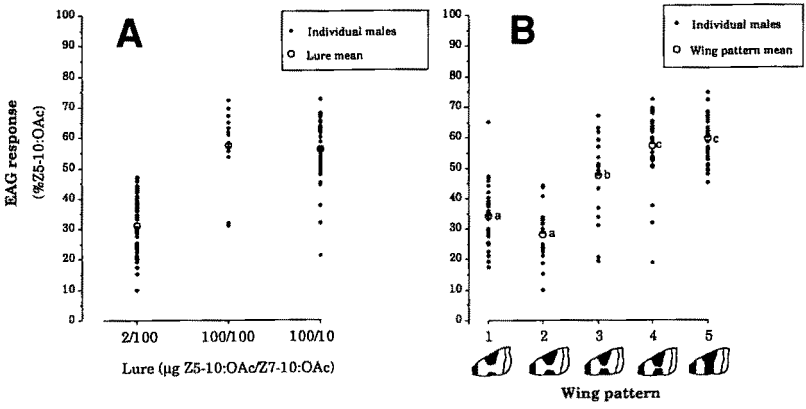


FIG. 5. Relative EAG responses of male *Diachrysia* trapped with different blends of Z5–10:OAc and Z7–10:OAc (A) and correlation between wing pattern type and EAG response among trapped males (B). Differences in mean EAG response between different groups in figure B are tested by one-way ANOVA followed by Fisher's protected LSD test ($P \leq 0.05$). Means accompanied by the same letters are not significantly different.

There was also a clear correlation between the wing pattern of field-trapped males and their relative EAG responses (Figure 5B). Relative responses to Z5–10:OAc of males with Wp 1 and 2 were significantly lower than those of males with Wp 4 and 5. The males with Wp 3 formed an intermediate group also with respect to pheromone response. However, this kind of statistical analysis may be misleading. Each type of male possessed a wide range of EAG responses, and it can be seen from the figure that the intermediate mean response of males with Wp 3 can be explained as the average response of males belonging to the two extreme types.

EAG response profiles for *D. chrysitis* and *D. tutti* were generated by screening 39 monounsaturated acetates, alcohols, and aldehydes on antennae of typical *chrysitis* and *tutti* males, respectively (Figure 6). Males of the two taxa differed not only in their relative response to the two decenyl acetate isomers but revealed a general preference towards delta-5 unsaturated (*D. chrysitis*) or delta-7 unsaturated (*D. tutti*) compounds in the series of compounds with shorter chain length.

Behavioral Observations in a Flight Tunnel. D. chrysitis males showed 63% completed responses including source contact to synthetic pheromone of the *D. chrysitis* type. Synthetic pheromone of the *D. tutti* type attracted only 5% (one of 20 males tested), whereas the intermediate synthetic pheromone was not significantly different from the *D. chrysitis* type with respect to landing response (Figure 7).

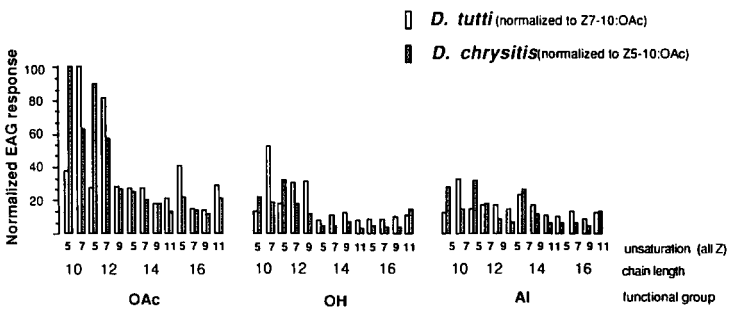


FIG. 6. EAG response profiles of *D. chrysitis* and *D. tutti* males. The responses of each male are normalized relative to the activity of the compound being most active for the respective taxa, i.e., Z5–10:OAc for *D. chrysitis* and Z7–10:OAc for *D. tutti* ($N = 5$).

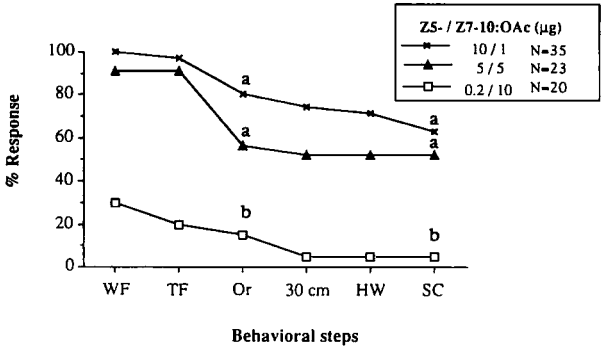


FIG. 7. Behavioral response of male *D. chrysitis* to three different blends of Z5- and Z7–10:OAc in a laboratory flight tunnel. WF = wing fanning, TF = taking flight, Or = orientation, 30 cm = upwind flight 30 cm from the release cage in the plume, HW = flying half the distance between the source and the release cage in the plume, and SC = source contact. For Or and SC, responses followed by the same letter indicate values that are not significantly different at the 95% confidence level, according to the method of adjusted significance levels for proportions (Ryan, 1960).

Each of six males from a *Diachrysia* family having wing pattern of the *tutti* type and females producing pheromone ratios between 4 and 34% Z5–10:OAc were tested to both the *D. chrysitis* and *D. tutti* mixtures. Three males were first tested on *chrysitis* pheromone, recaptured, and then after 15 min tested on the *D. tutti* pheromone. The other three males were tested on the two blends in reverse order. All six males responded to both types of pheromone with completed flights. The relative EAG responses (percent Z5–10:OAc) of these males varied between 28 and 44 (Figure 2B, family 3).

Density of Hind Wing Scale Ridges. The density of ridges on the hind wing scales of the males in family 3 varied between 567 and 500 ridges per millimeter (mean 539, SD 25) compared with 532 ridges per millimeters earlier reported for *D. tutti* and 628 ridges per millimeters for *D. chrysitis* (Bruun, 1987).

DISCUSSION

The results of our study confirm the occurrence of two distinct pheromone types in *D. chrysitis* s.l. with respect to female pheromone production and male electrophysiological and behavioral responses. In contrast, the most easily observable morphological character, the forewing pattern, seems to overlap significantly between *D. chrysitis* and *D. tutti* families. In spite of the clear dichotomy observed in these pheromone characters, our results suggest that cross-attraction between the two taxa may still occur under natural conditions.

The coupled gas chromatographic–electrophysiological analysis of *D. chrysitis* and *D. tutti* females and the extensive EAG screening of unsaturated acetates, alcohols, and aldehydes indicated no additional pheromone components to the attractants *Z*5–10:OAc and *Z*7–10:OAc as suggested earlier by Priesner (1985). Single sensillum recordings revealed two further receptor cells in both taxa, in addition to those responding to the pheromone components (Priesner, 1985). The additional cells responded to *Z*7–12:OAc and *Z*7–12:OH, respectively. These compounds are common pheromone components in other Plusiinae species, but they reduced rather than increased trap catches in the *Diachrysia* taxa (E. Priesner, unpublished results). In the case of *D. chrysitis*, the average percentage of *Z*5–10:OAc, 85%, corresponds well with the optimal lure containing 91% *Z*5–10:OAc as reported by Priesner (1985), considering that his series of ratios tested did not include any ratio between 76 and 91% *Z*5–10:OAc. Thus, in this case, the average female-produced ratio may be slightly superior to the standard 100:10 bait generally used for *D. chrysitis* and should be tested further. The average pheromone production for *D. tutti* found by us, 10% *Z*5–10:OAc, is also within the range of baits reported as being most attractive to *D. tutti*.

Our experiments on pheromone biosynthesis using deuterium-labeled precursors confirmed the biosynthesis of the two decenyl acetates from Δ11 desaturation of palmitic and myristic acids, respectively. Unfortunately, our failure to rear *Diachrysia* continuously in the laboratory put limitations on such experiments, but our results are in agreement with the suggested pathways (Figure 3). The identification of a compound with the characteristics of *E*11–14:Me in the total lipid extracts appears somewhat strange as no *E* isomer acetates are produced. However, in pyralid, yponomeutid, and tortricid moths the *E*- and *Z*11–14:acyl isomers often occur in combination (Wolf et al., 1981; Löfstedt

et al., 1991; Bjostad and Roelofs, 1986). The specific *E*/*Z* ratio appears to be produced by selective reduction of the precursors. In the European corn borer, *Ostrinia nubilalis*, both the *E* and the *Z* strains contain the *E*- and *Z*11–14:acyl precursors, but the *E* strain converts the *E* isomer and the *Z* strain the *Z* isomer selectively (Roelofs et al., 1987; Zhu et al., unpublished). In *Diachrysia* spp. the chain-shortening enzyme (or the reductase) may interact selectively with the *Z* isomers. The relative titer of *Z*11–14:acyl is higher in *D. tutti* than in *D. chrysitis*, which corresponds to the larger amount of *Z*7–10:OAc produced by *D. tutti*. Incubation with labeled precursors in naturally occurring ratios resulted in formation of labeled acetates close to native ratios in *D. chrysitis* but not in *D. tutti*. One explanation for this may be, as demonstrated in experiments with other moths, that acyl moieties from gland extracts are not only precursors but also leftovers, which biosynthetically may constitute different pools in the cells (Bjostad and Roelofs, 1986).

Looking at the range of ratios produced by females and the EAG responses of males in the laboratory-reared families, we found considerable individual variation. It may, however, be noted that there was no overlap in either pheromone production or male EAG response ratios between individual insects belonging to the families classified as *D. chrysitis* (1, 2, 4–10) and the remaining three (3, 11, 12) assigned *D. tutti*. Family 3 attracted our attention, as the mean percentage *Z*5–10:OAc produced by females from this family appeared to be higher than in the optimal blend for trapping of *D. tutti* (Priesner, 1985). Males from this family also showed a mixed response when tested in the flight tunnel and responded equally well to the *D. tutti* and the *D. chrysitis* blends. One possible interpretation is that this family was produced by hybridization of the two taxa. However, the density of ridges on the hind wing scale for this family was congruent with the data given by Bruun (1987) for typical *D. tutti*.

A prerequisite for hybridization to take place is that cross-attraction occur under natural conditions. Males trapped with the *chrysitis* blend seem to be predominantly of the *chrysitis* EAG type. Several of the males attracted by this blend, however, had an EAG response, based on the results with laboratory-reared insects, that could be classified as *D. tutti*. This is an indication of cross-attraction; but one cannot assume that there is perfect correlation between EAG response type and behavioral response type. In *O. nubilalis*, the best known moth with respect to pheromone genetics, it was found that electrophysiological characteristics of the male antenna were determined by an autosomal locus, whereas the male behavioral response type was determined by a locus on the Z chromosome (Hansson et al., 1987; Roelofs et al., 1987). The major difference in female pheromone production is determined by a third locus, and all factors segregate independently upon hybridization. The occurrence of males with *tutti*-type EAG in the traps baited with *chrysitis* pheromone can thus be interpreted in two ways: It may actually indicate cross-attraction, or some males of the

chrysitis behavioral response type may show *tutti* EAG response. In the latter case, the lack of correlation between electrophysiological and behavioral response may, however, just as well suggest cross-attraction and hybridization in earlier generations. We tried to establish laboratory cultures of *D. chrysitis* and *D. tutti* to dissect the genetics of the pheromone differences, but without success. Svensson et al. (1989) were unable to establish clear-cut allozyme differences between moths trapped by *D. tutti* and *D. chrysitis* pheromone and suggested that this could be due to mixing samples by cross-attraction. It would be interesting to investigate allozyme variation in family-reared moths as well as EAG-characterized field-trapped males. The probability of cross-attraction under natural conditions should be larger with females than with synthetic baits as the average insect-produced isomer ratios for the two taxa are more similar than the standard synthetic lures used. This issue may, however, be further complicated by differences in diel periodicity of sexual activity between the two taxa.

The indication of cross-attraction and potential hybridization between *D. chrysitis* and *D. tutti* is interesting but not surprising. Although the two taxa use *Z*5–10:OAc and *Z*7–10:OAc in an almost opposite ratio, the difference in pheromone composition between them is less than has been found between the two pheromone strains of *O. nubilalis*. In *O. nubilalis* the so-called *E* strain produces and responds preferentially to an approximately 99:1 mixture of *E*11- and *Z*11–14:OAc, whereas the *Z* strain uses the opposite mixture (Kochansky et al., 1975; Klun and cooperators, 1975). In spite of these extreme pheromone differences, cross-attraction is significant and hybrids are frequently formed in areas where the two strains cooccur (Klun and Huettel, 1988).

What will happen in a pheromonally mixed population is an open question. *D. chrysitis* and *D. tutti* are sympatric in large parts of their area of distribution, but differences in flight periods and habitat preferences have been noticed. Priesner (1985) and Rezbanyai-Reser (1985) concluded that the two taxa probably diverged allopatrically with respect to sex pheromones, flight phenology, and habitat preferences, but that isolation may be incomplete in areas of secondary contact. If there is any significant selection against cross-attraction depends on the incidence of cross-attraction and the fitness of potential hybrids. With no significant selection against hybridization, the two taxa may eventually merge as a result of hybridization in sympatry. This possibility was suggested by Rezbanyai-Reser (1985).

Male moths normally respond to a much broader range of pheromone component ratios than are produced by conspecific females (Löfstedt, 1990 and references therein). Males cannot afford to be "choosy" as females are the limiting sex. Complete pheromonal isolation between two taxa may not be expected to develop based on differences in ratios of two components. Accordingly, with respect to *D. chrysitis* and *D. tutti*, increased specificity of the sex pheromones would require the employment of additional pheromone components, for which we found no evidence so far.

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AGGREGATION PHEROMONES IN *Dryocoetes affaber* (Mann.) (COLEOPTERA: SCOLYTIDAE): STEREOISOMERISM AND SPECIES SPECIFICITY

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Abstract—Chemical analysis of whole body extracts and volatiles produced by feeding males *Dryocoetes affaber* (Mann.) disclosed (+)-*exo*-brevicomin and (+)-*endo*-brevicomin [(+)EXOB and (+)ENDOB], as the major insect-produced potential pheromones. Laboratory bioassays and field-trapping experiments demonstrated that (+)ENDOB is the main pheromone component, and (−)ENDOB has an inhibiting effect. EXOB either as (+) or (±) appears to be a multifunctional pheromone. It has a synergistic effect in blends of EXOB and ENDOB in ratios up to 1:1, and it is inhibitory at higher ratios. (−)EXOB was inactive. The most attractive blend for *D. affaber* was a 1:2 blend of (+)EXOB and (+)ENDOB. When this blend was compared with a 9:1 blend, the best known blend for *Dryocoetes confusus* Swaine, the responses by beetles of each of the two species were highly specific, providing evidence for pheromonal exclusion between the two congenerics. We conclude that the combined effect of chirality and the ratio of geometrical isomers of brevicomin determines both the level of response and the species-specificity of the chemical signal in *D. affaber*.

Key Words—Semiochemicals pheromones, *Dryocoetes affaber*, *Dryocoetes confusus*, Coleoptera, Scolytidae, enantiomers, diastereoisomers, *exo*-brevicomin, *endo*-brevicomin.

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INTRODUCTION

Dryocoetes affaber (Mann.) infests *Picea* spp., and has been reported from *Abies*, *Pseudotsuga*, *Larix*, and *Pinus* spp. It is the most widespread member of the genus in North America, ranging from Alaska and eastern Canada to New Mexico and North Carolina (Bright, 1963, 1976). The males are polygynous and are the first to attack the bole of weakened trees (Keen, 1952; Furniss and Carolin, 1977). The life cycle is poorly known, but in Colorado it appears to have one generation per year and overwinters as adults (McCambridge and Knight, 1972).

Furniss et al. (1976) observed attraction of *D. affaber* and *D. autographus (Ratzeburg)* to uninfested spruce logs and to one or more semiochemicals (*trans*-verbenol, verbenol, seudenol, and frontalin), produced by spruce beetles, *Dendroctonus rufipennis* (Kirby). Evidence for secondary attraction in other *Dryocoetes* spp. has been reported (Nilssen, 1979; Stock and Borden, 1983). European male *D. autographus* produce *exo*- and *endo*-brevicomin (7-ethyl-5-methyl-6,8-dioxabicyclo[3.2.1]octane); (+)-*endo*-brevicomin was attractive to females in field tests, while the antipode was inactive (Kohnle and Vité, 1984; Kohnle, 1985). (Herein, we use EXOB for *exo*-brevicomin and ENDOB for *endo*-brevicomin; blends are referred to as EXOB:ENDOB, in that sequence). Males of the western balsam bark beetle, *Dryocoetes confusus* Swaine, produce EXOB and ENDOB, mostly as (+) enantiomers (Schurig et al., 1983); *trans*-verbenol, verbenone, and myrtenol have also been identified from males in this species (Borden et al., 1987; Stock, 1991).

D. confusus is sympatric with *D. affaber* in the subalpine forests of British Columbia, wherein mixtures of (+)EXOB and (+)ENDOB at a 9:1 ratio optimally attract *D. confusus*. At a 1:1 ratio, either as (±) or (+) enantiomers, *D. affaber* was attracted rather than *D. confusus*, which suggested the existence of an isolation mechanism based on the chirality and the ratio of EXOB and ENDOB (Camacho et al., 1993).

Our objectives were: (1) to isolate and identify the major volatiles produced by feeding male *D. affaber*, (2) to determine the most attractive combination of these insect-produced volatiles, (3) to investigate the role of chirality in the system, and (4) to elucidate mechanisms of pheromone-based species specificity between *D. confusus* and *D. affaber*.

METHODS AND MATERIALS

Collection of Insects and Hosts. Bolts of Engelmann spruce, *Picea engelmannii* Parry, both healthy and infested with *D. affaber*, were obtained from trees felled near Merritt, British Columbia. The infested bolts were placed in

screened cages in the laboratory at 20–21°C. Emerging beetles were sexed and kept on moistened paper at 5°C until used in laboratory bioassays.

Collection and Analysis of Volatiles. Groups of male or female beetles were placed individually in preformed entrance holes in fresh spruce logs. The beetles were allowed to bore into the phloem tissue for three days and were then excised from the phloem. Extracts were prepared by crushing whole beetles in pentane held over Dry Ice; the liquid fraction was recovered and stored at −29°C. Another batch of spruce logs was infested in the laboratory with male or female *D. affaber* and placed in aeration chambers. The emanating volatiles were captured on Porapak-Q and recovered by extracting it with pentane (Pierce et al., 1981).

The whole-body extracts and the volatiles from infested logs were analyzed by gas chromatography (GC) using Hewlett Packard 5830A and 5880A instruments equipped with capillary inlet systems, flame ionization detectors, and open tubular glass columns (30 m × 0.5 mm ID) coated with SP-1000 (Supelco, Bellefonte, Pennsylvania). The temperature program was 70°C for 2 min, then 4°C/min to 180°C, and holding for 20 min. The enantiomeric composition of EXOB and ENDOB was determined by analysis of the volatiles from feeding-male whole-body extracts on a Chirasil-Dex (8) column (25 m × 0.25 mm ID) (V. Schurig, University of Tübingen, Germany). Coupled gas chromatography–mass spectrometry (GC-MS) was performed with a Hewlett Packard 5895A GC-MS fitted with a fused silica column (30 m × 0.33 mm ID) coated with SP-1000 (J&W Scientific, Inc., Folsom, California). Helium was the carrier gas for the GC and GC-MS.

Synthetic Pheromones. (±)EXOB (96.3% pure with 2.5% ENDOB) and (±)ENDOB (96.4% pure with 0.4% EXOB), were obtained from Phero Tech Inc., Delta, British Columbia. Optically pure brevicomins were synthesized by B.D. Johnston (Department of Chemistry, Simon Fraser University), according to the procedures developed by Johnston and Oehlschlager (1982) and Oehlschlager and Johnston (1987); formulations included (+)ENDOB (98.8% and 90.15% chemically and optically pure, respectively), (−)ENDOB (97.8% and 91% chemically and optically pure, respectively), and (−)EXOB (96.4% and 92.6% chemically and optically pure, respectively). For field experiments conducted in 1992, we also employed (+)EXOB (98.79% and 94.0% chemically and optically pure, respectively). The Sharpless asymmetric dihydroxylation (Sharpless et al., 1991) was used for the synthesis (E.K. Czyzewska, Department of Chemistry, Simon Fraser University, unpublished). Blends of EXOB and ENDOB were prepared by weight, and ratios referred to below are on a weight-to-weight basis.

Determination of Ratio in Vapor Phase. The ratio in vapor phase was determined for the 1:2 formulation of (±)EXOB:(±)ENDOB. Two glass cap-

illary tubes (1.0 mm ID) sealed at one end, containing 12 μl of the 1:2 blend, were kept inside open 400-μl polyethylene tubes at 24–26°C. Vapor-phase samples were taken from the plastic tube at 24, 40, 48 and 70 hr after formulation and analyzed by GC as above.

Laboratory Bioassays. Experiments on responses to EXOB and ENDOB and their blends were performed using walking beetles in an open arena olfactometer (Wood and Bushing, 1963; Stock and Borden, 1983). Groups of 10 beetles of either sex were exposed for 2.5 min. to an airstream (500 ml/min) containing volatile stimuli applied in 10 μl of pentane to a filter paper wick. The solvent was used as a control. Room temperature was 20–21°C and room lighting was diffuse and of low intensity (22.57 lux). A series of 1-pg stimuli consisting of (+)EXOB, (+)ENDOB, and binary blends of these compounds at ratios of 3:1 (the natural ratio), 2:1, 1:1 [reported attractive (Camacho et al., 1993)], 1:2, 1:3, and 1:6 were tested. We tried to cover a wide range of possible combinations excluding those with high content of (+)EXOB, reported attractive for *D. confusus* (Camacho et al., 1993).

Field Experiments. Trapping experiments were conducted in a forest of Engelmann spruce and subalpine firs, *Abies lasiocarpa* (Hook.) Nutt., 40 km west of Merritt, British Columbia. Multiple funnel traps (Lindgren, 1983) (Phero Tech Inc.), were placed 15 m apart in randomized complete blocks, with 9–20 replicates per experiment. The release rates, either as single compounds or as blends, were approximately 0.2 mg/24 hr at 27°C from each glass capillary (10. mm ID), as determined in the laboratory (Stock et al., 1990). In the forest, the release rates are temperature dependent.

Experiment 1, conducted in 1992, tested blends of (+)EXOB and (+)ENDOB at the following ratios: 3:1 (found in the aerations of male infested logs) 1:1 [attractive for *D. affaber* (Camacho et al., 1993)], 1:2 (from the results of laboratory bioassays), 1:10 (attractive to *D. affaber* in other field experiments, J.H. Borden, unpublished), and an unbaited control.

Experiments 2–4 investigated the question of enantioselectivity. In 1991, experiment 2 tested the attractiveness of (±), (−), and (+)ENDOB, and blends of EXOB:ENDOB in the following combinations of enantiomers (±):(±), (±):(−) and (±):(+). Experiments 3 and 4 in 1992, tested EXOB:ENDOB blends of (−):(+), (+):(+), and (±):(+) (experiment 3), and (−):(−), (+):(−) and (±):(+) (experiment 4). The (±):(+) combination was always present in experiments 2–4. In all cases, blends of EXOB:ENDOB were in a 1:1 ratio.

Experiment 5 utilized the best blends of EXOB:ENDOB established in previous experiments for *D. affaber* and *D. confusus* and challenged their capacity to maintain species specificity. We used (+)EXOB:(+)ENDOB at a 9:1 ratio for *D. confusus* (Camacho et al. 1993) and (±)EXOB:(+)ENDOB at a 1:1 ratio (i.e., a 1:2 ratio of (+) enantiomers) for *D. affaber*.

Statistical Analysis. Laboratory bioassay results were analyzed by one-way

analysis of variance (ANOVA) and the Ryan-Einot-Gabriel-Welsch multiple F or "REGWF" test (Schlotzhauer and Littell, 1987) utilizing percentages of positive responders converted to $p' = \text{arcsine } \sqrt{p}$, to approximate a normal distribution (Zar, 1984). Percent values of 0% were recorded as $1/4\,n$ to improve the transformation (Bartlett, 1937). For field trapping experiments we used two-way ANOVA and the REGWF test on numbers of beetles captured transformed by $x' = \log(x + 1)$, to remove heteroscedasticity (Zar, 1984). In all cases $\alpha = 0.05$. Treatments with zero catches were excluded from statistical analyses. All analyses employed SAS computer software (SAS Institute, 1990).

RESULTS AND DISCUSSION

Identification of Candidate Pheromones. (+)EXOB and (+)ENDOB in a 1.7:1 ratio were conspicuous insect-produced compounds found by GC-MS analysis in whole-body extracts of males. Volatiles emanating from male-infested logs also contained (+)EXOB and (+)ENDOB; in this case the ratio was 3.04:1 (Figure 1). Small amounts of EXOB of undetermined chirality were detected from feeding females in logs.

In laboratory bioassays, blends of (+)EXOB:(+)ENDOB in the ratio range between 2:1 and 1:2 elicited the highest levels of response from female *D. affaber*; males showed some preference for blends in the ratio range between

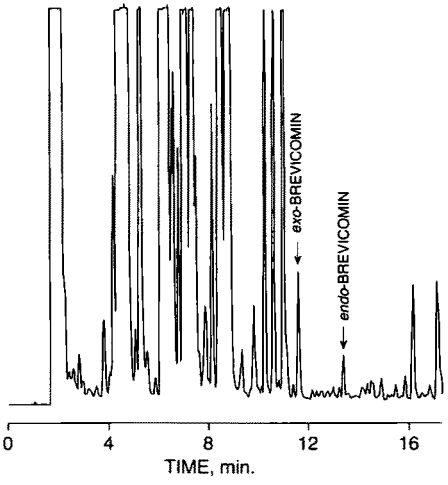

FIG. 1. Gas–liquid chromatogram of Porapak Q-trapped volatiles produced by male *Dryocoetes affaber* feeding in fresh bolts of *Picea engelmannii*, showing *exo*-brevicomin and *endo*-brevicomin in a 3:1 ratio.

3:1 and 1:2 (Figure 2). Both sexes showed the highest numerical response to the 1:2 ratio. The response to (+)EXOB or to (+)ENDOB presented individually was very low.

Ratio in Vapor Phase. The 1:2 blend of (+)EXOB:(±)ENDOB formulated by weight was confirmed by GLC analysis. The ratios ($\bar{X}$ ± SD) determined by GLC analyses of vapor phase samples after 24, 40, 48, and 70 hr were: 1:1.3 ± 0.015, 1:1.7 ± 0.03, 1:1.6 ± 0.015, and 1:1.7 ± 0.015, respectively. Differential volatility of EXOB and ENDOB caused only minor changes from the formulated ratio in the proximity of the release device. Modifications of this ratio are to be expected at further distances from the release point as a result of diverse environmental factors (e.g., temperature, turbulence) that affect the pheromone plume in the forest (Murlis et al., 1992).

Field Experiments. The highest response from both males and females in

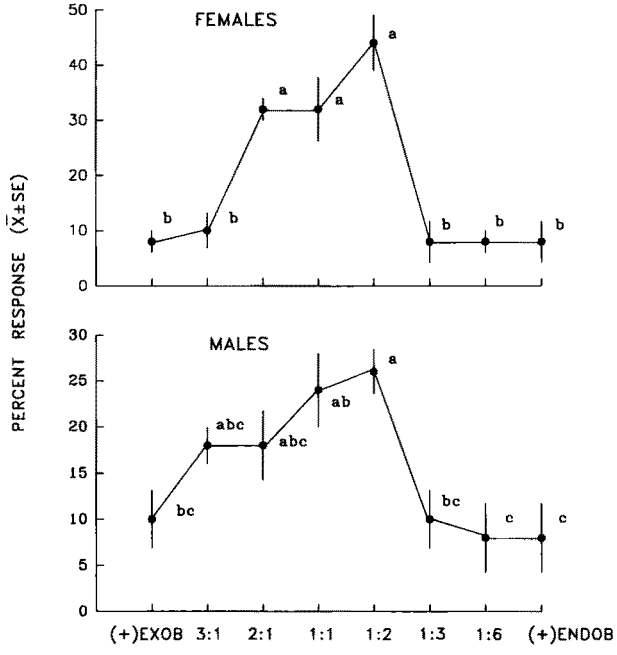

FIG. 2. Response of *Dryocoetes affaber* in laboratory bioassays to 1 pg stimuli of (+)-*exo*-brevicomin (EXOB), (+)-*endo*-brevicomin (ENDOB) and six blends of the two isomers at different ratios. Fifty beetles of each sex tested per stimulus. Response to pentane controls: male 4%, females 6%. Percents with the same letter are not significantly different, Ryan-Einot-Gabriel-Welsh multiple F test, $P < 0.05$.

experiment 1 was elicited by the (+):(+) blend at the 1:2 ratio (Figure 3). As in the laboratory (Figure 2), the natural 3:1 blend was poorly attractive. Blends of 1:1 and 1:10 (+)EXOB:(+)ENDOB attracted significantly less *D. affaber* of both sexes than did the 1:2 blend.

For Lepidoptera, it is generally accepted that optimum blends of pheromone components closely approximate the natural ratio emitted by the producing sex. However, production and reception genes are not linked (Roelofs et al., 1987). Response to different blends could indicate missing elements in the chemical message (Baker, 1989). It is possible that environmental factors could alter the pheromone plume from the natural 3:1 ratio to more attractive ratios. Other effects such as geographical and individual variation (Miller et al., 1989), physiological changes due to manipulation and storage, or mechanisms of avoidance

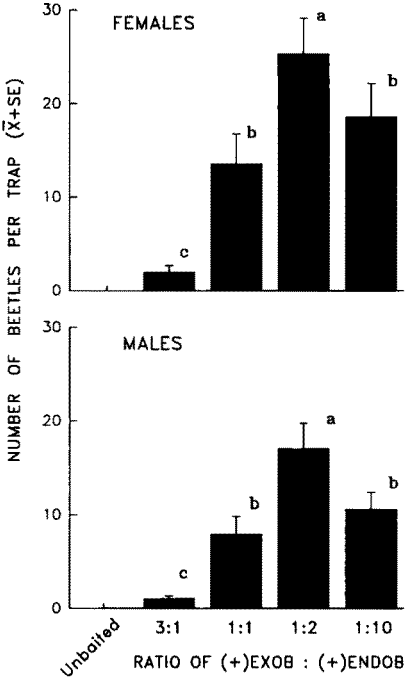

FIG. 3. Numbers of *Dryocoetes affaber* caught in experiment 1 in traps baited with blends of (+)-*exo*-brevicomin (EXOB) and (+)-*endo*-brevicomin (ENDOB) in four ratios; 10 replicates, July 7 to August 5, and 10 replicates August 5–20, 1992. Bars with the same letter are not significantly different, Ryan-Einot-Gabriel-Welsh multiple F test, $P < 0.05$.

of competition for pheromonal channels, could help to explain the observed difference between pheromone production and response in *D. affaber*.

Our results suggest that in *D. affaber* there is considerable tolerance to variation in ratios of pheromone components, as reported for other bark beetles (Schlyter et al., 1987; Byers, 1988) and moths (Linn and Roelofs, 1989). This plasticity could be of selective advantage for secondary bark beetles. For *D. affaber*, plasticity would be restricted to EXOB:ENDOB blends that comprised >50% ENDOB.

There was a decrease in response to ENDOB by both sexes in experiment 2, from (+) to (±) and finally (−), indicating that the (+) enantiomer is the active component; response to the blends indicates that the antipode is inhibitory (Figure 4). The 1:1 blend of (±)EXOB:(+)ENDOB was the most attractive, indicating that synergism occurs between EXOB and ENDOB, but not disclosing which enantiomer of EXOB is active. This question was resolved by experiment

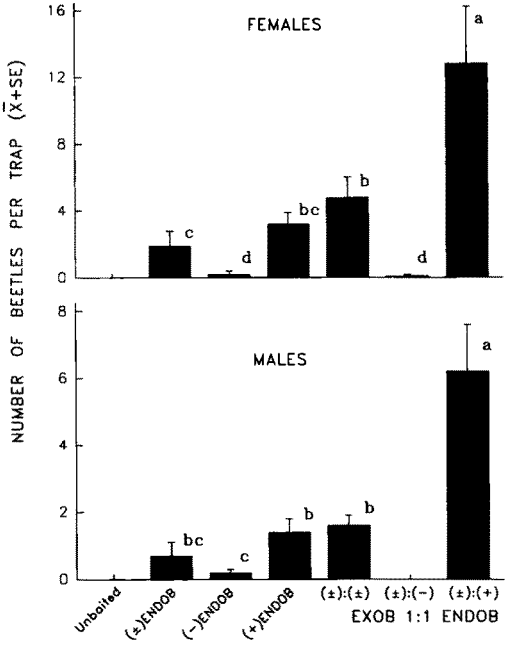

FIG. 4. Numbers of *Dryocoetes affaber* caught in experiment 2 to traps baited with (+), (−), and (±)-*endo*-brevicomin (ENDOB) and blends of (±)-*exo*-brevicomin (EXOB) and (ENDOB) mixed in a 1:1 ratio, in three enantiomeric combinations, nine replicates, August 9 to September 25, 1991. Bars with the same letter are not significantly different, Ryan-Einot-Gabriel-Welsh multiple F test, $P < 0.05$.

3, in which (+) or (±) EXOB in combination with (+)ENDOB elicited the highest levels of response (Figure 5). These results indicate that (+)EXOB is active and that (−)EXOB is inactive. The partial activity of the (−):(+) blend (Figure 5) can be attributed to the 7.4% (+)EXOB impurity. In experiment 4, binary blends containing only (−)ENDOB were not attractive (Figure 6), confirming that (+)ENDOB is the active enantiomer.

To facilitate interpretation of enantioselectivity, the pooled results of experiments 2–4 (all conducted in the same forest stand) were plotted as proportions (percentages), with response to the most attractive treatment [(±)EXOB:(+)ENDOB in a 1:1 ratio] normalized to 100% (Figure 7). It should be noted that a 1:1 ratio of (±)EXOB:(+)ENDOB results in a 1:2 ratio of the active (+) enantiomers, the most attractive ratio of geometrical isomers found in experiment 1 (Figure 3). Figure 7 shows evidence for the

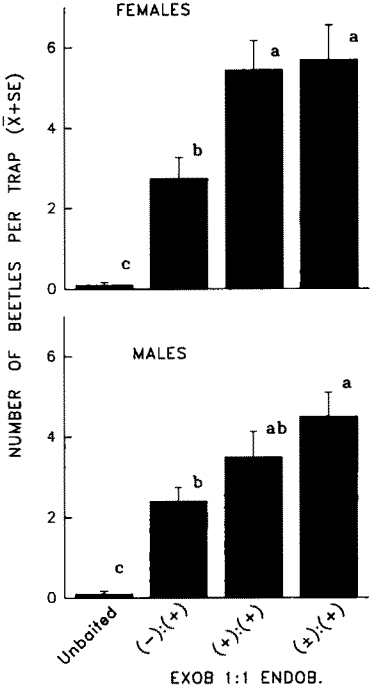

FIG. 5. Numbers of *Dryocoetes affaber* caught in experiment 3 to traps baited with chiral combinations of *exo*-brevicomin (EXOB) and (+)-*endo*-brevicomin (ENDOB) all in a 1:1 ratio, 10 replicates June 16 to July 7, and 10 replicates, July 7–23, 1992. Bars with the same letter are not significantly different, Ryan-Einot-Gabriel-Welsh multiple F test, $P < 0.05$.

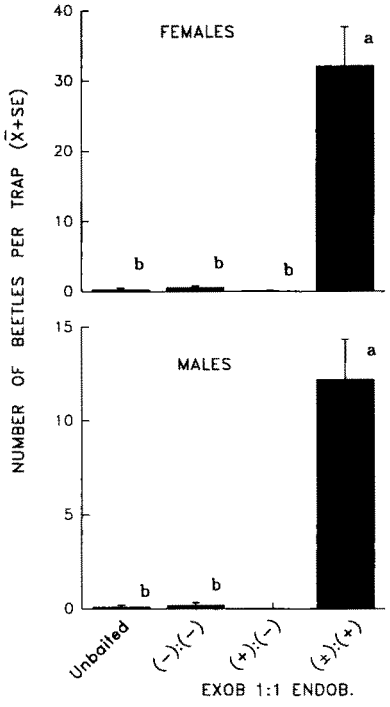

FIG. 6. Numbers of *Dryocoetes affaber* caught in experiment 4 to traps baited with enantiomeric combinations of *exo*-brevicomin (EXOB) and *endo*-brevicomin (ENDOB) all in a 1:1 ratio, 10 replicates, July 22 to August 20, 1992. Bars with the same letter are not significantly different, Ryan-Einot-Gabriel-Welsh multiple F test, $P < 0.05$

combined effect of optical and geometrical isomerism. (+)ENDOB (top row) is revealed to be the major component in the chemical signal. The capacity for (−)ENDOB to cause an inhibition of response to its antipode is shown in the second row from the top (Figure 7).

Synergism between (+)EXOB and (+)ENDOB, either as (+):(+) or (±):(+) blends, is disclosed in the two columns on the left of Figure 7. (+)EXOB is multifunctional, as at high ratios it is inhibitory (Figure 2), just as (+)ENDOB is for *D. confusus* (Camacho et al., 1993).

Cooccurrence of the (−) enantiomers of EXOB and ENDOB has an inhibitory effect for *D. confusus* (Camacho et al., 1993) and the European *D. autographus* (Kohnle and Vité, 1984). It is probable that a similar effect occurs in *D. affaber* (Figure 7).

When the best blends of EXOB:ENDOB for *D. confusus* (Camacho et al.,

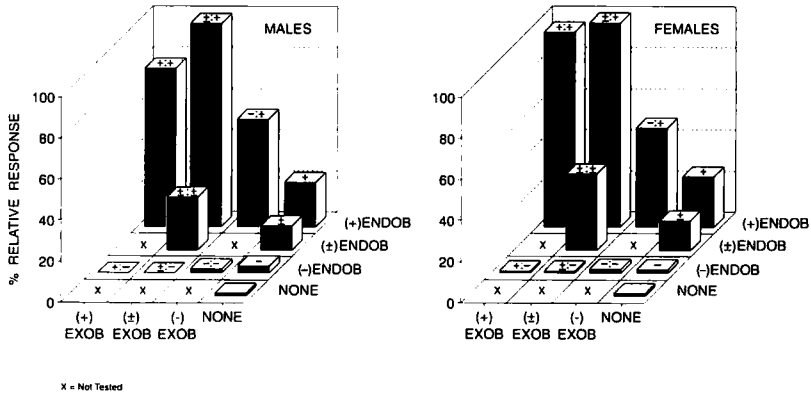

FIG. 7. Summary of pooled results obtained in experiment 2–4. Data normalized so that 100% response occurs to the blend of (+)-*exo*-brevicomin:(+)-*endo*-brevicomin in a 1:1 ratio.

1993) and *D. affaber* (Figures 2–7) were tested in the same location in experiment 5, there was a very clear demonstration that the responses of the two sympatric *Dryocoetes* spp. to blends of EXOB:ENDOB were highly species-specific (Figure 8). The numbers of *D. confusus* captured in response to the 1:2 blend, and of *D. affaber* attracted by the 9:1 blend were not statistically different from the captures obtained with unbaited traps (Ryan-Einot-Gabriel-Welsh multiple F test, $P < 0.05$). Our results support the hypothesis of semiochemical-based reproductive isolation advanced by Camacho et al. (1993), and we conclude that a mechanism of pheromonal exclusion based on the ratio of EXOB:ENDOB and on discrimination of enantiomers exists between *D. affaber* and *D. confusus*.

D. affaber is not sympatric with *D. confusus* over much of its range (Bright, 1963, 1976). The evolutionary forces operating in the development of fine tuning of pheromone channels when closely related species are in sympatry would not exist in allopathy. Therefore, we hypothesize that character displacement of pheromones could occur in *D. affaber* where it is sympatric with *D. confusus*. Studies on *D. affaber* pheromones in other areas might well disclose considerable variation in the production of and response to pheromones.

Practical Implications. Our results reaffirm that if attractive semiochemicals are to be used efficiently against *D. confusus*, e.g., to contain and concentrate infestations prior to logging (Stock et al., 1993), regulation of the composition and chirality of semiochemical baits is critical. Conversely, the evidence that ENDOB is a multifunctional pheromone for *D. confusus* (Camacho et al., 1993) is now stronger; it is involved in attraction at low ratios and is repellent at higher

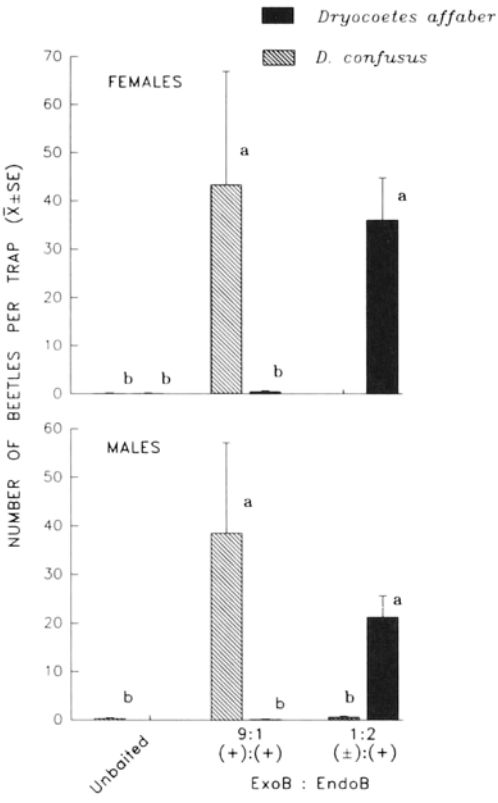

FIG. 8. Numbers of *Dryocoetes affaber* and *D. confusus* caught in experiment 5 to traps baited with optimal blends for each species of *exo*-brevicomin (EXOB) and *endo*-brevicomin (ENDOB), 10 replicates, June 16 to July 7, 1992. Bars with the same letter within each species are not significantly different, Ryan-Einot-Gabriel-Welsh multiple F test, $P < 0.05$. Note that a 1:1 ratio of (±)EXOB:(+)ENDOB results in a 1:2 ratio of (+) enantiomers.

ratios with EXOB (Figure 8). Thus according to our results, the use of ENDOB to prevent or deter attack by *D. confusus* in high hazard stands (Stock et al., 1990; Stock, 1991), requires a formulation with appropriate enantiomeric composition released at adequate rates.

Borden (1992) proposed some novel tactics for the use of semiochemicals, among them the use of pheromone-induced competitive displacement of tree-killing bark beetles by secondary species. This method of biological control was proposed for mountain pine beetles (MPB), *Dendroctonus ponderosae* Hopkins.

MPB can be effectively displaced by pine engravers, *Ips pini* (Say), which rapidly utilize all available phloem tissue (Rankin and Borden, 1991). *D. affaber* is often present in large number in hosts infested by the spruce beetle, *Dendroctonus rufipennis* (Kirby), and is involved in the mortality due to interspecific competition (McCambridge and Knight, 1972). If it can displace *D. rufipennis*, as occurs between *I. pini* and MPB, biological control of the spruce beetle through semiochemically induced competitive displacement with *D. affaber* may be possible.

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DIFFERENT FEEDING AND GUSTATORY RESPONSES TO ECDYSONE AND 20-HYDROXYECDYSONE BY LARVAE OF THE SILKWORM, *Bombyx mori*

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Abstract—The feeding and gustatory responses to ecdysone and 20-hydroxyecdysone were investigated in the silkworm, *Bombyx mori*. 20-Hydroxyecdysone reduced feeding response strongly in fourth- and fifth-instar larvae, whereas ecdysone had no effect on feeding response. 20-Hydroxyecdysone stimulated the R receptor, the receptor to feeding deterrents, to a great degree. By contrast, ecdysone was much less effective for stimulating the R receptor. These results indicate that ecdysone and 20-hydroxyecdysone have different effects on feeding response due to different interactions with mouthpart chemoreceptors.

Key Words—*Bombyx mori*, Lepidoptera, Bombiridae, silkworm, larvae, ecdysone, 20-hydroxyecdysone, feeding behavior, electrophysiology, sensilla response, chemoreceptors.

INTRODUCTION

Ecdysone and 20-hydroxyecdysone are the two major insect molting hormones, but ecdysone is usually thought to be the precursor of the active hormone, 20-hydroxyecdysone (Smith, 1985). However, different effects of the two ecdysteroids have been found in some cases (Oberlander, 1969; Clever et al., 1973; Quennedey et al., 1983; Robert et al., 1986; Perriere et al., 1993).

Recently, we succeeded in inducing 11 larval ecdyses, i.e., seven additional larval ecdyses (ultranumerary larval ecdyses), with low mortality by the application of ecdysone in the silkworm, *Bombyx mori* (Tanaka and Takeda, 1993a),

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and found that dietary supplements of ecdysone and 20-hydroxyecdysone affect larval development differently (Tanaka and Takeda, 1993b). Our results suggest that ingested ecdysteroids affect endocrine organs differently (Tanaka and Takeda, 1993b), but further detailed study is needed to explore the different effect of the two ecdysteroids on development.

20-Hydroxyecdysone affects insect development by inhibiting feeding behavior (Ma, 1972; Schoonhoven and Derksen-Koppers, 1973; Jones and Firn, 1978), whereas the effect of ecdysone on feeding behavior has not been investigated in detail (Jones and Firn, 1978). It is possible that ecdysone has a different effect on feeding behavior from 20-hydroxyecdysone, because our preliminary results show that the amount of feces from larvae reared on the diet supplemented with ecdysone was apparently different from that of larvae reared on the diet supplemented with 20-hydroxyecdysone.

In this study, we report on the different effects of ecdysone and 20-hydroxyecdysone on the feeding behavior of *Bombyx mori*.

METHODS AND MATERIALS

Insect Rearing. The C145 × N140 race of *Bombyx mori* was used in all experiments. Larvae were reared on artificial diet (Yakult Co. Ltd.) at 25 ± 1°C under a photoperiod of 12 hr light and 12 hr dark.

Feeding Response. The larvae used in the experiment were freshly molted larvae (second to fifth instar) within 6 hr after ecdysis. Ten larvae were used in each experiment, and each experiment was replicated three times. The amount of diet actually eaten was estimated by the difference in the dry weight of the diet before and after testing.

Chemicals. Ecdysone and 20-hydroxyecdysone (Sigma Chemical Company) were dissolved in 5% ethanol and added to the diets during diet preparation. Concentration of ecdysteroids were expressed as parts per million (ppm) of dry matter.

Electrophysiological Response. The larvae used in electrophysiological experiment were newly molted fifth instars. The chemoreceptor tested was the R receptor associated with one of the sensilla styloconica (called the Ss-II hair for easy reference) on the maxilla (Ishikawa, 1963).

The electrophysiological methods used in the experiment were the same as described by Ishikawa (1963). The isolated head was fixed on an indifferent, platinum wire electrode. Stimuli were aqueous solutions contained in the stimulating recording electrodes. Stimulation and recording were started simultaneously when the electrode was slipped over the tip of the hair by means of a micromanipulator. The signal was amplified, observed on a cathode ray oscilloscope, and displayed on paper by thermal array recorder. The period of stim-

ulation was 1 sec. Ecdysone and 20-hydroxyecdysone were dissolved in 2×10^{-2} M NaCl solution. Concentrations of ecdysone and 20-hydroxyecdysone were expressed as ppm in 2×10^{-2} M NaCl solution.

RESULTS

Feeding Response. The effects of ecdysone and 20-hydroxyecdysone on feeding behavior were investigated in fifth-instar larvae. Larvae were fed on a diet supplemented with 100 ppm ecdysone or 100 ppm 20-hydroxyecdysone for 36 hr following onset of feeding. The larvae fed on a diet supplemented with ecdysone entered larval apolysis (head capsule slippage occurred) within 36–48 hr, but the amount of diet eaten during the 24 hr following onset of feeding was not different from the control (Figure 1). By contrast, the larvae fed on a diet supplemented with 20-hydroxyecdysone did not enter apolysis. They did not begin spinning within 48 hr (data not shown) and continued to feed, but the amount of diet eaten was reduced compared with the control. Feeding activity was markedly diminished during the 12 hr following onset of feeding. The amount of diet eaten was reduced by 33% of the control. Feeding activity recovered to normal immediately following the first 12 hr.

Feeding activity during the 12 hr following onset of feeding decreased in accordance with the concentration of 20-hydroxyecdysone (Figure 2). 20-Hydroxyecdysone at more than 50 ppm was effective in reducing feeding,

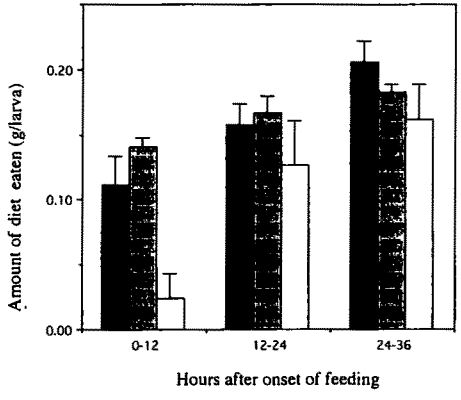

FIG. 1. Effect of ecdysone and 20-hydroxyecdysone on feeding activity of newly molted fifth-instar larvae. The amount of diet eaten is indicated as dry weight per 12 hr. Bars indicate the standard deviations. ■: control, □: ecdysone (100 ppm), □: 20-hydroxyecdysone (100 ppm).

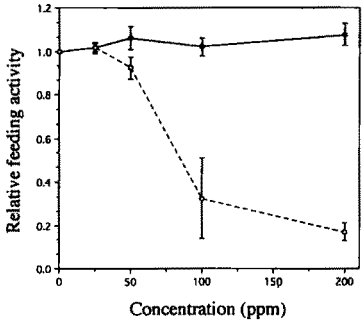

FIG. 2. Effect of different concentrations of ecdysone and 20-hydroxyecdysone in diet on feeding activity of newly molted fifth-instar larva. Relative feeding activity is indicated by the ratio of the amount of diet eaten to that of the control larvae during 12 hr following onset of feeding. Bars indicate the standard deviations. ●: ecdysone, ○: 20-hydroxyecdysone.

and ecdysone had neither an inhibitory nor stimulatory effect on feeding activity at any concentration tested.

Next, we investigated the relationship between the developmental stage of the larva and the feeding response to ecdysone and 20-hydroxyecdysone. Feeding activity during the 12 hr following onset of feeding at different concentrations of ecdysone and 20-hydroxyecdysone were investigated from the second to fifth instars (Figure 3). 20-Hydroxyecdysone tended to reduce feeding activity by second- and third-instar larvae but the deterrent effects were pronounced for fourth- and fifth-instar larvae. 20-Hydroxyecdysone at more than 100 ppm markedly reduced feeding in the fourth- and fifth-instar larvae. By contrast, ecdysone had little effect on feeding activity at any instar regardless of the concentration.

Electrophysiological Study. Electrophysiological responses of the R receptor also differed between ecdysone and 20-hydroxyecdysone (Figure 4). 20-Hydroxyecdysone at 100 ppm in 2×10^{-2} M NaCl solution stimulated the R receptor to discharge many impulses (R impulses), but ecdysone at 100 ppm was much less effective (Figure 4). The discharge frequency of R impulses increased with increasing concentrations of 20-hydroxyecdysone (Figure 5). Even 1 ppm 20-hydroxyecdysone stimulated the R receptor to a great degree. On the other hand, ecdysone only slightly stimulated the R receptor. The discharge frequency slightly increased according to the increase in the concentration of ecdysone, but the response to 100 ppm ecdysone was much lower than the response to 1 ppm 20-hydroxyecdysone.

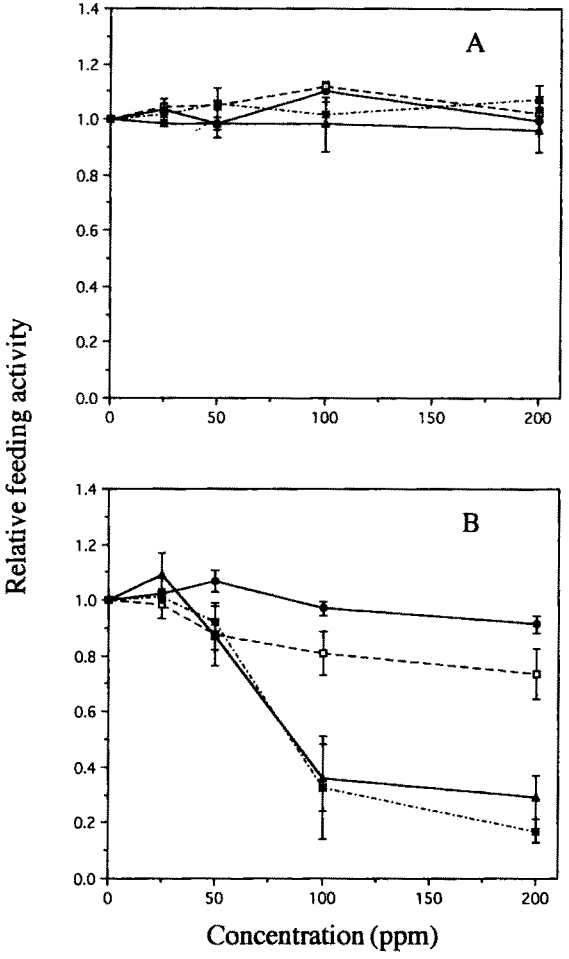

FIG. 3. Effect of different concentrations of ecdysone and 20-hydroxyecdysone on feeding activity from the second to fifth instar. Relative feeding activity is indicated by the ratio of the amount of diet eaten to that of the control larvae during the 12 hr following onset of feeding at each instar. Bars indicate the standard deviations. (A) ecdysone, (B) 20-hydroxyecdysone. ●: second instar, □: third instar, ▲: fourth instar, x: fifth instar.

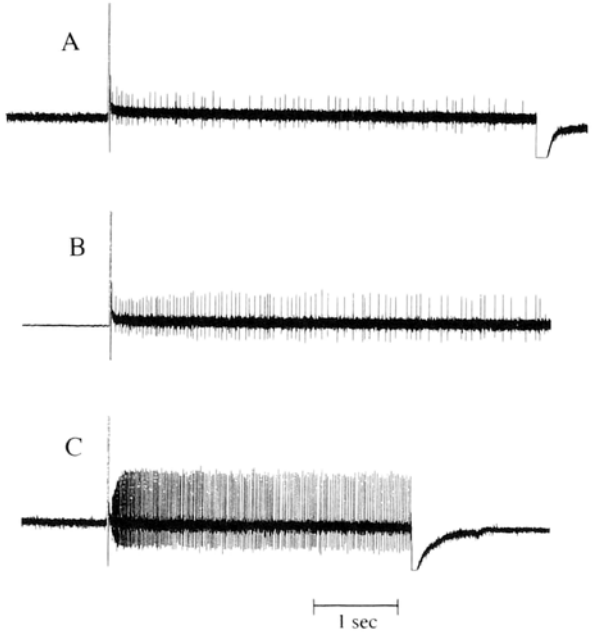

FIG. 4. Recorded impulse from medial styloconic sensillum (the R receptor). All records are from same sensillum. (A) Control (2×10^{-2} M NaCl solution), (B) ecdysone (100 ppm in 2×10^{-2} M NaCl solution), (C) 20-hydroxyecdysone (100 ppm in 2×10^{-2} M NaCl solution).

DISCUSSION

Ecdysteroids have hormonal effects after oral intake, and feeding behavior is influenced by endocrine factors, e.g., hemolymph ecdysteroid level as well as neural input from peripheral chemoreceptors. When ecdysteroid titer in hemolymph begins to increase, larvae cease feeding and enter apolysis or metamorphose; as a result, feeding activity decreases (Dominick and Truman, 1984). Indeed, in larvae exposed to 100 ppm ecdysone, feeding activity declines during the 24–36 hr after onset of feeding as the larvae enter apolysis during this time period. However, in larvae exposed to 20-hydroxyecdysone at more than 100 ppm, feeding was markedly reduced, although all the larvae continued to feed and did not enter larval apolysis, nor did they begin spinning within 48 hr after onset of feeding. Furthermore, the hemolymph ecdysteroid level of larvae exposed to 20-hydroxyecdysone was lower than that of larvae exposed to ecdysone during the 36 hr following onset of feeding (data not shown). These results

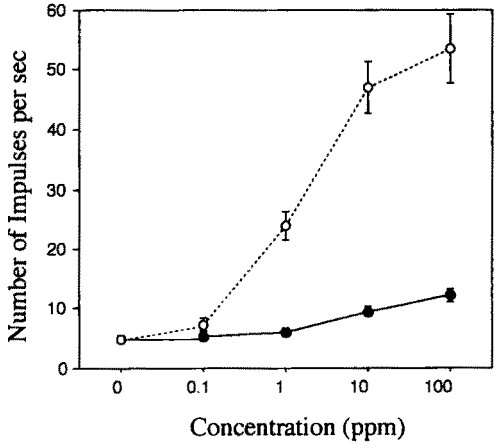

FIG. 5. Comparison of the R receptor responses to ecdysone and 20-hydroxyecdysone. Concentrations of ecdysone and 20-hydroxyecdysone were expressed as parts per million in 2×10^{-2} M NaCl solution. Each value is the mean of three replicates. Bars indicate the standard errors. ●: ecdysone, ○: 20-hydroxyecdysone.

suggest that the reduction in feeding activity during the 12 hr following onset of feeding was not caused by a hormonal effect of 20-hydroxyecdysone.

The R receptor is stimulated by bitter substances, for example, alkaloids, salicin, and phlorizin (Ishikawa, 1966), and the sensory input from R receptors inhibits feeding, specifically by inhibiting biting behavior (Hirao, 1978). In our experiments, 20-hydroxyecdysone stimulated R receptors and reduced feeding activity just after ecdysis, but ecdysone did not stimulate R receptors nor did it reduce feeding. These results suggest that the two ecdysteroids have different effects on feeding behavior because they act on peripheral chemoreceptors differently and that hydroxylation at C-20 is important for inhibition of feeding in larvae of *Bombyx*. This system in *Bombyx* may be a useful tool for structure–deterrent activity study.

In larvae exposed to 20-hydroxyecdysone, feeding activity recovered immediately following the first 12 hr, as shown in Figure 1. The mechanism of recovery has not been investigated yet, but habituation to feeding deterrents may occur, as reported by Jermy et al. (1982), or starvation may have some effect on feeding behavior in the larvae.

Ecdysteroids (phytoecdysteroids) have been found in many species of plants, and the most abundant phytoecdysteroid is 20-hydroxyecdysone (Lafont and Horn, 1989). By contrast, ecdysone is much less common than 20-hydroxyecdysone, and ecdysone has been isolated only from ferns (Kaplanis et al., 1967; Heinrich and Hoffmeister, 1967; Takemoto et al., 1973). Mulberry leaf, *Morus*

alba, which is a sole food plant for *Bombyx*, also contains 20-hydroxyecdysone and inokosterone but does not contain ecdysone (Takemoto et al., 1967). These data suggest that ecdysone is not effective for protecting plants against phytophagous insects, but it has been reported that both ecdysone and 20-hydroxyecdysone act as antifeedants in *Pieris brassicae* (Jones and Firn, 1978). We must await further investigation in other insects to adequately test this hypothesis.

Phytophagous insects have developed very efficient protective mechanisms against ingested ecdysteroids, i.e., the absorption of ingested ecdysteroids into body tissues is slow and limited, whereas the excretion and catabolism is rapid (Hikino et al., 1975). Thus, ingested ecdysteroids have little effect on insect development, except in some cases (Robbins et al., 1968; Shigematsu et al., 1974; Singh and Russell, 1980; Kubo et al., 1983). However, the larvae can ingest large amounts of ecdysone, which cannot be metabolized or excreted rapidly, and is enough to induce ecdysis within a short period because ecdysone has no inhibitory effect on feeding behavior. Furthermore, we are now considering that the high level of ecdysteroids in the hemolymph of ultranumerary-ecdysed larvae (Tanaka and Takeda, 1993a) are mainly caused by exogenous ecdysteroids, not by endogenous ecdysteroids, because of the modest activation of prothoracic glands (data unpublished). Thus, the lack of inhibitory effect of ecdysone on feeding behavior may be one of the important factors for inducing ultranumerary larval ecdyses by ecdysone.

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USCHARIN, THE MOST POTENT MOLLUSCICIDAL COMPOUND TESTED AGAINST LAND SNAILS

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Abstract—*Calotropis procera*, is a shrub with broad ovate fleshy leaves that grows wild in the Egyptian deserts. The plant was discovered to be highly toxic to the land snails *Thepa pisana*. The active ingredient responsible for the molluscicidal activity was isolated from its latex by solvent extraction and partitioning and was finally purified by fractional crystallization from 95% aqueous ethanol. The purity of the isolated material was monitored by TLC. Chemical identification was carried out using mass, infrared, and proton magnetic resonance spectroscopic methods. The active compound was found to be uscharin, and its identity was confirmed by comparing its spectroscopic data with the literature values. The isolated compound was 128 times more toxic than methomyl to the snails tested.

Key Words—*Calotropis procera*, land snails, *Theba pisana*, molluscicides, methomyl, uscharin, cardenolides, land mollusks, mexacarbate, methiocarb, glycosides.

INTRODUCTION

The white garden terrestrial snail *Theba pisana* (Muller) causes great damage to ornamental plants, shrubs, vegetables, fruits, and citrus trees. It is considered one of the most harmful terrestrial Mollusca to the economic production of citrus, ornamentals, and cereals (Cairaschi and Lecomte, 1973; Miller et al., 1988; Baker, 1986). In spite of the economic importance of such land snails, few attempts have been successful in discovering non-hazardous molluscicides

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of natural origin against land snails (Hussein and El-Wakil, 1993). Metaldehyde (r-2, C-4, C-6, C-8-tetramethyl-1,3,5,7-tetraoxocane) and many of the synthetic carbamate compounds such as carbaryl (1-naphthyl methylcarbamate), methomyl [*S*-methyl *N*-(methylcarbamoyloxy)thioacetimidate], methiocarb(4-methylthio-3,5-xylyl methylcarbamate), and mexacarbate(4-dimethylamino-3,5-xylyl methylcarbamate) have been widely used to control land molluscs in toxic baits, but, unfortunately they are used in very high concentrations (0.5–5%), which causes a great threat to human health, the environment and nontarget organisms. In this study we report for the first time the promising molluscicidal activity of uscharin, isolated from the latex of *Calotropis procera* against the land snail *Theba pisana* in comparison to that of methomyl.

METHODS AND MATERIALS

Isolation of Uscharin. The latex of *C. procera* was collected from the plants grown in the eastern desert of Egypt during the summer of 1992, in brown glass bottles by cutting at the tip leaf–stem juncture. Classification and identification of the plant was carried out by the Botany Department, University of Cairo, Cairo, Egypt. The latex (60 ml) was stirred with 100 ml ethanol and filtered (filtrate A), and the precipitate was stirred with 100 ml aq. ethanol (50%) and filtered (filtrate B). Both filtrates A and B were combined and concentrated at 40°C under vacuum until the solution appeared cloudy. This solution was kept at 4°C for 24 hr and was filtered to give a clear yellow filtrate, which was extracted three times with hexane (3 × 150 ml), diethyl ether (3 × 150 ml), and chloroform (3 × 150 ml). The chloroform extract was dried over anhydrous sodium sulfate and evaporated to dryness. The residue was reextracted with chloroform–benzene (1 : 1) (benzene is carcinogenic and should be handled with caution) three times (each 30 ml), evaporated, and then extracted with benzene alone. The benzene extract was shaken with 20% methanol, and the benzene layer was evaporated and extracted with diethyl ether (2 × 30 ml). Thin-layer chromatography of the ether extract showed one major and three minor spots. Further purification of the ether fraction by one washing with hexane–acetone (9 : 1) and three washings with ethanol (1 ml), gave a single spot by the thin-layer chromatography, using 0.1-mm precoated silica gel Kodak chromagram sheets with fluorescent indicator from Eastman Kodak Company, on three solvent systems, chloroform–ethanol (8:2) saturated with water; chloroform–ethanol (2:8), and hexane–acetone (9 : 1). The R_f values of the isolated compound in the indicated solvent systems were 0.81, 0.75, and 0.0, respectively. Detection was carried out by exposure of the plates to iodine vapor, UV light, and by spraying with Raymond reagent (1% *m*-dinitrobenzene in ethanol, followed by 20% NaOH). The melting point of the isolated compound was 290°C with decomposition.

Molluscicidal Activity. Adult animals of *Thepa pisana* were chosen for testing (average weight was 0.93 g). Three replicates were used for each dose; in every replicate 10 animals were kept in a 0.5-liter glass jar covered with cloth netting and secured with a rubber band. The highest tested dose of the isolated compound was prepared by dissolving 2 mg in 1.8 ml of DMSO (dimethylsulfoxide), which was diluted to 24 ml with H_2O. Lower dosages were prepared by further dilutions with H_2O (a much lower volume of DMSO can be used, but we used this volume to avoid any possible precipitation). Control animals were treated with the same solvent. The tested dose contained in 30 μl of these solutions was gently applied on the surface of the snail body inside the shell using a micropipet. The snails were provided with lettuce leaves to feed on after 24 hr of treatment. Dead animals were detected 24, 48, and 72 hr after treatment by loss of response to a thin stainless steel needle according to the WHO (1965) procedure. Methomyl 90% (methavin 90 sp Rhone Poulenc A.G.), was used to compare its molluscicidal activity to that for the purified compound in the same way. Ethanol (95%) was used to prepare the aqueous tested dosages of methomyl, where the highest concentration used of ethanol the same as that of DMSO, 7.5%.

Instruments and Conditions. Melting point is uncorrected. ^{1}H NMR spectrum was recorded in $CDCl_3$ on a 300-MHz GE NMR spectrometer. Electron impact mass spectroscopy was performed on a Finnigan-3300 spectrometer at 70 eV. The infrared spectrum was recorded on Perkin Elmer 2000 FT-IR spectrophotometer.

RESULTS AND DISCUSSION

The unusual tolerance of *Theba pisana* snails to most known synthetic organophosphate and carbamate pesticides renders their control difficult in areas where they overpopulate. Trials are being continued to select effective pesticides against this species. In 1988, Miller et al. tested many bait and sprayable molluscicides against this snail and found that methomyl (1%) bait caused only 16% mortality after 12 days in the lab and 30.3% mortality after 14 days in small field cages. The most effective sprayable molluscicides they tested in the lab were mesurol (which caused 32% mortality after 12 days when sprayed at rate of 4.48 kg active ingredient/hectare), and zectran, which caused 40% mortality at concentration of 0.48%. In our study we used the topical application method which was found reproducible, easy, and rapid for screening any compound that may be used by spray application. The LD_{50} of methomyl was found to be 105 μg/snail, while that for uscharin was 0.82 μg/snail after only 24 hr, which means that, the snail has to come in contact with 30 μl of 0.0027% uscharin solution, which is considered very small when compared with the concentrations used of

synthetic molluscicides. Table 1 shows toxicity data of uscharin, methomyl, and other carbamates tested against *T. pisana* snails. The glycosides of *C. procera* were isolated many decades ago (Hesse et al., 1939; Hesse and Ludwig, 1960), but this is the first report of such a strong molluscicidal activity for one of these glycosides. Hussein and El-Wakil (1993) isolated an extract highly toxic to *Theba pisana* from the latex of this plant, and many workers reported on the nematicidal, insecticidal, and rodenticidal properties of this plant (Verma et al., 1989; Sharma, 1985; Pahwa and Chatterjee, 1988).

The mass spectrum of the isolated compound shows the molecular ion peak (M^+) at *m/z* 587, corresponding to the molecular formula $C_{31}H_{41}NO_8S$. The molecular ion loses CO and $2H_2O$ simultaneously, giving rise to a fragment at *m/z* 523. The fragment ion at *m/z* 404 represents the genin (G) fragment, which indicates the loss of the sugar moiety containing nitrogen. The fragment ions at *m/z* 386, 368, and 358 represent G-H_2O, G-$2H_2O$ and G-H_2O-CO, respectively. These data show that the sugar part most probably contains a thiazolidine ring, which is confirmed from the IR absorption band at 1630 cm^{-1} (C=N stretching) and ^1H NMR signals at δ = 7.5 ppm, s, 1H (N=CH) and δ = 3.87 ppm, s, 2H ($S-CH_2$). Moreover, an aldehydic proton is observed at δ = 10.00 ppm,

TABLE 1. EFFICACY OF USCHARIN, METHOMYL, AND SPRAYABLE MOLLUSCICIDES AGAINST *T. pisana* SNAILS IN LABORATORY TESTS

Toxicant	Active ingredient (kg/ha)	Conc. (%)	Mortality (%)
Uscharin[a]		0.0083	100
		0.0066	80
		0.0033	60
		0.0027	50
		0.0017	20
Methomyl[a]		0.35	50
Control			0
Slug-N-Snail Special Spray (50%) metaldehyde**	4.7	0.503	10
Zectran 2EC (mexacarbate)**	4.48	0.48	40
Mesurol 75 WP (methiocarb)**	4.48	0.96	32
Lance 480 g/liter (cloethocarb)**	2.24	0.48	28
Control			04

[a]Toxicants tested according to our method.
[b]Results obtained by Miller et al. (1988), where conc. (%) was calculated based on the volume of water used to spray the toxicant.

s, 1H, confirming the IR absorption band at 2860 cm^{-1} typical for an aldehyde group. The ^1H NMR also shows the presence of two methyl signals at δ = 0.82 ppm, s, 3H and δ = 1.29 ppm, s, 3H, the latter being attached to the oxygen function of the sugar moiety. The downfield value of the signal at δ = 5.87 ppm, s, 1H shows that a proton is attached to a O—C=O group, which is in agreement with the absorption bands in the IR spectrum at 1788 cm^{-1}, 1745 cm^{-1}, and 1630 cm^{-1} characteristic of a butenolide system.

The data obtained were compared to those of uscharin (Figure 1), a cardenolide previously isolated from *Calotropis procera* (Bruschweiler et al., 1969), and were found to be identical. The ^1H NMR chemical shift values are given in Table 2.

Many workers isolated uscharin with different melting points according to the final solvent used in its purification (Seiber et al., 1982; Hesse et al., 1939).

We have studied the molluscicidal properties of uscharin. The rest of the *C. procera* glycosides and other cardiac glycosides from other plants should be evaluated for their molluscicidal activity. We expect many of them to be active in this field because they have almost the same mode of action.

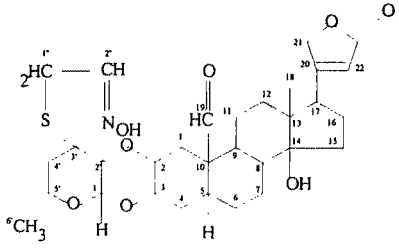

FIG. 1.

TABLE 2. ^1H NMR CHEMICAL SHIFT VALUES OF USCHARIN

Proton	Chemical shift (ppm)
H_3-18	0.82, s
H-19	10.00, s
H-21	4.85, dd, J = 18, 20 Hz
H-22	5.87, br.s
H_2-1′	5.06, s
H_3-6′	1.22, d, J = 6 Hz
H_2-1″	3.87, s
H-2″	7.51, s

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SEX PHEROMONE ACTIVITY IN A SINGLE COMPONENT OF TERGAL GLAND EXTRACT OF *Lutzomyia longipalpis* (DIPTERA: PSYCHODIDAE) FROM JACOBINA, NORTHEASTERN BRAZIL

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Abstract—The sex pheromone component of male *Lutzomyia longipalpis* tergal gland extract was isolated and its activity confirmed by bioassay. Whole tergal gland extract was analyzed by HPLC and fractions were collected as they eluted from the detector. Each fraction was tested in an attraction bioassay with virgin unfed female *Lutzomyia longipalpis*. HPLC analysis showed that whole extract contained several peaks; one large peak, one small peak and several minor peaks. Purity of the HPLC fractions was determined by GC analysis. The bioassays revealed that the large peak was responsible for most of the observed female behavior. The addition of the small peak to the large peak improved the response although by itself the small peak failed to elicit any significant behavior. Minor peaks failed to elicit any response. Chemical analysis revealed the large peak to be a relatively nonpolar hydrocarbon.

Key Words—sex pheromone, tergal gland, *Lutzomyia longipalpis*, Diptera, Psychodidae, gas chromatography, high-pressure liquid chromatography.

INTRODUCTION

The sandfly, *Lutzomyia longipalpis* (Lutz & Neiva) is the only vector of the protozoan parasite *Leishamnia chagasi*, the causative agent of visceral leishmaniasis (VL) in South and Central America. Of all the cases of VL recorded

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from South America, 97% are from Brazil, and of these over 67% are from the northeast of the country (Adler, 1964; Ward et al., 1983).

L. longipalpis exists as a species complex and has been the subject of pheromone investigations since 1961, when Barth described the presence of an "odiferous gland" on males. He believed that these glands were involved in stimulating the female before copulation.

Mangabeira (1969) described two morphologically distinct forms of male *L. longipalpis*; one had pale patches of cuticle on the third and fourth tergites, and the other had pale patches on the third tergite only. Lane et al. (1985), showed that the pale patches contained numerous mammiform papules 3–3.5 μm in diameter, with central pores 0.25 μm in diameter. Lane and Bernardes (1990) showed that the papules were associated with underlying glandular tissue. The individual cells of this tissue possessed a complicated end apparatus and a central reservoir that was connected to the papule pore via a cuticular duct. It was suggested by Lane et al. (1985) that these glandular areas produced a sexual attractant pheromone that was distributed by the male before and during mating by the fanning of the males' wings.

Clear evidence for the role of these glandular areas in the production of sex pheromones was given by Ward et al. (1989), who demonstrated that females could be attracted from distances of up to 60 cm to filter paper disks impregnated with whole tergal gland extracts.

Two populations of *L. longipalpis* were identified when extracts made from the glandular patches were examined by GC-MS (Lane et al., 1985, Phillips et al., 1986). One population produced a compound with the general formula $C_{16}H_{26}$ and with a mass spectrum similar to farnesene or homofarnesene. Sandflies belonging to this population were from the Lapinha and Sobral regions of Brazil. The other population produced a compound with the general formula $C_{20}H_{32}$ and a mass spectrum similar to a diterpene. Sandflies belonging to this population were found in four regions of Brazil: Sobral, Santarem, Marajo, and Morado Nova.

Recently Hamilton and Ward, (1991) showed that there are at least six different populations of *L. longipalpis* in South America that appear to fall into three chemically distinct classes and possibly represent sibling species. GC analysis showed that the chemical composition of tergal gland extract varied both qualitatively and quantitatively between the populations examined and that glandular extracts contained more compounds than had been previously described by Lane et al. (1985).

Although sex pheromone activity has been associated with whole tergal gland extract, it is not known which, if any, of the observed compounds contributed to the attraction of female flies in the bioassays. It is not known if the farnesene/homofarnesene or diterpene compounds described by Lane et al. (1985) and Philips et al. (1986) are pheromonally active. The aim of the current study

was to relate specific compounds present in the glandular extract to pheromonal activity. Chemicals produced by sandflies from the Jacobina region of Brazil, which were used in this study, are believed to be distinct from the farnesene/homofarnesene- and diterpene-producing populations of sandflies (Hamilton and Ward, 1991).

High-pressure liquid chromatography (HPLC) was used to isolate specific components of whole male sandfly tergal gland extract. Collected fractions were then used in an attraction bioassay to determine which compounds elicited activity from female flies. This was seen as a necessary prerequisite to a full structural determination of the active compounds.

METHODS AND MATERIALS

Sandflies. Sandflies used in this study were from Jacobina, Bahia State, northeastern Brazil. They were colonized according to the method of Modi and Tesh (1983) at a temperature of 26°C, 98% relative humidity, and a 12:12 hr light–dark photoperiod.

Extract Preparation. Adult males were removed from stock cages four to five days after emergence. Pheromone glands have been shown to be highly vacuolated in flies of this age (Boufana, 1990). Thirty males were prepared for each extract by placing them in a −70°C freezer. The third and fourth tergites were then removed under a binocular dissecting microscope and placed into acid-cleaned 2-ml vials containing 150 μl of pesticide-grade hexane (BDH, Poole, England). The volume of hexane was reduced to 30 μl under a gentle stream of N_2. This gave a standard concentration of one male equivalent (1 ME) per microliter of solvent. To minimize loss of volatiles and ensure the complete extraction of material, samples were stored overnight at −70°C in sealed vials before analysis.

High-Pressure Liquid Chromatography (HPLC). HPLC analysis was performed on a Waters HPLC system comprised of a 600E multisolvent pump and gradient controller, U6K injector, and 991 photodiode array detector (Waters, Watford, England). Data were acquired and analyzed using a dedicated NEC APC IV personal computer running Waters 991 software. Analysis was carried out on a Resolve C18 analytical column (3.9 × 300 mm) heated to 30°C by a Waters steel column heater. The mobile phase was acetonitrile–isopropanol 70:30 at 1 ml/min (isocratic flow), sparged with helium at 25 ml/min prior to and during analysis. Standards used for calibration and monitoring of separations were caryophyllene and caryophyllene oxide (Aldrich, Gillingham, England) (1 μg/μl). Fractions for bioassay and gas chromatographic analysis, generated by the HPLC, were collected manually in 10-ml acid-cleaned beakers as they eluted from the HPLC detector. The nonpolar gland extract was recovered from

the relatively polar mobile phase by a liquid–liquid separation with hexane as the nonpolar phase. Ultrapure water was added to the polar mobile phase solvents to aid phase separation. Purity of collected material was confirmed by gas chromatography.

Gas Chromatography (GC). GC analysis was carried out using a J&W DB-1 column (Alltech, Carnforth, Lancashire) (30 m × 0.32 mm ID, 0.25 μm film thickness) in a Shimadzu model 15-A gas-chromatograph (Shimadzu Corp., Kyoto, Japan) linked to a Shimadzu CR-5A Data-pac integrator. The GC was fitted with a flame ionization detector (FID) operating at 310°C. Injection of unmodified extract was via a Grob split/splitless injector in the splitless mode (sampling time was set at 0.6 min). The carrier gas was helium at 2 ml/min. The injection block temperature was set at 180°C, detector temperature 310°C. The GC was programmed from an initial temperature of 45°C, held for 2 min, to 250°C at 15°C/min. The temperature was held for a further 2 min and then allowed to rise at 10°C/min to 310°C, where it was held for 4 min. One male equivalent (ME) of extract was injected for each analysis. The system was calibrated with caryophyllene (C_{15}), (*E*)-β-farnesene (C_{15}) and *n*-alkane standards (C_{14} and C_{15}) to give relative retention times and to ensure accurate retention time comparisons. Comparative retention time data were obtained by analyzing standards and *L. longipalpis* tergal gland extract at 130°C (isothermal).

Column Chromatography. A silic-gel microcolumn was prepared by placing 240 mg of Bio-Sil A (20–44 μm) (BioRad, Hemel Hempsted, Hertfordshire) in a Pasteur pipet. The column was precleaned by eluting with hexane (10 ml, pesticide grade) (BDH, Poole, Dorset) and diethyl ether (10 ml, chromatography grade) (BDH). Extract (30 ME) was loaded onto the top of the column and then eluted in 3 column volumes of hexane and 3 column volumes of diethyl ether.

Bioassays. Bioassays were carried out using 5-day-old unfed virgin female *L. longipalpis*. Newly emerged females were separated from males in the colony rearing pots before rotation of the male genitalia. This ensured that females were unmated. These virgin females were then maintained in Barraud cages (15 × 15 × 15 cm) in a humid environment (90% relative humidity) and given access to a saturated sugar solution.

Bioassays were performed as described by Hamilton (1992) and are a modification of those originally described by Ward et al. (1989). Two anesthetized hamsters were placed side by side underneath the bioassay cage. Hamsters were anesthetized with a 12 mg/ml solution of sodium pentobarbitone. Two filter paper disks, (2 cm diam.) were placed inside the cage directly over the hamsters. One test disk was treated with the tergal gland extract or fractions of it and the other was the solvent control. In addition to the fractions generated by HPLC, whole male tergal gland extract and hexane as a control were tested in the bioassay. The concentration of extract or purified components of extract in each

bioassay was 3 ME. During the bioassay, the cage was covered with plastic sheeting to reduce the influence of the observers' host odor or air movements. The number of contacts made by females with the test and control disks was recorded over a 20-min period. Observations were recorded every 2 min. Each experiment was replicated 10 times. Extracts and fractions were prepared freshly for each bioassay replicate. Data were analyzed by one-way analysis of variance and unpaired *t* tests.

RESULTS

HPLC Analysis. HPLC analysis resolved the crude extract into a number of peaks that could be collected for analysis by GC and bioassay. A typical HPLC chromatogram is shown in Figure 1. The chromatogram is characterized by one large peak (a) with a retention time (R_t) of 5–6 min, with a smaller peak (b) eluting slightly before it at 5.2 min. Two small unresolved peaks (c and d) may be seen before and after the main peak. A further small peak (e) elutes at 7.5 min. A larger peak (f) elutes at 10.19 min; this is equivalent to the R_t of cholesterol. Early peaks, up to 4 min, are believed to be nonpolar sandfly-derived compounds and solvent injection effects. Five fractions were collected for bioassay: fraction 1 (F1), material up to 5 min; fraction 2 (F2), small peak; fraction 3 (F3), large peak; fraction 4 (F4), small and large peak together (doublet); fraction 5 (F5), remaining material up to 12.5 min. UV spectra were obtained for peaks a and b, λ_{max} was found to be 204.3 nm for these unknowns, 208.2 nm for caryophyllene and 209.5 nm for caryophyllene oxide. Retention times for caryophyllene and caryophyllene oxide were 4.74 and 3.26 min, respectively.

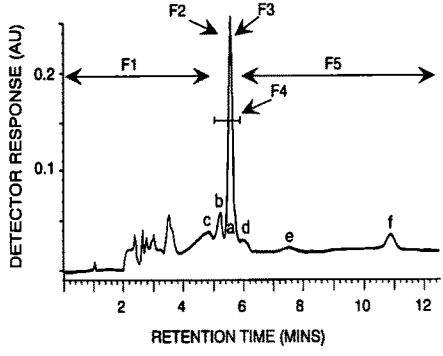

FIG. 1. HPLC chromatogram (UV max) of tergal gland extract (30 ME). Fractions collected are indicated as F1–F5.

GC Analysis. GC analysis showed that HPLC collected peaks were free from contamination with other glandular material and correlated with peaks seen in the GC analysis of tergal gland extract with HPLC peaks. A typical GC chromatogram of unfractionated, tergal gland extract is presented in Figure 2A. Two dominant peaks were found at 10.0 and 10.6 min, respectively. Several other smaller peaks were observed before and after the large peaks. The area of the smaller peak was found to be 17.9% of the area of the larger peak. The small HPLC peak (b) corresponded to the small GC peak (1) (Figure 2C, R_t 10.6 min). The large HPLC peak (a) corresponded to the large GC peak (2). Both peaks in F2 and F3 appeared to be almost 100% pure by GC analysis

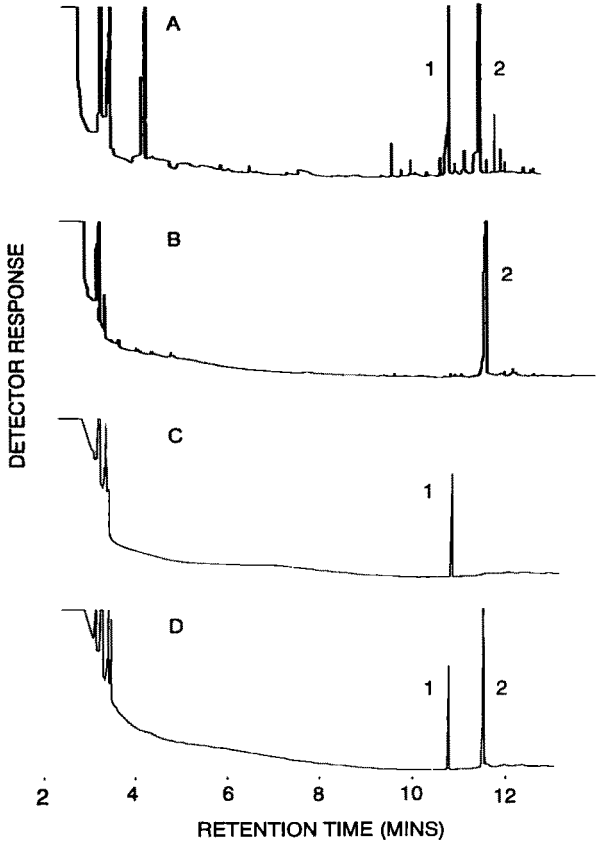

FIG. 2. GC analysis of tergal gland extract. A = whole extract; B = HPLC collected large peak (F3); C = HPLC collected small peak (F2); D = HPLC collected large and small peaks (F4).

(Figure 2B and C). Fraction 4, which contained both the large and the small HPLC peaks (1 and 2), had a slightly lower percentage of small peak (when compared to the unfractionated crude extract) Figure 2D. Chromatograms for F1 and F2 are not presented. GC analysis also indicated that each male produced approximately 70 ng of the principle component and 8 ng of the secondary compound. The C_{14} and C_{15} *n*-alkane standards had retention times of 6.99 and 10.98 min, respectively. The caryophyllene standard had a retention time of 7.57 min. The large peak (peak 1) had a retention time of 10.42 min and the small peak (peak 2) had a retention time of 8.41 min. The farnesene standards had a retention time of 8.6 min, which is significantly different from both the large and small peaks.

Column Chromatography. The hexane fraction (hydrocarbon fraction) contained both peaks 1 and 2, which are normally seen in the crude extract (Figure 2A).

Bioassays. Bioassay results are shown in Figure 3. When virgin female sandflies were exposed to F3 from tergal gland extract, they responded in a way that was not significantly different from their response to the whole tergal gland extract. The whole extract gave a response of 273 $\pm$ 102.4 contacts in the 20-min observation period. The large peak (F3) gave a response of 219 $\pm$ 29.1 and the doublet (F4) gave a response of 242 $\pm$ 39.0.

The activity exhibited by female *L. longipalpis* to whole extract was not significantly different from activity exhibited to the doublet (F4) ($F = 5.34$, $P > 0.01$). There was no significant difference between the response to the large peak (F3) and the doublet (F4), ($F = 2.97$, $P > 0.01$). However, there was significant difference between the females' response to whole extract and the single large peak (F3) ($F = 15.9$, $P = 0.0017$). This indicated that the small peak may have had a significant biological effect, however, when this peak was analyzed by itself, it was found to induce a response significantly less than either whole extract, F4 or F3.

The responses to the small peak (F2), early peaks (F1), late peaks (F5), and hexane blends are all significantly less than for whole extract, doublets, and large peak by itself. Within this group of observations, however, the response to F1, the prepeaks, was found to be significantly greater than the response to the small peak ($F = 11.46$, $P = 0.002$) and the late peaks ($F = 41.85$, $P = 0.0001$). None of these responses are significantly different than to the hexane double control [small peaks (F2) $F = 1.83$, $P = 0.22$); prepeaks (F1) ($F = 5.42$, $P = 0.06$); late peaks (F5) ($F = 6.68$, $P = 0.02$].

DISCUSSION

These experiments clearly demonstrated that the largest peak (F3) of the *L. longipalpis* male tergal gland extract is responsible for the sexual behavior exhibited by females. GC analysis revealed that the tergal gland extract contained at

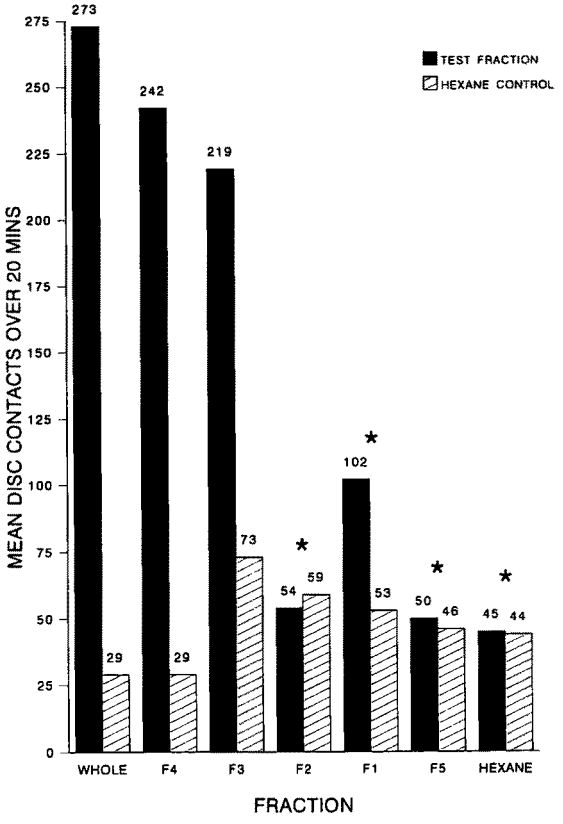

FIG. 3. Bioassay results of HPLC fractions of tergal gland extract. Not significantly different from hexane controls.

least 11 minor components not described by Lane et al. (1985) and Phillips et al. (1986). These minor compounds do not appear to play a significant role in the attraction of females to treated filter paper disks.

The experimental procedure allowed the comparison of compounds collected from HPLC analysis with compounds observed in GC chromatograms. Analysis and fractionation by HPLC allowed us to collect much larger quantities of material for bioassay than would have been possible by preparative GC without the possibility of thermal degradation or rearrangement of collected material. Reextraction of sandfly components from the HPLC eluent gave a high yield of pure material for bioassay and was an efficient procedure.

The chemical data presented here indicates that the main active peak is a

hydrocarbon. UV data indicate that there may be one or two double bonds. GC and HPLC retention time comparisons support this view and suggest that if the compound is biosynthetically related to the farnesene/homofarnesene compound described by Lane et al. (1985), it should be cyclic or polycyclic. Neither peak appears to be (*E*)-β-farnesene.

It was clear from observations made during the bioassay that behavior released on perception of the isolated pheromone (F3) closely resembled that of the unfractionated tergal gland extract. Females in contact with a pheromone-treated disk or a disk treated with F3 performed characteristic courting behavior: wing fanning (rapid wing fluttering), arrestment on filter paper disk (females remain in contact with filter paper disk for a long period of time), abdomen dipping (posterior end of abdomen is moved to touch the filter paper disk), aggression displayed towards other females, and strike behavior exhibited toward the filter paper disk.

Male sex pheromones are not uncommon but are considerably less so than those of females (Tamaki, 1985). They have been reported from at least 17 species of Diptera belonging to the families Calliphoridae, Chironomidae, Culicidae, Drosophilidae, Muscidae, Psychodidae, Sarcophagidae, and Tephritidae. They usually act as short-range aphrodisiac pheromones stimulating the female for copulation after the sexes have been brought together by the female sex pheromone and other auditory and visual cues. Thornhill (1979) and Trivers (1972) predicted that males would produce sex pheromones when they were providing a significant investment in the offspring, for example, a nutritional investment of some sort that the female can use to manufacture eggs (Boggs and Gilbert, 1979). It is interesting to note that female sandflies are attracted to a combination of host odor and sex pheromone. Male *L. longipalpis* often form mating leks in association with a host odor source, for example, on the backs of cattle or around chicken coops (Morton and Ward, 1989; Ward et al., 1989). Males of certain species of *Drosophila* aggregate at food and oviposition sites (Spieth, 1974). Male-produced aggregation pheromones normally released at such sites may be synergistic with food odors for female attraction (Bartlett et al., 1988). The papaya fruit fly, *Toxotrypana curvicauda*, puffs its pleural abdominal pouches, releasing pheromone, while perched on papaya fruit in the field (Landolt and Hendrichs, 1983).

It is not unusual for male pheromones to be single components, as in the case of tsetse fly pheromone (Carlson et al., 1978) or housefly pheromone, or simple blends as in the Mediterranean fruit fly (Baker et al., 1990). Two compounds, methyl (*E*)-6-nonenoate and nonen-1-ol, act as the sex pheromone of the Mediterranean fruit fly, *Ceratitis capitata* (Jacobson et al., 1973). A single compound, 15,19,23-trimethylheptatriacontane, acts as the nonvolatile aphrodisiac contact pheromone of the tsetse fly, *Glossina morsitans morsitans* (Langley et al., 1975; Carlson et al., 1978).

It remains to be seen if any of the other compounds present in the male tergal gland extract play a role in courtship behavior when presented over a longer distance, or, indeed, if their effect may be exerted over shorter distances. In general, male calling may be influenced by external factors such as time of day, temperature, light intensity, and location or occurrence of food or oviposition sites (Burk, 1983; Landolt and Sivinski, 1992). The importance of such factors in the regulation of *L. longipalpis* mating must be determined by experiments in laboratory and field environments before a complete understanding of pheromone-induced mating can be achieved. Experiments will include the entrainment of compounds and their testing in wind tunnels and traps for monitoring and perhaps control of populations in the field.

It has already been established that females are attracted to host odor (Nigam and Ward, 1991). The experiments described above relied on the presence of host odor and females give a significantly improved response to the male pheromone when host odor is present (Hamilton, 1992).

Now that the active component of the tergal gland extract has been identified it will be possible to proceed with a full structural characterization of the compound.

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PHEROMONAL SECRETIONS FROM GLANDS ON THE 5TH ABDOMINAL STERNITE OF HYDROPSYCHID AND RHYACOPHILID CADDISFLIES (TRICHOPTERA)

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Abstract—Extracts of different body parts of adult Trichoptera were tested for electrophysiological activity. Extracts of the IVth and Vth abdominal sternites of female *Hydropsyche angustipennis*, *Rhyacophila nubila*, and *R. fasciata*, containing a paired exocrine gland, elicited significant electroantennographic responses when tested on conspecific male antennae. The paired gland occurs also in males of all the species, and in *H. angustipennis*, extracts from males were more active than female extracts when tested on male antennae. Female and male extracts from all species were analyzed by gas chromatography with simultaneous flame ionization and electroantennographic detection (EAD). EAD-active peaks in female extracts, stimulating male antennae, were identified in *H. angustipennis* as nonan-2-one; and in *R. nubila* and *R. fasciata* as heptan-2-one, heptan-2-ol, nonan-2-one, and nonan-2-ol. EAD-active components from male *H. angustipennis* stimulating male antennae were octan-2-one, nonan-2-one (major peak), (*Z*)-6-nonen-2-one, decan-2-one, and a methylbranched decan-2-one. Female extracts and synthetic mixtures of compounds identified from female *H. angustipennis* and *R. fasciata* were tested for attractivity in the field. High catches with control traps obscured the results, but a synthetic mixture of the four identified compounds was significantly attractive and not different from female extracts for attracting male *R. fasciata*. In *H. angustipennis*, a synthetic six-component male blend, in which nonan-2-one was the major component, attracted significant numbers of male and female *H. angustipennis*. Extracts of male *R. nubila* and *R. fasciata* contained acetophenone and hexanoic and octanoic acids but did not have any electrophysiological or behavioral activity on either male or female

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antennae of conspecifics. The occurrence of a female sex pheromone in *Rhyacophila* and an aggregation pheromone in *Hydropsyche* corresponds to earlier described differences in mating behaviors in the Rhyacophilidae and Hydropsychidae.

Key Words—Trichoptera, Rhyacophilidae, sex pheromone, aggregation pheromone, *Rhyacophila nubila*, *Rhyacophila fasciata Hydropsyche angustipennis*, electroantennogram, heptan-2-one, octan-2-one, nonan-2-one, decan-2-one, (*Z*)-6-nonen-2-one, heptan-2-ol, nonan-2-ol, GC-EAD, swarming.

INTRODUCTION

A critical event in sexual reproduction is location or recruitment of a mate. In a number of insect groups, the necessary movements in time and space are mediated by pheromones. Sex pheromones occur in at least 10 orders of insects but have been studied predominantly in the Lepidoptera, Coleoptera, Hymenoptera, Orthoptera, Diptera, and Homoptera since, in these groups, chemical attraction is a major means of sexual recruitment. Females are usually the emitters and males the receivers (Cardé and Baker, 1984; Birch and Haynes, 1982).

Although evidence has indicated that members of the order Trichoptera use some form of chemically mediated communication (e.g., Kelner-Pillault, 1975), pheromones have only recently been demonstrated for caddisflies (Wood and Resh, 1984). In that study, whole body extracts of females of the sericostomatids *Gumaga griseola* (McLachlan) and *G. nigricula* (McLachlan) were shown to attract conspecific males but not females. The significance of these findings lies not only in the fact that information on chemical communication systems may be important in understanding adult behavior, but may also provide insights into systematic and ecological relations.

Taxonomically, the Trichoptera has been considered a specialized member of the neuropteroid orders, and Ross (1967) regarded the caddisflies as a sister order of the Lepidoptera. Several studies of caddisfly adults have focused on structures that have been presumed to be scent-producing glands (e.g., Cummings, 1914; Mosely, 1923), and anatomical studies (Moretti and Bicchierai, 1981) have referred to the androconial organs that occur throughout the order but only in adult males, as scent- or pheromone-producing glands. The first experimental demonstration of a specific site associated with sex pheromone production was by Resh and Wood (1985). These workers conducted field tests using extracts from females of *G. nigricula* and *G. griseola*, and the limnephilid *Dicosmoecus gilvipes* (Hagen). Extracts of the fifth abdominal sternite attracted conspecific males, thus indicating this region as the site of pheromone production. Microscopic examination revealed paired exocrine glands located at this site, with a pair of muscles attached to the neck of each gland, probably regulating the release of substances from the gland reservoir. Solem (1985) claimed

pheromones to be present in extracts from the fourth abdominal sternite in female *Rhyacophila nubila* (Zetterstedt), although scanning electron microscopy did not show any structure that could be identified as the gland reservoir. Other studies have focused on the potential defensive function of secretions from these trichopteran glands (Duffield, 1981; Ansteeg and Dettner, 1991).

Electrophysiological techniques have proved valuable in the study of insect pheromone systems. The insects' peripheral olfactory system—the antenna—is easily accessible, and electroantennograms (EAGs) have provided a rapid and convenient screening procedure for pheromones (Roelofs, 1976). Interfaced with capillary gas chromatography (GC), electroantennographic detection (EAD) has been a valuable tool for the isolation and identification of pheromone components in moths (Struble and Arn, 1984). Electroantennogram assays were used in the present study to determine the site of pheromone production in two hitherto uninvestigated species of Trichoptera, *Hydropsyche angustipennis* (Curtis) (Hydropsychidae) and *Rhyacophila fasciata* (Hagen) (Rhyacophilidae), as well as in *R. nubila* (Rhyacophilidae), previously studied by Solem (1985). Identification of the electrophysiologically active components was carried out by gas chromatography with electroantennographic detection, and subsequent mass spectrometric (MS) analysis of the active components. Field bioassays for *R. fasciata* and *H. angustipennis* were conducted to evaluate the behavioral significance of the identified compounds.

METHODS AND MATERIALS

Collection and Rearing of Insects. Mature pupae in their cases were collected from the surfaces of small rocks and stones immersed in shallow, fast-running streams at four principal sites in south Sweden. *R. fasciata* was taken from a small stream in the vicinity of the Stampen field station, about 30 km SE of Lund (55°35′N, 13°30′E). *R. nubila* were taken from a stream at S. Åsum (north Sjöbo), about 35 km E of Lund (55°39′N, 13°40′E). *R. fasciata* were also collected at Järsöströmmen, the outlet of Lake Erken in the province of Uppland (59°51′, 17°22′E). *H. angustipennis* were collected near the village of Övedskloster, about 30 km E of Lund (55°41′N, 13°39′E). Adults of *R. nubila* and *R. fasciata* were collected by sweep-netting in the stream shore vegetation, at the same localities where their pupae were collected.

The pupae of each species were kept in aerated water baths in a rearing chamber with a light–dark cycle similar to outdoor conditions at the time of the year, and at a temperature varying between 21 and 25°C. Polystyrene rafts were positioned in the water baths to provide a support for the emergence of the adult insects. Adults were removed and kept separately for identification and sexing prior to experimentation.

Identification and Sexing. *R. fasciata* and *R. nubila*, are distinguished by wing-shape and pattern and by certain characteristics associated with the genital segment and shape of the ovipositor. *R. nubila* has a more angular hind wing, while *R. fasciata* has more conspicuous wing markings (e.g., Esben-Petersen, 1916).

The locality for collection of *H. angustipennis* was inhabited by only one other known species of *Hydropsyche* (*H. siltalai*), comprising approx. 1% of the population and not emerging until June (L. Petersen, personal communication). The identity of the adult males was confirmed using a key developed by Tobias (1972a,b).

Preparation of Extracts. Extracts of individual body parts used in stationary EAG recording experiments and combined GC-EAD recording experiments were prepared by dissecting live adults or adults stored in the freezer for a few days (−20°C). The insects were dissected under a light microscope and the tissues were placed into separate glass vials containing approximately 30 μl of dichloromethane (for EAG) and vials containing dichloromethane or hexane (for GC-EAD). To avoid contamination, dissecting instruments were carefully cleaned with distilled acetone between dissections. Extracts of body parts and synthetic mixtures of EAG-active components used in field bioassays were dissolved in 100 μl dichloromethane. The stimulation of the male antenna by hexanone (present as an impurity in the hexane solvent) shows that the choice of solvent used in the preparation of extracts and synthetic mixtures is important, since the introduction of possible inhibitory or masking compounds may influence experiments intended to show attraction of insects in the field.

Electroantennography. In each of the three species tested, the tip of an excised male antenna was cut off and the antenna was mounted between two pipet electrodes containing Beadle-Ephrussi Ringer (155 mM NaCl, 5 mM KCl, 3 mM $CaCl_2$). The electrode holding the base of the antenna was connected to ground via an Ag–AgCl wire and the distal end of the antenna was connected in the same way to a high-impedance amplifier (10^{12} Ω). A puff of approximately 2 ml of air from a plastic syringe was by an injection device (Syntech) rapidly flushed through a Pasteur pipet containing 2 μl of extract and dispensed into a charcoal filtered and humidified airstream continuously flushing over the antennal preparation at a speed of 0.5 m/sec. Each stimulation with an extract was followed by a control stimulation with solvent only. The resultant electroantennograms were monitored on a storage oscilloscope and analyzed using a Macintosh computer equipped with Maclab software (Chart version 2.5) and hardware.

Gas Chromatography with FID and EAD. A GC effluent splitter (approximate split ratio 1 : 1) and transfer system, which enabled the simultaneous recording of EAG and flame ionization detector (FID) responses, was used. For each species tested, 4 μl of active extract was injected splitless into the GC, equipped either with a DB-1 or a DB-wax (30-m × 0.25-m-ID) fused silica

capillary column (J&W, Folsom, California 95630). Hydrogen carrier gas was supplied at approximately 50 cm/sec linear velocity. The injector temperature was 250°C and the split valve opened 1 min after injection. The column temperature was maintained at 40°C for 5 min following injection and then heated to 220°C at a rate of 10°C/min.

Gas Chromatography–Mass Spectrometry. Mass spectrometry with electron impact ionization, scanning the mass range *m/z* 31–250, was performed on a Hewlett Packard model 5970B GC-MS system interfaced with a 5890 GC, equipped with a DB-wax (30-m × 0.25-mm-ID) fused silica capillary column. Helium carrier gas was supplied at approximately 30 cm/sec linear velocity. Conditions of chromatography were the same as for GC-EAD analysis.

Field Tests. Field tests were conducted at Erken and Stampen (*R. fasciata*) and Övedskloster (*H. angustipennis*). Sticky traps of the Lund II-type described in detail by Anderbrant et al. (1989) were made from two pieces of flat, white cardboard (30 × 30 cm) with 8-cm spacing and positioned on bamboo sticks 60 cm above the ground and not more than 1 m from the stream edge. Treatments diluted in 100 μl dichloromethane were infused into rubber septa (red 5 × 9-mm septa from Arthur H. Thomas Co.) and suspended from the ceiling of each sticky trap. For most experiments, the traps were checked and rerandomized several times during the trapping period. Each reading of a trap catch was then treated as a replicate in the statistical analysis. The catch data were analyzed by a Kruskal-Wallis analysis of variance followed by pairwise comparisons using the Fligner-Policello robust Mann-Whitney U test (Fligner and Policello, 1981).

The first field bioassay was conducted for *R. fasciata* from September 8–24, 1986. Traps baited with five female equivalents (5 FE) of extracts of IVth and Vth abdominal sternites were compared with unbaited control traps and traps baited with 5 FE of a synthetic four-component mixture of the electrophysiologically active gland constituents heptan-2-one, heptan-2-ol, nonan-2-one, and nonan-2-ol in a 100:10:10:1.5 ratio. The major component heptan-2-one amounted to 6.7 μg/bait in this and in all the following experiments. A mixture of 2 μg heptan-2-ol and 0.08 μg of nonan-2-ol was also included in the comparison. Three traps with the same treatment were operated simultaneously and checked three times, thus adding up to nine replicates for each treatment.

In a second experiment, August 10–September 12, 1987, the same four-component mixture was compared with mixtures of the alcohols and ketones, respectively, female sternite extracts (5 FE), and blank traps. This experiment was repeated October 6–November 4, 1987. The total number of replicates for each treatment varied (see Figure 7 below) because of occasionally destroyed traps and a shortage of female extracts. Finally the synthetic female four-component mixture (amount approximately equal to 5 FE) was compared with extracts of male IVth and Vth abdominal sternites (5 ME) and controls. Five

traps were operated from October 19 to November 10, 1988 and checked once ($N = 5$).

In a first experiment with *H. angustipennis* June 17–20, 1988, the attractivity of extracts of female IVth and Vth abdominal sternites (3 FE) was compared to that of nonan-2-one (10 μg) ($N = 6$). In a second experiment, from August 22 to 29, 1988, nonan-2-one and a six-component blend consisting of heptan-2-one, octan-2-one, nonan-2-one, decan-2-one, nonan-2-ol, undecan-2-one in a 0.01 : 0.03 : 100 : 1.5 : 0.25 : 0.65 ratio were compared with control traps. The amount of nonan-2-one in these treatments was 50 μg, corresponding to approximately five male equivalents (5 ME). Five-traps of each treatment were operated simultaneously and checked twice during the experiment ($N = 10$). In a third experiment from June 12 to 16, 1989, male IVth and Vth abdominal sternite extracts (5 ME) were compared with the six-component blend and controls. Six traps with each treatment were operated simultaneously and checked six times during the trapping period.

Chemicals. Samples of heptan-2-ol and nonan-2-ol were a gift from the Laboratory of Chemical Ecology, Göteborg University. (*Z*)-6-Nonen-2-one was a gift from W. Francke, Hamburg. Other compounds were purchased from Fluka.

RESULTS

EAGs of Different Body Parts. In *R. fasciata* and *H. angustipennis*, clear EAG responses were recorded from both male and female antennae when stimulated with body extracts of conspecifics of both sexes. When extracts were prepared from different parts of the body, the highest EAG response was always obtained from extracts of sternites IV–V (Figure 1). During the initial EAG experiments, hexane was used as solvent. The hexane did, however, contain hexan-2-one as an impurity, and this compound released a significant EAG response from the antenna. The hexane was therefore substituted by dichloromethane, which showed no interactions with antennal receptors.

Anatomical Observations of R. nubila. Externally, ventrolateral on the fifth abdominal sternite, two similar structures that appeared to be gland openings and that did not occur on the other segments were found. The structures were not conspicuous, but appeared as a small, slightly glossy protuberance. Internally, a sac/reservoir was found that was connected to the openings. The sacs/reservoirs were also loosely attached to the integument and extended into the fourth segment. We showed by fine dissection and subsequent GC analyses of the extracts that the electrophysiologically (and presumably also behaviorally) active constituents of the sternite extracts (see below) are contained in these reservoirs.

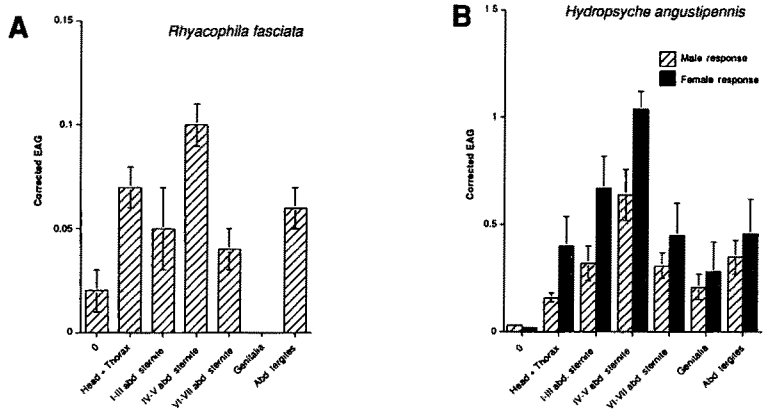

FIG. 1. Electroantennographic responses from male *R. fasciata* antennae stimulated with extracts (4 FE) from different parts of the female body (A) and from male and female *H. angustipennis* antennae stimulated with extracts (5 ME) from different parts of the male body (B).

Identification of Electrophysiologically Active Gland Constituents and Chemically Related Compounds in R. nubila *and* R. fasciata. GC-EAD analysis of female *R. nubila* extracts with a conspecific male antenna as detector gave four reproducibly active peaks on a DB-1 column, corresponding to large FID peaks (Figure 2). Male extracts produced no EAD-active peaks when either male or female antennae were used as detectors. However, two large tailing peaks were produced by male extracts in the FID chromatograms. GC-EAD analysis of female *R. fasciata* extracts with a conspecific male antenna as a detector gave four reproducibly active peaks on DB-1 column (Figure 3). The retention times of these compounds were the same as for compounds I–IV in *R. nubila*. Again, male extracts produced no EAD-active peaks when either male or female antennae were used as detectors, but two large FID peaks similar to the ones found in *R. nubila* were present.

Female and male extracts were subjected to GC-MS analysis. In the female extracts, compound I had a mass spectrum, *m/z* 114(M^+, 4%) 99(2), 85(2), 71(9), 58(49), and 43(100), identical to that of synthetic heptan-2-one. The spectrum of compound II, *m/z* 101(3), 98(M−18, 3%), 83(6), 70(4), 55(16), and 45(100), was similar to that of synthetic heptan-2-ol. The spectrum of compound III, *m/z* 142(M^+, 7%), 127(3), 113(2), 99(2) 85(3), 71(16), 58(86), and 43(100), corresponded to nonan-2-one and compound IV, *m/z* 129(2%), 126(M−18, 2%), 111(3), 98(7), 69(11), 55(16), and 45(100), was similar to that of synthetic nonan-2-ol. The retention times of these compounds were identical to those of synthetic references on both the DB-1 and the more polar

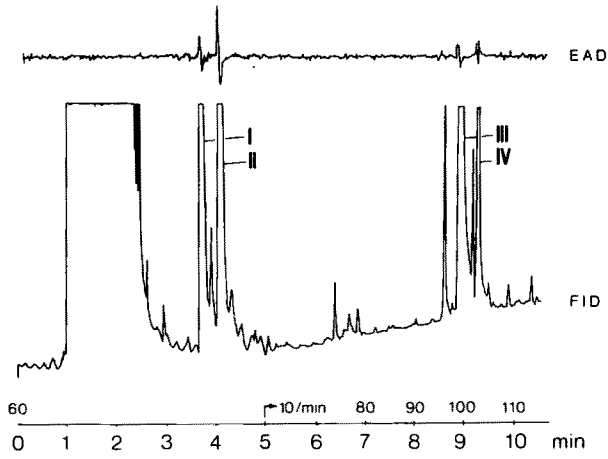

FIG. 2. Gas chromatographic analysis on a DB-1 column of extracts of IVth and Vth abdominal sternites of female *R. nubila* with simultaneous FID and EAD (male *R. nubila* antenna).

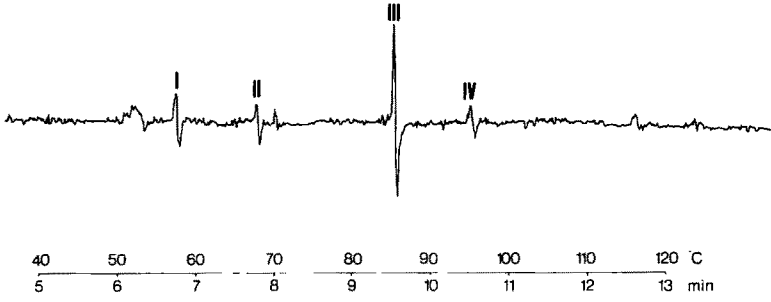

FIG. 3. Gas chromatographic analysis on a DB-1 column of extracts of IVth and Vth abdominal sternites of female *R. fasciata* monitored by EAD (male *R. fasciata* antenna).

DB-wax column. The two major male components had the mass spectra and retention times of hexanoic and octanoic acid, respectively [diagnostic fragments *m/z* 87($M-29$, 12%), 73(43), 60(100), 55(14), 45(21), 41(32) and *m/z* 144(M^+, 1%), 115($M-29$, 11%), 101(25), 73(63), 60(100)]. In addition, a peak eluting before the two acids in the male extracts was identified as acetophenone, containing the characteristic fragments *m/z* 120(M^+, 40%), 105(100), 77(70), and 51(29).

The retention times of all suggested compounds were identical with those of synthetic references on both the DB-1 and a more polar DB-wax column.

GC-EAD of female extracts using a DB-wax column for separation and a male antenna as detector revealed no new active peaks, but confirmed the earlier assignments.

Based on GC analyses of batches of insects that emerged in the laboratory, female *R. nubila* contained about five times as much of heptan-2-one as did *R. fasciata* (approximately 250 and 50 ng/female, respectively). Ratios between heptan-2-one, heptan-2-ol, nonan-2-one, and nonan-2-ol were, on average, 100:120:119:12 in *R. nubila* (six samples each containing one to five females) and 100:338:40:12 in *R. fasciata* (seven samples containing one to three females each). However, these ratios varied considerably between individual insects, and females of the two species could not be identified positively based on the ratios of pheromone components. Analysis of an extract obtained from 15 female *R. fasciata* collected with a net in the field gave the clearly different proportions 100:10:10:1.5.

Identification of Electrophysiologically Active Gland Constituents in H. angustipennis. GC-EAD analysis of female extracts using male antennae as detectors revealed one electrophysiologically active peak (Figure 4A). The mass spectrum of this compound corresponded to that of nonan-2-one.

When male extracts were subjected to GC-EAD analysis with a male antenna as detector, at least five electrophysiologically active components were pinpointed (Figure 4B). The mass spectrum of compound I was identical to that of octan-2-one: *m/z* 128(M^+, 7%), 113(3), 99(1), 85(5), 71(11), 58(69), and 43(100); compound II was identified as nonan-2-one (see above), compound III had a spectrum *m/z* 140(M^+, 4%), 125(4), 111(6), 97(4) 82(41), 67(86), 55(18),

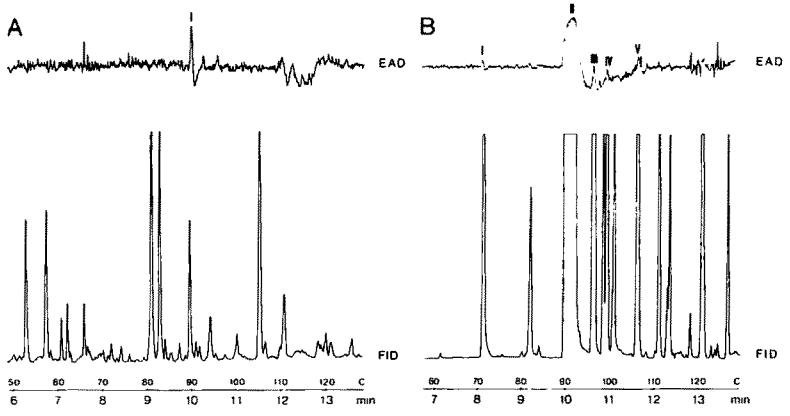

FIG. 4. Gas chromatographic analysis on a DB-wax column of extracts of IVth and Vth abdominal sternites of female (A) and male (B) *H. angustipennis* with simultaneous FID and EAD (male *H. angustipennis* antenna).

and 43(100) identical to that of synthetic (Z)-6-nonen-2-one, compound IV with a spectrum *m/z* 156 (M^+, 4%), 141(1), 123(3), 113(1) 98(3), 85(3), 82(10), 71(20), 58(77), and 43(100) was tentatively assigned a methyl-branched decan-2-one, and compound V had a spectrum identical to that of synthetic decan-2-one, *m/z* 156 (M^+, 7%), 141(3), 127(2), 113(2) 96(3), 85(4), 71(22), 58(91), and 43(100). A large number of additional compounds with related chemical structures were found. Some of these could be identified based on their mass spectra, such as heptan-2-one, nonan-2-ol, and 2-undecanone [*m/z* 170 (M^+, 7%) 155(3), 127(3), 113(2), 85(7), 71(24), 58(96), and 43(100)]. The identical retention times of synthetic standards analyzed under the same conditions confirmed these assignments for both females and males. The quantity of nonan-2-one was about 500 ng/female and 10 μg/male.

EAG Responses to Synthetics. In a dose-response experiment, male *H. angustipennis* antennae clearly showed a dose-related response to the main component in male and female extracts and to a mixture of six compounds identified in male extracts that were available in the laboratory as synthetics (heptan-2-one, octan-2-one, nonan-2-one, decan-2-one, and nonan-2-ol, undecan-2-one in ratios 0.01:0.03:100:1.5:0.25:0.65 (Figure 5). No difference was observed between the two curves.

Field Tests, R. fasciata. Extracts of abdominal sternites IV and V from

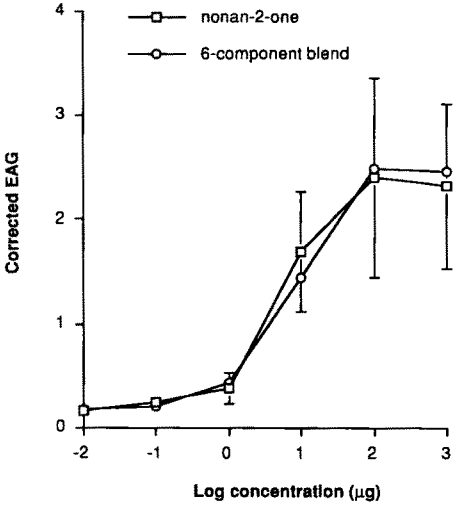

FIG. 5. EAG dose-response curves from male *H. angustipennis* antennae stimulated with nonan-2-one and a six-component synthetic blend of heptan-2-one, octan-2-one, nonan-2-one, decan-2-one, nonan-2-ol, undecan-2-one in a ratio of 0.01:0.03:100:1.5:0.25:0.65 ($N = 5$).

females and males were tested for attractancy towards males and females. The attractancy of mixtures of synthetic compounds identified in the female extract was compared with the female extract. The traps baited with female extract attracted conspecific males (Figures 6 and 7), whereas male extracts attracted neither sex (Figure 8). Both the female sternite extract and the four component mixture of heptan-2-one, heptan-2-ol, nonan-2-one, and nonan-2-ol were significantly more attractive than controls in all experiments (Figures 6–8). Traps baited with mixtures of the two ketones or the two alcohols performed no better than control traps under the same conditions.

Field Tests, H. angustipennis. Extracts and synthetic mixtures of compounds identified in extracts of the female and male abdominal sternites IV and V were tested for attractancy. In a first experiment, traps baited with nonan-2-one trapped in total 105 males and 22 females and female extracts trapped 201 males and 23 females, whereas control traps caught 45 and 16 males and females, respectively. However, the differences in attractivity were not statistically significant (Kruskal-Wallis analysis of variance, $P > 0.05$). In a following experiment the synthetic mixture of six ketones and alcohols caught significant numbers of both males and females (Figure 9A). Large numbers of males were caught with controls as well as treatments and in a final experiment in 1989 the different treatments were not significantly superior to the unbaited control traps (Figure 9B).

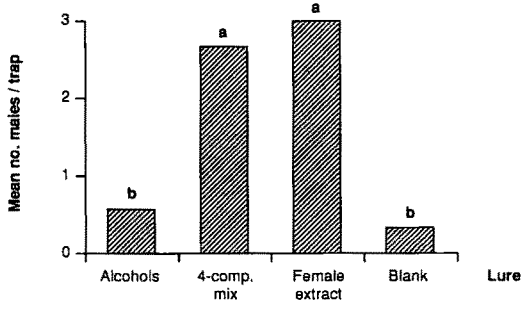

FIG. 6. Catches of male *R. fasciata* in traps baited with extract of female abdominal sternites IV and V (5 FE) and synthetic mixtures September 8–24, 1986 ($N = 9$). The synthetic four-component mixture consisted of heptan-2-one, heptan-2-ol, nonan-2-one, and nonan-2-ol in a 100 : 10 : 10 : 1.5 ratio. The major component heptan-2-one amounted to 6.7 μg/bait. The alcohol mixture consisted of the two alcohols. Catches followed by the same letters are not significantly different according to a Kruskal-Wallis analysis of variance followed by pairwise comparisons using the Fligner-Policello robust Mann-Whitney U test ($P \leq 0.05$).

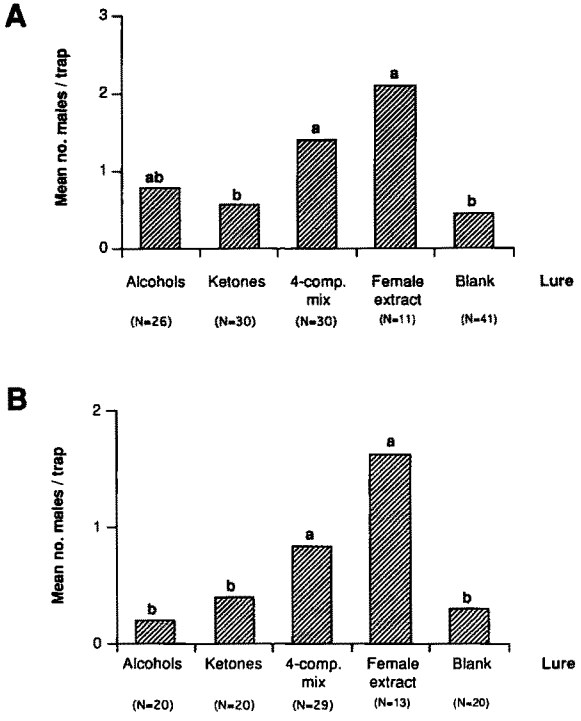

FIG. 7. Catches of male *R. fasciata* in traps baited with extract of female abdominal sternites IV and V (5 FE) and synthetic mixtures August 10–September 12, 1987 (A), and October 10–November 4, 1987 (B). The synthetic four-component mixture consisted of heptan-2-one, heptan-2-ol, nonan-2-one, and nonan-2-ol. The alcohol and ketone mixtures consisted of the two alcohols and ketones, respectively. Catches followed by the same letters are not significantly different according to a Kruskal-Wallis analysis of variance followed by pairwise comparisons using the Fligner-Policello robust Mann-Whitney U test ($P \leq 0.05$).

DISCUSSION

Previous studies of pheromone production in caddisfly adults have identified either the female abdominal fourth sternite (Solem, 1985) or the female abdominal fifth sternite, and most likely the pair of exocrine glands located there (Resh and Wood, 1985) as the site of pheromone production in a number of caddisflies. Anatomical observations confirmed the presence of these glands in both sexes of all three species studied by us. Extracts of the IVth and Vth abdominal sternites of female *Rhyacophila* and female and male *Hydropsyche*

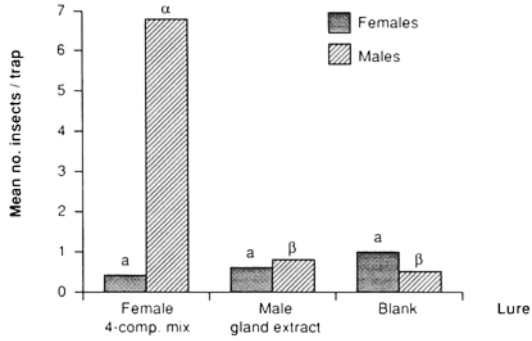

FIG. 8. Trap catches of male and female *R. fasciata* in traps baited with synthetic female pheromone or extracts of abdominal sternite IV and V of conspecific males October 19–November 10, 1988 ($N = 5$). Catches followed by the same letters are not significantly different according to a Kruskal-Wallis analysis of variance followed by pairwise comparisons using the Fligner-Policello robust Mann-Whitney U test ($P \leq 0.05$).

were electrophysiologically active when tested on conspecific males. This indicates that the males have receptor cells on their antennae, tuned to secretions produced by these structures. Furthermore, compounds identified from some of the extracts were behaviorally attractive when tested in the field. This is, to our knowledge, the first identification of long-range pheromones in Trichoptera.

A four-component blend consisting of heptan-2-one, heptan-2-ol, nonan-2-one, and nonan-2-ol was as attractive as female gland extracts in the attraction of male *R. fasciata*. However, further experiments are needed to show if all of these compounds are needed to obtain the full attraction or if a reduced blend would do as well. The enantiomeric composition of the secondary alcohols also remains to be investigated before the pheromone is fully characterized. For *H. angustipennis*, not all of the electrophysiologically active compounds in the male extracts were available to us as synthetics for field experiments. The synthetic six-component mixture used for field trapping included three of the five compounds with significant EAD activity in male extracts plus two other ketones and one alcohol, which also occurred in male extracts. (*Z*)-6-Nonen-2-one had a high EAD activity, especially considering that it eluted immediately after the major active peak and may thus have behavioral activity if included. Nonan-2-one on its own was significantly attractive to females but not to males. A subtractive assay starting with the complete blend isolated from males should resolve the activity of the individual components.

Solem (1985) could not find any glands associated with the IVth abdominal sternite in female *R. nubila*, although he reported that extracts of this segment were attractive to males. The resolution to our somewhat conflicting results may

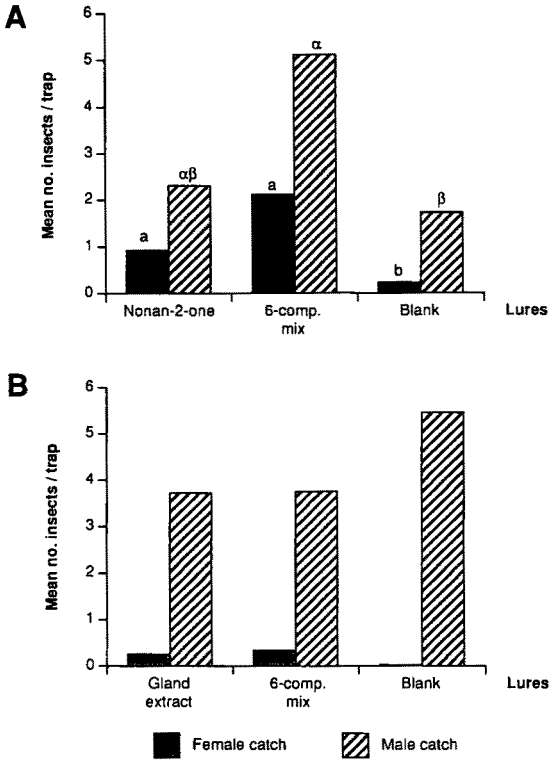

FIG. 9. Trap catches of male and female *H. angustipennis* in traps baited with synthetic compounds or male extracts August 22–29, 1988 (N = 10) (A) and June 12–16, 1989 (N = 36) (B). Catches in A followed by the same letters are not significantly different according to a Kruskal-Wallis analysis of variance followed by pairwise comparisons using the Fligner-Policello robust Mann-Whitney U test ($P \leq 0.05$). Catches in B were not different according to a Kruskal-Wallis analysis of variance (P = 0.134 for females and P = 0.920 for males).

lay in our anatomical and electrophysiological observations. The reservoirs of the glands opening on the Vth segment are at least partly located under the IVth abdominal sternite, an observation that is confirmed by the parallel study by Ansteeg and Dettner (1991). According to our experience, active extracts are best obtained by extracting both the IVth and Vth sternites.

High catches of caddisflies in control traps, more than low catches with treatments, was a major problem in our field tests of extracts and synthetic pheromone blends. High-density populations and catches by the control traps suggested that treatment interaction may also be an important factor. *H. angus-*

tipennis has been observed to form aerial swarms (L. Petersen, personal communication), which may well be pheromonally mediated. In this respect attraction of a swarm to a treatment trap in close proximity to control traps would likely result in random catches of insects in these traps. Random catches have previously been observed by Resh and Wood (1985) and Solem (1985). Such catches of insects in controls are important to consider in analyzing data, since, if these insects emit pheromones, the control effectively becomes a treatment.

Thus, we are inclined to conclude that the ambiguous behavioral results obtained by us in some field tests are due to the mating behavior of the investigated species, in which pheromones do play a role for attraction, but a less important one than in the well-known Lepidoptera. *Rhyacophila* and *Hydropsyche* differ in many ways with respect to their biology. Members of *Rhyacophila* are almost entirely nocturnal, whereas hydropsychids frequently are active during the day. In *Hydropsyche*, males have been reported to aggregate in mating swarms (Gruhl, 1960; Schumacher, 1969; Benz, 1975; Sullivan, 1981; Becker, 1987), a behavior that has not been observed in *Rhyacophila*. This implies that there are some basic differences between the mating systems of *Hydropsyche* and *Rhyacophila* and that pheromones might have different roles in the two species. Because the substances found in the glands of *H. angustipennis* attracted both male and female conspecifics, and the extracts from males were more active than those from females, the pheromone in this species probably functions as an aggregation pheromone. In *R. nubila*, Solem (1985) did report that males are attracted to the female pheromone, and we found the same in *R. fasciata*. This suggests that in *Rhyacophila* the pheromone functions as a traditional sex pheromone, like those reported in the majority of Lepidoptera.

All of the three species investigated by us produce pheromone components and/or gland constituents with chemically related structures: aliphatic ketones, aldehydes, alcohols, and acids with 6- to 11-carbon-atom skeletons. Independently, Ansteeg and Dettner (1991) also reported heptan-2-ol as the major constituent of female gland secretions in *R. nubila*. The same authors, in agreement with our findings, reported acetophenone, hexanoic acid, and octanoic acid in males of *R. nubila* and *R. fasciata*. Nonan-2-ol was found in the nearctic *R. fuscula* (Duffield, 1981). No species of *Hydropsyche* have been investigated before. Other species have been found to produce primary short-chain alcohols or various aromatic structures (Ansteeg and Dettner, 1991; Duffield et al., 1977). The similarity of many of the compounds found in Vth abdominal sternite glands in Trichoptera indicate that they are produced along common biosynthetic pathways. Interestingly, males and females of *Rhyacophila* produce different chemicals in morphologically similar glands. Another case of such sexual dimorphism was reported by Ansteeg and Dettner (1991) in *Polycentropus flavomaculatus*. In *H. angustipennis* nonan-2-one is the major gland constituent in both males and females, but, whereas males contain a rich bouquet of other ketones and

alcohols in large amounts, females have fewer compounds and much smaller amounts of them.

The chemical composition of the secretions from *R. nubila* and *R. fasciata* were remarkably similar, and the compounds produced by both sexes of *H. angustipennis* were identical to what was found in a preliminary analysis of *H. siltalai* (Löfstedt and Petersson, unpublished). The enantiomeric composition of the secondary alcohols was not determined in the present study or in any of the previous studies of trichopteran gland secretions. Thus, differences in enantiomeric composition could hypothetically contribute to species specificity of the signals.

At present, it is premature to conclude that pheromone communication is common and widespread in the Trichoptera. Nielsen (1980) reported that about half of the 26 Trichoptera that he examined possessed paired glands on the Vth abdominal sternite, but from a systematic point of view he found the occurrence to be very irregular. Furthermore, when glands do occur, they are not necessarily associated with pheromone production. Duffield et al. (1977) reported the occurrence of *p*-cresol, indole, and skatole in these glands in the limnephilid *Pycnopsyche scabripenniis*, but suggested that they function as defense compounds. Ansteeg and Dettner (1991) also focused on the possible role of the gland constituents as defensive compounds. They demonstrated a high fumigant activity of acetophenone, pentanol, and 3-methyl-heptan-2-one, and moderate toxicity of several other compounds identified from different Trichoptera. Thus, it appears likely that the secretions from the sternite glands have a mixed function; they may serve as either pheromones or defensive compounds depending on species, sex, and circumstances.

The pheromone components identified by us in Trichoptera are chemically different and are produced in different glands compared to what has been reported for Lepidoptera (Arn et al., 1992; Löfstedt, 1991; Percy-Cunningham and MacDonald, 1987), a sister group of Trichoptera. Most sex pheromones in Lepidoptera have 10–18 carbons in the carbon skeleton, and they are produced by the females in glands located on the ovipositor. The moth pheromone components normally contain one or more double bonds and most commonly have an alcohol, acetate, or aldehyde functional group.

We conclude that electrophysiology, especially in combination with gas chromatography, is a very useful tool for further elucidation of pheromone communication in Trichoptera, just as it has proven to be in the Lepidoptera. An overview of gland secretions of Trichoptera supplemented with behavioral observations of swarming and mating behavior could provide further insight into the importance of chemically mediated behavior in this group of insects.

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compounds and helpful discussion. Valuable suggestions from two anonymous reviewers are also acknowledged. A. Richards participated in this study as an exchange student from Imperial College, London, Great Britain, carrying out his third-year project at the University of Lund. A.R. wants to express his thanks to Drs. Lillemor Lewan and K.L. Alvin for arranging and organizing his stay. The study was supported by grants from the Swedish Natural Science Research Council, The Bank of Sweden Tercentenary Foundation, and the Crafoord Foundation.

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CODLING MOTH (*Cydia pomonella*): DISRUPTANTS OF SEX PHEROMONAL COMMUNICATION

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Abstract—In a small section of an apple orchard, six traps were placed each in control and test areas and baited with live virgin female codling moths. Gray elastomer septa were used to dispense communication disruptants around the traps. Dyed male codling moths were released in control and test areas, and the numbers of males captured in control and test traps were compared. In 1991, linear regression curves of percent communication disruption versus logarithm of dose were obtained for three compositions: (*E,E*)-8,10-dodecadien-1-ol, codlemone (1); codlemone + dodecan-1-ol + tetradecan-1-ol (2); and an equilibrium mixture of the four isomers of 8,10-dodecadien-1-ol (30, (61% *EE*, 14% *ZE*, 20% *EZ*, and 5% *ZZ*). All three regressions gave r^2 values greater than 0.90. At the 95% confidence limits, slopes and intercepts of compositions 1 and 2 were equivalent, and different from that of composition 3, which produced the greatest percentages of disruption at all doses. In 1992, five treatments were compared at a single dose: 1, 3, none (4), (*Z,E*)-8,10-dodecadien-1-ol (5), (*E,Z*)-8,10-dodecadien-1-ol (6). Compositions 5 and 6 gave the greatest and similar percentages of disruption and were different from codlemone (1) and 4 (95% confidence), but not from composition 3. Communication disruption produced by composition 3 was greater than (codlemone), which was greater than 4.

Key Words—Codling moth, *Cydia pomonella*, Lepidoptera, Tortricidae, communication disruption, mating disruption, sex pheromone, (*E,E*)-8,10-dodecadien-1-ol, (*E,Z*)-8,10-dodecadien-1-ol, (*Z,E*)-8,10-dodecadien-1-ol, dodecan-1-ol, tetradecan-1-ol.

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INTRODUCTION

The technique of reducing insect populations by disrupting sex pheromonal communication with semiochemicals offers the potential of controlling insect pests without the problems associated with insecticide use: accidental poisoning of farm workers, environmental contamination, development of insect resistance to insecticides, and disruption of biological control systems. At the present time, sex pheromones are the only class of semiochemicals actively under investigation for communication disruption. It is believed that an exact replicate of the natural sex pheromone will be a more effective communication disruptant than a single pheromone component or an "off ratio" blend of pheromone components (Minks and Cardé, 1988; Roelofs, 1978). This belief is based mainly on a consideration of the possible mechanism of communication disruption. Because of the difficulty of obtaining information on comparative efficacy of mating disruptants, direct experimental support for this viewpoint is limited. No theory on the relative efficacy of nonpheromone versus pheromone disruptants has been developed. In general, nonpheromone disruptants were not superior to pheromone disruptants when the two were compared experimentally (e.g., McLaughlin et al., 1974; Hathaway et al., 1979).

Our study was undertaken to determine the relative efficacy of potential communication disruptants for control of codling moth, *Cydia pomonella* (L.), and was directed at evaluating sex pheromone components and isomers of (*E*,*E*)-8,10-dodecadien-1-ol (codlemone). Compounds reported to be behaviorally active sex pheromone components are (*E*,*E*)-8,10-dodecadien-1-ol (codlemone), dodecan-1-ol, and tetradecan-1-ol (Roelofs et al., 1971; Einhorn et al., 1984; Arn et al., 1985; Bartell et al., 1988). During the summers of 1991 and 1992, we tested codlemone alone, codlemone in combination with the other two alcohols, an equilibrium mixture of isomers of codlemone, and, individually, the *EZ* and *ZE* isomers of codlemone. To carry out these tests, we developed a method derived from earlier reports of studies utilizing small field plots and female-baited traps to attract and capture males (Tamaki et al., 1983; Roelofs and Novak, 1981; Sanders, 1981; Hathaway et al., 1979).

METHODS AND MATERIALS

Field Test Areas. The laboratory apple orchard was used for the test (Figure 1). The intrarow distance between adjacent trees was 5.5 m, and the separation between rows was 6.1 m. This orchard had not been sprayed for several years, and 100% of the apples were infested every year since 1989. Traps were placed in the stippled and white trees (Figure 1). The control area (white trees) was northwest of the test area, and the prevailing winds during the late afternoon and early evening, when flight occurred, were from the northwest. The trees

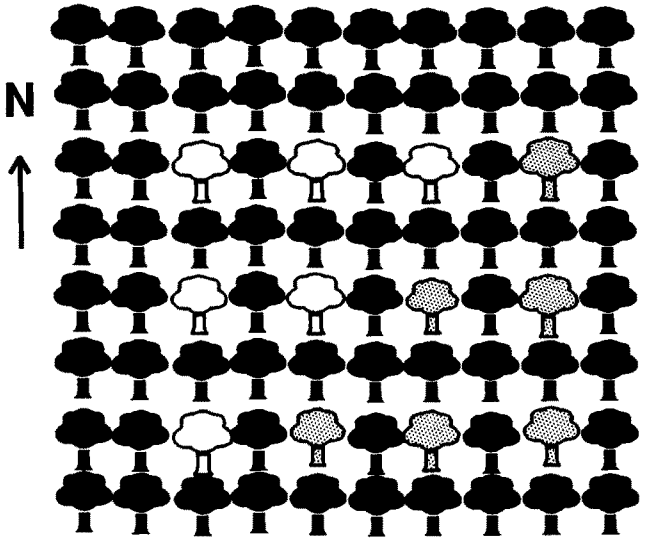

FIG. 1. Map of southeast corner of apple orchard used in communication disruption tests: stippled trees are in test plot and white trees are in control plot.

were about 3.5 m high and about 4 m in diameter. To facilitate placement and removal of the traps, a 1.9-cm-OD steel pipe was driven into the ground next to each tree trunk. The steel pipe extended to a height of 3.0 m. A metal shelf bracket was attached to the top of the pipe by means of a steel hose clamp. Traps were attached to the shelf bracket by means of a wire loop hooked to the bracket and wire hooks on the traps and on the end of a long pole.

Traps. A picture of the trap is shown in Figure 2. The trap was a modified Phercon 1 (Trece Corp., Salinas, California). The usual trap spacers were cut to produce an opening of about 0.5 cm and the corners of the traps were bolted with a wing nut and an additional spacer. A loose-leaf paper fastener (No. 624, AACO International Inc., Chicago, Illinois) was connected between the bolts to stiffen the trap edges. The cutaway of this trap shows the cage (8 cm diameter $\times$ 3 cm high) for confining the 10 virgin females used to attract males. Ten virgin females per trap were used to obtain consistent attractancy among traps. Wires holding the disruptants were 2 mm in diameter and were welded together to produce two perpendicular horizontal axes and one vertical axis perpendicular to the plane of the horizontal wires. The dispensers were connected to the six ends of the wires 20 cm from the center of the trap.

Insects. Pupae were obtained from a laboratory colony reared on artificial diet (Howell, 1972; Toba and Howell, 1991). Individual pupae were placed in

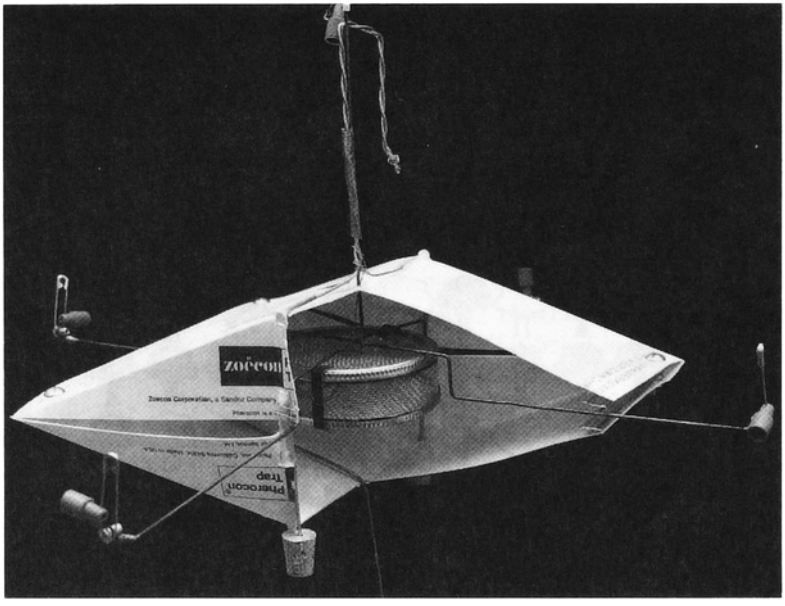

FIG. 2. Picture of a cutaway version of the traps used in communication disruption tests. Note the cages for females, wires for septa, and the wing nut and loose-leaf brace used to control trap opening.

a 15 × 50-mm vials and maintained at 20°C and 68% relative humidity on a 10:14 hr dark–light cycle. In all experiments, both male and female moths were used two to four days after eclosion.

Disruptant Dispensers. Gray elastomeric septa were used to dispense disruptant compositions because they minimize isomerization of conjugated dienes (Brown and McDonough, 1986) and their release rates were known for the compounds of interest (McDonough et al., 1993). When the dispensers were dosed with codlemone + dodecan-1-ol + tetradecan-1-ol, the ratios in the septa were chosen so as to produce the same ratios in the emitted vapor (100:64.8:9.3, respectively) as had been found in the female effluvium (Arn et al., 1985). In 1991, septa doses of disruptants were 0.3, 1.0, 3.0, 10.0, and 30.0 mg for the mixture of isomers and 1.0, 3.0, 10.0, and 30.0 for the other two compositions. The compositions were added to the cup of the septum as a solution in dichloromethane. The maximum dose that would absorb into the septum was 10 mg. For the 30-mg dose three septa were connected to each wire end (18 total/trap). Blank septa were placed on the control traps.

Chemicals. Dodecan-1-ol and tetradecan-1-ol (Aldrich Chemical Co., Mil-

waukee, Wisconsin) were used as received; (*E*,*E*)-8,0-dodecadien-1-ol (Bedoukian Research Inc., Danbury, Connecticut) was recrystallized three times from pentane and was 99.7% isomerically pure by capillary gas chromatography. (*E*,*Z*)-8,10-Dodecadien-1-ol was synthesized by the method of McDonough and Smithhisler (1989) for the synthesis of (*E*,*Z*)-10,12-tetradecadien-1-ol except that 1,8-octandiol was used in place of 1,10-decandiol. (*Z*,*E*)-8,10-Dodecadien-1-ol was synthesized by the method of McDonough et al. (1982) for the synthesis of (*Z*,*E*)-10,12-tetradecadien-1-ol except that 1,8-octandiol was used in place of 1,10-decandiol. For both syntheses, the *E*,*E* isomer was removed by reaction with tetracyanoethylene (McDonough and Smithhisler, 1989). The equilibrium mixture of isomers of 8,10-dodecadien-1-ol was prepared as previously reported (McDonough et al., 1993). The isomeric content was 61% *EE*, 20% *EZ*, 14% *ZE*, and 5% *ZZ*.

Test Protocol. Each test consisted of the average of two three-day test periods: Tuesday–Thursday and Friday–Sunday. Male and female moths, septa, and traps were put out on Tuesdays and Fridays midday and removed when trap catches were counted at approximately the same time on Fridays and Mondays. The males and females in their vials were chilled in a refrigerator to facilitate their transfer: 10 females each into the 12 cages (total, 120) and 30 males each into the 12 cylindrical cartons (total, 360) containing the appropriate powdered dye. The dyes were nontoxic (Moffitt and Albano, 1972) and were applied to the wall of the cartons with a spatula, and the excess was wiped off with a soft paper. The males were placed in their cartons for an hour and were then placed in galvanized wire-screen holders (11 cm in diameter and 13 cm high) with a galvanized wire-screen lid to prevent bird predation, attached to the test and control trees at a height of about 1.5 m, and the lids of the cartons were removed. On the days when the traps were removed, the number of males remaining in the cartons and the number of dead females in the cages were counted. Dyes (Day-Glo Color Corp. NALCO Chemical Co., Cleveland, Ohio) used to identify the males were horizon blue in the test plot and signal green in the control plot; these colors were alternated weekly with lavender in the control plot and blaze orange in the test plot. For each replicate, different compositions were tested consecutively so that each composition was tested throughout the season. After a test, the traps were hung in the laboratory for a week before reuse.

Calculating the Percent Disruption. For each treatment, total captures in test and control areas were compared. Because any estimation of percent communication disruption presupposes equal numbers of males in test and control areas, different colored moths were released in the test and control areas so that any disparity in migration between these areas could be taken into account when calculating the percent communication disruption. For this situation, the following formula was derived to calculate the percent communication disruption, *D*:

$$D = 100 - \frac{100\ (N_{tt} - N_{tc})}{N_{cc} - N_{ct}} \tag{1}$$

where N_{tt} is the number of males caught in the test area that were released in the test area; N_{tc} is the number of males caught in the test area that were released in the control area; likewise N_{cc} and N_{ct} are similarly defined. This formula was derived from the fact that the total numbers of each color of moth released were the same. If x is the fraction of males in the test area that succeeded in finding the females in the traps, then:

$$N_{cc} + N_{tc}/x = N_{ct} + N_{tt}/x$$

$$X = \frac{N_{tt} - N_{tc}}{N_{cc} - N_{ct}}$$

and

$$D = 100 - 100x$$

When the percent communication disruption was calculated in terms of the native population of males, the formula was:

$$D = 100\,\frac{(N_c - N_t)}{N_c} \tag{2}$$

where N_c and N_t are the numbers caught in the control and test areas, respectively.

RESULTS AND DISCUSSION

Development and Methodology. Because of the proximity of the male release stations to the female baited traps, nonbaited traps were tested to determine the degree of inadvertent captures. When the standard release protocol was used with standard Pherocon IC traps but without disruptant, 8.5% of the released moths were recaptured in the unbaited traps. Since we only expected 40–70% recovery of released males in baited traps, this percentage represented considerable error. This source of error was eliminated by modifying the trap (Figure 2) to reduce the entrance space from a height of 3.5 cm to 0.5 cm. Then the efficacy of this trap was tested with the standard protocol, except only half of the traps were baited with females and no disruptant was used. No dyed or native moths were caught in the unbaited traps, while 220 dyed males were caught (61% of those released) and 311 native males were caught in the baited traps.

The major problem with this type of methodology is that there may be a differential migration of the males out of the areas in which they were released.

Differential movement between areas was taken into account by releasing different colored moths in test and control areas and then calculating the percent disruption with equation 1. However, differential movement out of the areas can not be taken into account and was expected to be the main source of error.

To estimate the potential seriousness of differential male migration, two tests were conducted in 1991. In the first test, males were released as in the standard protocol, except that only the traps in the southeasterly area were baited with females. The number caught from those released in the southeasterly area was 191 (53% of those released); the number caught from those released in the northwesterly area was 135 (38% of those released). This test also was repeated except that only the traps in the northwesterly area were baited. Then the number caught from those released in the northwesterly areas was 182 (51% of those released); the number caught from those released in the southeasterly was 131 (36% of those released). In this experiment, the percentages migrating and the percentages recovered were remarkably similar. In the second test, males were released in both test (southeast section) and control (northwest section) areas, but the dispensers were not treated. Thus, if the populations remained the same in the test and control areas, D would calculate as 0%. Based on the dyed males, the application of equation 1 gave $D = -17.4\%$, and based on native males, the application of equation 2 gave $D = -2.1\%$. The more negative number deviates more than desired, but deviations of this magnitude may be unavoidable.

Mortality of the laboratory-reared moths used in these experiments was quite low. For the entire 1991 and 1992 seasons, 3.8% of the caged females were dead when the traps were removed from the trees, and 1.9% of the males failed to leave their cartons.

1991 Tests. The percent disruption (equation 1) versus logarithm of dose was evaluated for three compositions (Figure 3), according to the equation:

$$D = m \ln \text{dose} + y_0$$

where D is the percent disruption, m is the slope, and y_0 is the percent disruption when the dose was 1 mg. The regression parameters are summarized in Table 1. At the intercept, y_0, the isomeric mixture (composition 3) produced a significantly higher percent disruption at the 95% confidence limit than codlemone, composition 1 ($E8$, $E10$–12:OH), or composition 2 ($E8$, $E10$–12:OH + 12:OH + 14:OH), and compositions 1 and 2 were equivalent. The slope for the line of composition 3 was different from composition 2, but not from composition 1. The slopes for composition 1 and 2 were equivalent. In an earlier reported study of male responses in a flight tunnel (McDonough et al., 1993), compositions 1 and 2 produced identical responses over the total concentration range (0.1–100,000 μg $E8$, $E10$–12:OH) that produced responses. The present study also shows no difference in behavior to these compositions.

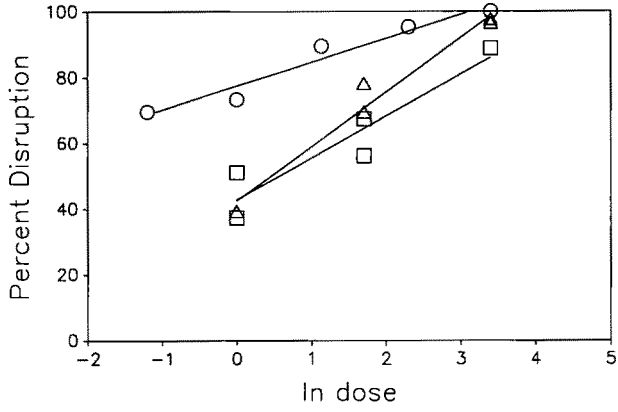

FIG. 3. Regression curves for percent disruption, D, versus logarithm of dose of 8,10-12:OH for three compositions: □, $E8$, $E10$-12:OH; △, $E8$, $E10$-12:OH + 12:OH + 14:OH; ○, equilibrium mixture of isomers of 8,10-12:OH (61% *EE*, 20% *EZ*, 14% *ZE*, and 5% *ZZ*). In the condensed nomenclature $E8$, $E10$-12:OH is (*E*, *E*)-8,10-dodecadien-1-ol and so forth.

TABLE 1. REGRESSION ANALYSIS PARAMETERS FOR PERCENT DISRUPTION VERSUS LOGARITHM OF DOSE (mg/septum) FOR 3 COMPOSITIONS[a]

Composition	Slope	y_0	r^2
(1) $E8$, $E10$-12:OH (codlemone)	12.4204, ab	42.99 a	0.905
(2) 1 + 12:OH + 14:OH	16.5200, a	42.44 a	0.968
(3) 8, 10-12:OH isomers	7.1914, b	77.44 b	0.947

[a] y_0 = values of percent disruption when ln dose = 0. Numbers followed by different letters are different at the 95% confidence limit.

The isomers of $E8$, $E10$-12:OH (codlemone) are known to inhibit trap catch of codlemone (Roelofs et al., 1972) and upwind flight in a flight tunnel (McDonough et al., 1993), and the isomers might be expected to decrease the communication disruption efficacy of codlemone. Therefore, these results were surprising and raised the question of whether the increased disruption was due to the mixture or whether the individual isomers were disruptants. To further explore this finding, the isomers, $E8$, $Z10$-12:OH and $Z8$, $E10$-12:OH, were synthesized in substantially pure form and then tested individually.

1992 Tests. We compared four compositions at a dose of 1 mg/septum of 8, 10-12:OH and blank septa. The compositions were $E8$, $Z10$-12:OH, $Z8$, $E10$-12:OH, an equilibrium mixture of isomers of 8, 10-12:OH (61% *EE*,

20% *EZ*, 14% *ZE*, and 5% *ZZ*), and *E*8, *E*10–12:OH (Table 2). Table 2 shows data both for released dyed males and native males. At the 95% confidence limit, the *EZ* and *ZE* isomers were equivalent to each other in both tabulations and were superior to codlemone and to the blanks. The equilibrium mixture of isomers produced numerically less disruption than the *EZ* and *ZE* isomers, but the differences were not significant at the 95% confidence limit. The mixture of isomers was superior to codlemone, which was superior to the blanks at the 95% confidence limits.

Therefore, the 1992 results confirm the 1991 results for codlemone and the mixed isomers and establish that both the *EZ* and *ZE* isomers individually disrupt sexual communication of codling moths to a greater degree than codlemone in these tests. Further tests are needed to determine the practical value of these results. The principal question is whether the *EZ* or *ZE* isomer will be superior to codlemone under practical conditions of commercial communication disruption where spacing of females, males, and disruptant will vary.

A change in spatial factors can alter the relative effectiveness of communication disruptants. For some insects, compounds that inhibit response to the pheromone when mixed with the pheromone, completely fail to inhibit response when placed just a few centimeters from the pheromone (McLaughlin et al., 1974; Liu and Haynes, 1992; Rumbo et al., 1993). These authors proposed that, if the males can temporally resolve the signals from the pheromone and inhibitor, the males will become habituated to the inhibitor, which is detected by antennal receptors different from the pheromone, and then respond to the pheromone in the normal manner. In the case of *Trichoplusia ni* (Hübner), the inhibitor, (*Z*-7-dodecen-1-ol,) when mixed at a concentration of 0.1% of the sex pheromone, reduced trap catch (Tumlinson et al., 1972). In field tests, Kaae

TABLE 2. PERCENT COMMUNICATION DISRUPTION OF RELEASED AND NATIVE MALE CODLING MOTHS FOR DIFFERENT TREATMENTS[a]

Treatment	Calculated % communication disruption	
	Released males	Native males
*E*8, *Z*10–12:OH	80.9 a	91.9 a
*Z*8, *E*10–12:OH	80.8 a	96.4 a
8, 10–12:OH isomers	63.1 a	89.2 a
*E*8, *E*10–12:OH (codlemone)	42.4 b	55.2 b
None	18.9 c	17.3 c

[a]Numbers followed by different letters are different at the 95% confidence limit.

et al. (1974) reported 43% communication disruption from (Z)-7-dodecen-1-ol at an evaporation rate 100 times that of the pheromone rate that was required for 84% communication disruption. When the amount of (Z)-7-dodecen-1-ol was 10 times that of the pheromone that was required for 84% communication disruption, no disruption was obtained.

These results are in marked contrast to our results with the codling moth. Here the *ZE* and *EZ* isomers of codlemone disrupted communication to a higher degree than the pheromone at the same evaporation rate. In an earlier study of communication disruption of codling moth (Hathaway et al., 1979), 98% reduction of trap catch in a field test was achieved either by codlemone evaporating at a rate of 15 mg/ha-hr or the inhibitor, (*E*,*E*)-8,10-dodecadien-1-ol acetate, evaporating at a rate of 25 mg/ha-hr. Preiss and Priesner (1978) showed that male codling moths perceived codlemone and the inhibitor by separate antennal receptors. The evident lack of habituation to the inhibitor or to the *EZ* or *ZE* isomers of codlemone indicates that the physiological factors controlling the response of the codling moth and *T. ni* may be different.

Spatial factors could nevertheless be important in another way. If a non-attractive disruptant is widely separated from a calling female, the disruptant concentration might be insufficient to inhibit male response. However, in the same situation, the synthetic pheromone might be more attractive than the female, and the male would fly up the trail of the synthetic pheromone toward the source until he became habituated. Since the trail-following mechanism is probably unimportant in the tests in the study reported here, an increase in its importance might change the relative order of disruptant efficacy. This factor might not be as important for the mixed isomers of codlemone as for the individual *EZ* and *ZE* isomers, because the mixed isomers still are significantly attractive, especially at low concentrations (McDonough et al., 1993). Obviously further field studies are needed to fully determine the potential of these compounds for controlling codling moth.

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INDUCED RESISTANCE IN SOYBEAN TO *Helicoverpa zea*: ROLE OF PLANT PROTEIN QUALITY

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Abstract—Resistance in soybean to *Helicoverpa zea* is comprised of both constitutive and inducible factors. In this study, we investigated the induction of resistance by *H. zea* in both greenhouse and field studies. In a greenhouse experiment, fourth-instar *H. zea* growth rates were reduced by 39% after 24 hr feeding and by 27% after 48 hr when larvae fed on previously wounded V3 foliage (cv. Forrest) compared with undamaged foliage. In a field study, the weight gain by larvae was more than 52% greater when larvae fed for 72 hr on undamaged R2/R3 soybean plants (cv. Braxton) compared to those that fed on previously wounded plants. A significant component of the induced resistance is due to a decline in the nutritional quality of foliar protein following foliar damage by *H. zea*. Foliar protein was extracted from damaged and undamaged foliage and incorporated into artificial diets. Larval growth was reduced 26% after four days and 49% after seven days on diets containing protein from damaged plants compared to larvae feeding on foliar protein from undamaged plants. Chemical analyses of protein quality also indicated a decline in quality in damaged plants compared to unwounded plants. Increases in lipoxygenase activity (53%), lipid peroxidation products (20%), and trypsin inhibitor content (34%) were observed in protein from wounded plants. Moreover, a 5.9% loss in free amines and 19% loss in total thiols occurred in protein from wounded plants. Larval feeding causes a significant increase in foliar lipoxygenase activity that varied among genotypes. Lipoxygenase isozymes were measured at pH 5.5, pH 7.0, and pH 8.5 in V3 stage plants of Forrest, Hark, D75-1069, and PI 417061 genotypes. Lipoxygenase activity in each genotype was significantly increased after 72 hr of larval feeding at each pH level tested, with the exception of lipoxygenase isozymes at pH 5.5 in genotype PI 417061. Larval feeding on R2/R3 stage plants (field-grown cv. Braxton) for six days also increased foliar lipoxygenase activity.

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Key Words—Induced resistance, protein quality, *Helicoverpa zea*, soybean, *Glycine max*, protease inhibitor, lipoxygenase, Lepidoptera, noctuidae.

INTRODUCTION

The expression of host plant resistance is affected by both abiotic and biotic factors. Prior wounding by insects or mechanical means may induce resistance to insects in many crop plants (Smith, 1989). In some cases, the biochemical bases of induced resistance have been identified. Wound-induced responses in plants may be part of a general defensive reaction, due to increased activities of oxidative enzymes such as lipoxygenase (Hildebrand et al., 1989, Croft et al., 1990), peroxidase (Bronner et al., 1991), or polyphenol oxidase (Felton et al., 1992a). In other instances, induced resistance has been correlated with the enhanced synthesis of secondary metabolites such as phenolics, hydroxamic acid, alkaloids, coumarins, etc., or with synthesis of primary gene products such as protease inhibitors (Baldwin, 1988; Haukioja, 1990; Khan and Harborne, 1991; Morse et al., 1991; Olson and Roseland, 1991; Broadway and Colvin, 1992).

Induced resistance in soybean, *Glycine max* (L.) Merr., has been demonstrated previously (Kogan and Fischer, 1991). Mexican bean beetles, *Epilachna varivestis* (Mulsant), were deterred from feeding on genotype PI 227687 when plants were previously injured by Mexican bean beetle feeding 72 hr prior to the test (Chiang et al., 1986). Feeding by two-spotted spider mite on soybean induces resistance that effectively limits a further increase of the mite population (Hildebrand et al., 1986). Mechanical damage of soybean foliage also raises the level of resistance to the soybean looper, *Pseudoplusia includens* (Walker), in the foliage of an insect-resistant cultivar (Smith, 1985). Wound-induced resistance in soybean (cv. Williams 82) significantly retarded the development and growth of both soybean looper and Mexican bean beetle (Lin and Kogan, 1990).

In several instances, induced resistance in soybean has been correlated with increased levels of several plant natural products. Induced resistance to Mexican bean beetle herbivory in PI 227687 soybean leaves was positively correlated with total phenolic content and temporally increased L-phenylalanine ammonia-lyase and L-tyrosine ammonia-lyase activities (Chiang et al., 1987). Phenolic compounds arise from cinnamic acid or *p*-coumaric acid, which in turn are derived from phenylalanine or tryosine in a biochemical pathway mediated by phenylalanine ammonia-lyase or tyrosine ammonia-lyase (Fischer and Kogan, 1989). Soybean phytoalexins and simple phenolic acids are wound-inducible and are active feeding deterrents for the Mexican bean beetle (Kogan and Fischer, 1991). Mexican bean beetle herbivory induces trypsin inhibitor in soybean leaves (Kraemer et al., 1987). Soybean trypsin inhibitor has been shown to reduce

larval growth of *Manduca sexta* (Johannson) (Shukle and Murdock, 1983) and *Helicoverpa zea* (Boddie) (Broadway and Duffey, 1986). A large increase in lipoxygenase activity is induced by wounding of soybean leaves, either mechanically or by feeding of the two-spotted spider mite *Tetranychus urticae* Koch (Hildebrand et al., 1989). Shukle and Murdock (1983) first presented evidence that lipoxygenase could function as a plant defense against insects such as *M. sexta*. Further research is necessary to clarify the role(s) of lipoxygenase in plant defense against insects (Duffey and Felton, 1991).

The system chosen for this study includes the corn earworm, *H. zea*, and the soybean plant. *H. zea* is considered the major insect pest of soybean in the southern United States (Stinner et al., 1980). Soybean defoliation and pod feeding by *H. zea* lead to yield and quality reduction (Eckel et al., 1992). Densities of *H. zea* larvae are typically higher on flowering plants where flowers removed by *H. zea* larvae result in a delay in pod set. Subsequent feeding on pods reduces the number of seeds per pod (Eckel et al., 1992).

Resistance to *H. zea* has been observed in several accessions of soybean, but the biochemical bases of resistance have not been elucidated (Clark et al., 1972; Hatchett et al., 1976; Rowan et al., 1991; Nault et al., 1992). The present study was undertaken to elucidate some of the biochemical mechanism(s) of induced resistance in soybean to *H. zea*. The specific objectives were: (1) to determine if *H. zea* feeding on soybean foliage induces resistance, (2) to assess the impact of *H. zea* feeding on the nutritional quality of foliar protein, (3) to determine if lipoxygenases are induced in response to feeding by *H. zea*, and (4) to determine if genotypic differences exist in lipoxygenase induction.

METHODS AND MATERIALS

Insects and Plants. Eggs of *H. zea* were obtained from the University of Arkansas Insect Rearing Facility. Larvae were maintained on artificial diet (Burton, 1969), unless otherwise noted.

The soybean cultivars or accessions used in the experiment were Forrest, Braxton, Hark, PI 417061 and D75-1069. The seeds were soaked in water for 6 hr, then incubated at 28°C for 24 hr. Germinated seeds were sown in 430-ml polystyrene foam cups in the greenhouse. Cups were arranged in a randomized complete block experimental design. Plants were watered every two days. Fertilizer (N:P:K = 20:20:20) was applied weekly. Greenhouse conditions were: (1) 14-hr photophase, using high-pressure sodium light (400 and 1000 W) and (2) day temperature, 33 ± 2°C and night temperature, 20 ± 2°C.

In a field experiment, the cultivar Braxton was planted in 10 rows at the Agricultural Experiment Station of the University of Arkansas, Fayetteville. Row spacing was 0.96 m; row length was 20 m. Plants were spaced at 0.25-m intervals.

Experiment 1—Induction of Resistance. A test was conducted in the greenhouse with 32 V3 (V = vegetative stage as described by Fehr et al., 1971) soybean plants (cv. Forrest). Sixteen unwounded plants were designated as the control group, and one fourth-stage *H. zea* larva was placed on each of the other 16 plants for three days. Larvae were starved for 24 hr prior to infesting the plants. The levels of defoliation in the wounded plants were generally less than 30% (Kogan and Kuhlman, 1982).

After larvae were removed from the plants, fully expanded leaves of each plant were excised at the proximal ends of the petioles using a single-edge razor blade. The excised leaves were taken to the laboratory and placed in a clear plastic container (10 cm diameter, 7 cm high and with two holes of 2 mm diameter in the plastic cover) with two layers of moist filter paper (Whatman No. 1, one original layer, and 24 hr later another layer was added) in the bottom of the container. Each leaf sample consisted of two fully expanded trifoliate and one monofoliate leaves with their petioles pointed downward to the filter papers. A newly molted fourth-stage *H. zea* larva was placed in each container. Containers were randomly placed into an incubator at 28 $\pm$ 0.5°C. Each larva was weighed at the beginning of the test and again after 24 hr and 48 hr. There were no significant differences ($P = 0.85$) between the initial weights of the fourth-instar *H. zea* used in the treatment and control of experiment. The test was repeated two times.

In a second test conducted in the field, ten R2/R3 (R = reproductive stage) Braxton plants were used: five control and five treated plants, respectively. Each was covered with an organdy-covered cage (1 × 1 × 1 m) in the field in Fayetteville, Arkansas as described above. Plants were wounded by placing five fifth-stage *H. zea* larvae on each treated plant at the respective plant stage, and three days later, six additional fifth instars were added. After six days from the original wounding, original larvae had pupated or died. The uppermost fully expanded leaf was excised from each plant and assayed for lipoxygenases at pH 5.5, pH 7.0, and pH 8.5 as described below.

Five fourth-instar larvae were then placed on each control and treated plant. There were no significant differences ($P = 0.71$) between the initial weights of the larvae used in the experimental treatment and control. Larvae were collected and weighed after 72 hr.

Experiment 2—Impact of Insect Feeding on Nutritional Quality of Foliar Protein. Both soluble and ionically bound proteins were extracted to determine the effect of *H. zea* feeding on the nutritional quality of soybean foliar protein. The proteins were isolated from 200 V3 stage soybean plants (cv. Forrest). One hundred of the plants had been exposed to third-instar larvae for three days, and the other 100 plants served as controls. The larvae were starved for 24 hr prior to placement on plants. After larvae were removed, the uppermost expanded leaves (2 g fresh wt/plant) were harvested. The harvested leaves were imme-

diately frozen at −10°C for 4 hr. The frozen foliar tissue was homogenized in 0.05 M potassium phosphate ($KH_2PO_3 \cdot 3H_2O$), pH 8.0, containing 0.8 M potassium chloride (KCl) and 1% polyvinylpolypyrrolidone (PVPP), with 10 ml for each gram of foliage. A subsample of 100 mg plant tissue/plant was analyzed for protein content following Stoscheck (1990) with the addition of 1% soluble polyvinylpolypyrrolidone to the Coomassie reagent. Phosphoribulokinase (Sigma Chemical Co., St. Louis, Missouri) was used as a standard.

The homogenate was filtered through miracloth after stirring in a beaker for 60 min at 4°C in a chromatography refrigerator. The filtrate was centrifuged at 5000*g* for 30 min. The resulting supernatant was again filtered with miracloth, and lipoxygenase activity of the filtrate at pH 7.0 was determined. The protein from the filtrate was precipitated by slowly adding 20% TCA until the pH reached 3.5. The mixture was again stirred in a beaker for 60 min at 4°C followed by centrifugation at 5000*g* for 30 min. The pellets were frozen and freeze-dried. The freeze-dried samples were dialyzed for 48 hr with ca. 10,000 molecular weight cut off dialysis tubing against repeated exchanges of deionized water at 4°C. The samples were again frozen and freeze-dried.

To determine the nutritional value of pooled foliar protein extracts, the protein was incorporated into artificial diet at 1% wet weight. The 1% wet weight represented the mean foliar level found in preliminary analyses. Artificial diet (100 g) contained: 1 g soybean foliar protein extract; 5.215 g cellulose; 0.685 g Vanderzant vitamins; 2.400 g agar; 0.200 g wheat germ oil; 3.370 g dextrose; 2.750 g wheat germ; 0.900 g Wesson salts; 0.425 g alginic acid; 0.365 g ascorbate; 0.180 g cholesterol; 0.090 g choline chloride; 0.010 g streptomycin; 0.120 g aureomycin; 0.200 g methyl paraben; 0.90 g sorbic acid; and 82 g distilled water.

Neonate *H. zea* larvae were individually placed in 18.5-ml clear plastic cups containing the appropriately treated diet. Twenty larvae were tested per treatment, and the experiment was replicated three times for a total number of 60 larvae per treatment. Larvae were weighed to the nearest 0.1 mg after four days and seven days, respectively.

The free amine groups, thiol content, lipid peroxides, carbonyl formation, and trypsin inhibitor in the foliar protein extract were assayed as described below.

Chemical Analyses of Foliar Protein. To estimate lipid peroxidation in foliar protein, a thiobarbituric acid assay was used (Stewart and Bewley, 1980). Fifty milligrams of dried foliar protein isolate in 1 ml 0.1 M potassium phosphate, pH 7.0, was incubated in 1 ml 1% thiobarbituric acid and 1 ml 0.1 M sodium hydroxide (NaOH) in an 80°C water bath for 120 min. Samples were centrifuged at 10,000*g* for 10 min. The absorbance of the supernatants was read at 532 nm. Assays were replicated three times.

To determine the free amine content in foliar protein, the picrylsulfonic

acid assay was used (Fields, 1972). Twenty-five milligrams of protein extract was dissolved in a 1.0-ml mixture of 0.1 M sodium borate ($Na_2B_4O7 \cdot 10H_2O$) and 0.1 M sodium hydroxide (NaOH) followed by incubation for 5 min in 0.020 ml 0.1% picrylsulfonic acid. The reaction was stopped by a working solution containing 0.1 M sodium sulfite (Na_2SO_3) and 0.1 M sodium phosphate (NaH_2PO_4). Absorbance of the resulting solution was read at 420 nm. Assays were replicated three times.

To assess the formation of protein carbonyl adducts, 25 mg foliar protein extract was precipitated in 1 ml 10% TCA and centrifuged at 10,000*g* for 15 min. The resulting pellet was incubated in 2 ml 0.1% dinitrophenylhydrazine for 60 min at room temperature. Two milliliters of 10% TCA was then added to precipitate the protein, and it was centrifuged in −2°C at 10,000*g* for 15 min. The pellet was washed three times in 2 ml 1:1 ethanol–ethyl acetate solution and resuspended in 6 M guanidine. The absorption of supernatant was read at 370 nm (Levine et al., 1990). Assays were performed in triplicates.

To determine the content of protein thiols, a 0.5-mg protein extract was vortexed in 1 ml distilled water. One milliliter of 6 N sodium hydroxide was added, and the extract was incubated for 30 min at 20°C. Two milliliter 2.0 M phosphoric acid containing 0.02 mM EDTA was added to neutralize the base. The color was developed by adding 0.25 ml 2.5 mM 5,5′-dithio-bis-(2-nitrobenzoic acid) and 0.25 ml 0.02 M sodium acetate, pH 5.5. The colored solution was read at 412 nm as described by Anderson and Wetlaufer (1975). Assays were replicated three times.

To determine the amount of trypsin inhibitor in the protein extracts, bovine trypsin and soybean trypsin–chymotrypsin inhibitor (Sigma Chemical Co.) were used. One milligram of trypsin was dissolved in 1 ml 0.001 M hydrochloric acid. The substrate was 1.6 mM Na-*p*-tosyl-L-arginine methyl ester (TAME) in 1 mM potassium phosphate, pH 7.0. The rate of change in absorbance at 247 nm in the presence of soybean inhibitor was measured to develop a standard curve (Broadway and Colvin, 1992). The inhibitor content in the foliar protein extract was determined based upon the standard curve. Assays were replicated five times.

The protein content of the protein extract was determined using a modified Bradford (1976) assay. Five milligrams of protein extract was dissolved in 5 ml 0.1 M sodium hydroxide. One percent soluble PVPP was added in the Coomassie brilliant blue G-250 reagent. The reaction mixture was read at 595 nm. Phosphoribulokinase (Sigma Chemical Co.), was used as a standard. Assays were replicated five times.

Experiment 3—Induction of Lipoxygenase and Effect of Genotype. To determine if feeding by *H. zea* causes an increase in the activity of soybean foliar lipoxygenases, soybean plants (genotypes: Forrest; Hark, PI 417061, and D75-1069) were grown in the greenhouse. At the V3 stage, one fourth-instar *H. zea*

was placed on each plant, which was placed in a screen cage to prevent larval escape. Cages were 1 × 1 × 2 ft and were purchased from BioQuip Products (Gardena, California). For each genotype, five plants were treated by placing larvae on plants, and five control plants were identically treated except that larvae were excluded. After three days, fully expanded leaflets were excised from the uppermost node and assayed for lipoxygenases at pH 5.5, 7.0, and 8.5, as described below. The experiment was replicated three times.

The effect of larval feeding on lipoxygenases in field-grown cv. Braxton was assessed as described in experiment 1.

Lipoxygenase Assay. To assay for foliar lipoxygenases, 1 g foliage was homogenized in 10 ml 0.1 M potassium phosphate, pH 7.0, containing 1% PVPP and 0.5 mM EDTA (ethylenediaminetetraacetic acid). The homogenate was centrifuged in −2°C for 20 min at 10,000*g*. The supernatant was used immediately as the enzyme source. Linoleic acid (0.25 mM) was used as a substrate, and the rate of change in absorbance at 234 nm was measured with a Milton Roy Spectronic 3000 Array Spectrophotometer with kinetic software. Lipoxygenases were assayed at pH 5.5, 7.0 (0.1 M potassium phosphate adjusted with phosphoric acid), and 8.5 (0.1 M sodium borate adjusted with hydrochloric acid or sodium hydroxide), respectively (Grayburn et al., 1991). Assays were performed at the three pH levels due to the presence of the multiple isozymes with differing pH optima (Grayburn et al., 1991). Assays were performed in triplicate for each plant sample.

Statistics. Students *t* test in one-way completely randomized ANOVA was used to analyze the data in this study.

RESULTS

Experiment 1—Induction of Resistance. Feeding by *H. zea* larvae on V3 soybean foliage (cv. Forrest) significantly induced resistance to subsequent larval attack in a greenhouse experiment. Larval growth rates were reduced 39% ($P < 0.05$) after 24 hr and 27% ($P < 0.01$) after 48 hr as compared with larvae ingesting control foliage (Figure 1).

Feeding by *H. zea* larvae for 72 hr on R2/R3 soybean plants (cv. Braxton) in the field also induced resistance (Table 1). The weight gain by *H. zea* was 52% greater ($P < 0.01$) when larvae fed on control plants compared to those fed on previously wounded plants. Foliar lipoxygenase levels were significantly higher in the wounded plants compared to control plants. Activity of foliar lipoxygenases from wounded plants was 1.34× higher at pH 5.5 ($P < 0.01$), 156× at pH 7.0 ($P < 0.01$), and 1.73× at pH 8.5 ($P < 0.05$) compared to unwounded plants (Table 1).

Experiment 2—Impact of Insect Feeding on Nutritional Quality of Foliar

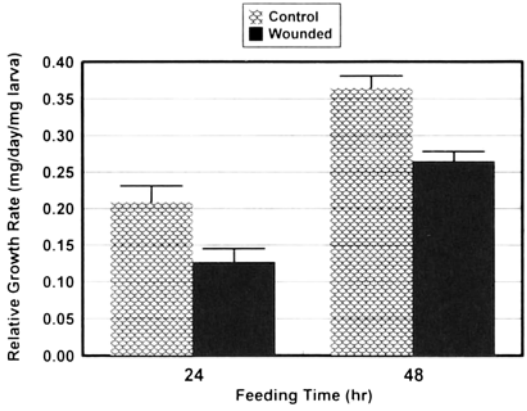

FIG. 1. The effect of wounding V3 soybean plant on *H. zea* larval growth. Fourth-instar *H. zea* larvae fed on previously wounded V3 soybean (cv. Forrest) leaves or unwounded leaves. Larval growth rate was significantly lower at 24 hr ($P < 0.05$) and at 48 hr ($P < 0.01$) when larvae fed on previously wounded leaves compared to control leaves. Errors bars represent 95% confidence limits.

TABLE 1. EFFECT OF WOUNDING ON LARVAL MASS GAIN AND FOLIAR LIPOXYGENASES IN FIELD-GROWN CV. BRAXTON[a]

Treatment	Mean larval mass (mg)	Lipoxygenase (nmol/min/g foliage)		
		pH 5.5	pH 7.0	pH 8.5
Unwounded	405(10)b	600(23)a	477(32)a	167(16)a
Wounded	323(23)a	809(51)b	747(51)b	289(36)b

[a]Means in columns followed by a different letter are significantly different at $P < 0.05$. Numbers in parentheses following means are standard errors.

Protein. Larval feeding slightly decreased the level of foliar protein ($P < 0.03$; data not shown). Protein levels in unwounded foliage averaged 0.99% but were 0.89% in wounded tissues. Feeding by *H. zea* larvae also decreased the nutritional quality of foliar protein as indicated by 53% higher lipoxygenase activity at pH 7.0 ($P < 0.001$), greater than 20% increase in lipid peroxidation products ($P < 0.001$), 34% higher trypsin inhibitor content ($P < 0.05$), 5.9% loss of free amines ($P < 0.02$), and 19% loss of total thiols ($P < 0.04$) in comparison with those in the foliar protein from unwounded plants (Table 2). Larval growth after four days was reduced 26% ($P < 0.05$) and after seven days 49% ($P < 0.01$) when larvae fed on diets containing foliar protein from wounded plants

TABLE 2. IMPACT OF *H. zea* FEEDING ON NUTRITIONAL QUALITY OF FOLIAR PROTEIN[a]

	LOX at pH 7.0[b]	Lipid peroxidation[c]	Protein (%)	Free amines (μmol/mg protein)	Total thiols (ABS/mg protein)	TI (μg/mg protein)[d]	Carbonyl (nmol/mg protein)	Relative nutritional value[e]	
								4 days feeding	7 days feeding
Unwounded	158 a	0.061 a	80.93 a	1.86 b	0.131 b	37 a	5.97 b	100 b	100 b
	(23)	(0.002)	(0.91)	(0.02)	(0.008)	(5)	(0.04)	(13)	(11)
Wounded	338 b	0.077 b	81.61 a	1.75 a	0.106 a	56 b	5.19 a	74 a	51 a
	(17)	(0.002)	(0.69)	(0.02)	(0.009)	(3)	(0.21)	(4)	(9)

[a] Means in columns followed by a different letter are significantly different at $P < 0.05$. Numbers in parentheses beneath means are standard errors.
[b] LOX at pH 7.0 expressed as nmol/min/g leaf tissue.
[c] Lipid peroxidation expressed as μmol/mg isolated protein sample measured by thiobarbituric acid method.
[d] TI: trypsin inhibitor level.
[e] Relative nutritional value: expressed by relative larval weight.

compared to larvae fed protein from control plants. There was no change in the percent protein between protein extracts from treatment and control. However, the carbonyl concentration was decreased by 13% ($P < 0.006$) in foliar protein from wounded plants (Table 2).

Experiment 3—Induction of Lipoxygenase and Effect of Genotype. Induction of lipoxygenases was significantly influenced by soybean genotype (Table 3). Feeding by *H. zea* on V3 soybean foliage (cv. Forrest) for 72 hr significantly increased the activity of lipoxygenases by 2.1× at pH 5.5 ($P < 0.05$), 2.27× at pH 7.0 ($P < 0.01$), and 2.05× at pH 8.5 ($P < 0.05$) compared with activity measured in control foliage. The results suggest that all of the foliar lipoxygenase isozymes may be inducible in this genotype.

D75-1069 showed the highest induced level of lipoxygenase at pH 8.5 compared with the commercial cultivars Forrest, Hark, and accession PI 417061. Activity was 1.51×, 2.20×, and 3.79× higher at pH 5.5, 7.0, and 8.5 on wounded plants compared to controls, respectively ($P < 0.05$). In the cultivar Hark, lipoxygenase was 1.54× higher at pH 5.5, 1.77× at pH 7.0, and 2.61 × at pH 8.5 in wounded plants compared to controls ($P < 0.05$). However, in the accession PI 417061, activity was not significantly higher at pH 5.5 ($P > 0.05$) but was significantly higher at pH 7.0 (1.76×) and pH 8.5 (1.81×) ($P < 0.05$).

TABLE 3. EFFECT OF GENOTYPE ON INDUCTION OF LIPOXYGENASES (LOX) IN V3 SOYBEAN[a]

	LOX activity (nmol/min/g leaf)		
Cultivar	pH 5.5	pH 7.0	pH 8.5
Forrest			
Constitutive	328(44)a	176(24)a	64(10)a
Induced	658(73)b	400(50)b	131(22)b
Hark			
Constitutive	709(66)a	400(51)a	153(12)a
Induced	1091(76)b	708(98)b	400(110)b
PI 417061			
Constitutive	261(15)a	285(24)a	100(19)a
Induced	317(21)a	503(58)b	181(18)b
D75-1069			
Constitutive	556(87)a	450(46)a	175(31)a
Induced	841(33)b	989(128)b	664(77)b

[a]Means in columns within genotype followed by a different letter are significantly different at $P < 0.05$. Numbers in parentheses following means are standard errors.

DISCUSSION

Several laboratories have investigated induced resistance in soybean to insects (e.g., Chiang et al., 1987; Liu et al., 1992). Kogan and Fischer (1991) reported that soybean phytoalexin glyceollins were wound inducible and were feeding deterrents for the Mexican bean beetle. Induced resistance in soybean to Mexican bean beetle and soybean looper previously was correlated with increased phenolic content and increased levels of phenylalanine lyase and tryosine ammonia lyase (Chiang et al., 1987; Neupane and Norris, 1991a,b). Lin and Kogan (1990) also investigated induced soybean resistance and found that prior herbivory by soybean looper caused reductions in relative growth rate and developmental times of Mexican bean beetles and soybean loopers. They found that induced resistance inhibited the relative growth rates of loopers by 3.4% and beetles by 10.7%. The magnitude of the induced resistance to *H. zea* observed in our study was much greater (i.e., 39% and 27% reduction in relative growth rate after 24 hr and 48 hr feeding, respectively) and may be due to plant genotypic differences. Furthermore, certain herbivores, such as *H. zea*, may be more effective at inducing resistance than others, such as Mexican bean beetles and soybean loopers (Felton, unpublished data).

Induced resistance in soybean is a multicomponent plant response and is not due to a single biochemical mechanism (Kogan and Fischer, 1991). Our data indicate that a substantial component of induced resistance to *H. zea* is present in the leaf protein fraction (Table 2). In addition to a small quantitative reduction in foliar proteins in wounded plants, there was a significant decline in the quality of foliar protein. The decline in the nutritional quality of protein may be attributed to an induction of defense proteins (e.g., trypsin inhibitor) and/or chemical damage to constitutive proteins. Kraemer et al. (1987) reported that Mexican bean beetle feeding induced higher levels of foliar proteinase inhibitors. We observed a statistically significant increase in the trypsin inhibitor level in the foliar protein. However, both the constitutive and induced levels of trypsin inhibitor in our protein isolates were quite low and when reconstituted in diet would be $\leq 0.056\%$ wet weight. This level of inhibitor is below that required to observe a reduction in the larval growth of *H. zea* in artificial diets (Felton, unpublished data).

The damage-induced decline in the nutritional quality of soybean foliar protein is likely due to an accumulation of lipid peroxidation products associated with the protein. We observed a significant increase in lipid peroxidation products associated with protein from wounded plants. Further indication that the lipid peroxidation products may have damaged leaf protein is shown by the decrease in free amines (primarily the ϵ-amino group of lysine) and the loss in thiol groups. These amino groups are partially susceptible to oxidative damage resulting from reactions with lipid peroxidation products (e.g., lipid hydro-

peroxides, aldehydes, and epoxides) (Gardner, 1991). Lysine and thiol amino acids are particularly limiting amino acids for larval growth of noctuids (Felton et al., 1992b). The accumulation of lipid peroxidation products associated with leaf protein is likely due to the large increase in foliar lipoxygenases observed in wounded plants (Tables 2 and 3). Lipoxygenases (linoleate: oxygen oxidoreductase, EC 1.13.11.12) are a class of enzymes found in all higher plants and animals (Hatanaka et al., 1987; Mack et al., 1987; Schewe et al., 1986; Vick and Zimmerman, 1987), which catalyze the hydroperoxidation of polyunsaturated lipid containing *cis*,*cis*-pentadiene structure. The principle substrates for lipoxygenases (LOXs) in higher plants are linoleic (C18:2) and linolenic (C18:3) acids (Hatanaka et al., 1987; Vick and Zimmerman, 1987).

Lipoxygenases may be important components of resistance to herbivores, and may function as plant defense proteins by affecting insect growth and development in a variety of direct and indirect manners. The products of lipoxygenase may be repellent to insect feeding and operate as antixenosis bases of resistance. Mohri et al. (1990) found that the products, linoleic acid hydroperoxide and hexanal, acted as feeding repellents to several beetle species. The products of lipid peroxidation may be toxic and function in antibiosis-based resistance. The initial products of lipoxygenase activity are fatty acid hydroperoxides. Shukle and Murdock (1983) showed that soybean lipoxygenases or linoleic hydroperoxide retards the larval growth of *M. sexta*. The hydroperoxides then may form an array of reactive aldehydes, malondialdehyde, γ-ketols and epoxides (Gardner, 1991). Many of these products can form Schiff base adducts with proteins or act as potent alkylating agents of macromolecules (Gardner, 1991). Thus dietary nutrients such as amino acids and/or carotenoids may be destroyed by lipoxygenase products (e.g., Hildebrand and Kito, 1984; Hildebrand et al., 1986). In addition, the enzyme substrate linoleic acid, another essential dietary component of insects (Dadd, 1973), is destroyed in the process. Moreover, lipoxygenase reactions may produce reactive oxygen radicals (Vick and Zimmerman, 1987). Free radicals are implicated in numerous pathologies associated with protein, lipid, and DNA damage (Halliwell, 1991).

Lipoxygenases may also be involved in indirect forms of pest resistance in plants such as intra/interplant communication. Lipoxygenase is involved in the early biosynthetic steps of jasmonic acid (JA) and methyl jasmonate (JA-Me) production (Ueda and Kato, 1980; Vick and Zimmerman, 1987; Enyedi et al., 1992). JA and JA-Me induce the synthesis of several plant defensive proteins or chemicals including proteinase inhibitors, phenylalanine ammonia lyase, and alkaloids (Enyedi et al., 1992; Hamberg and Gardner 1992). Recently, it was shown that the biosynthetic precursors of JA, α-linolenic acid, 13(*S*)-HPOT [13(*S*)-hydroperoxy-9(*Z*),11(*E*),15(*Z*)-octadecatrienoic acid] and 12-oxo-PDA (12-oxophytodienoic acid), also served to induce proteinase inhibitors in tomato leaves in a manner similar to JA and JA-Me (Farmer and Ryan, 1992).

In summary, induced resistance in soybean is a complex phenomenon and is associated with induction and/or activation of several diverse biochemical pathways (Chiang et al., 1987; Hildebrand and Hamilton-Kemp, 1988; Kogan and Fischer, 1991; Felton and Summers, 1993). The activation of lipoxygenases by insect feeding may be an early event in the induced response of soybean that induces not only the production of lipoxygenases, but also the induction of proteinase inhibitors and enzymes for phenolic biosynthesis. We suggest that lipoxygenases function in plant defense to insects by directly initiating oxidative and nutritional stress in herbivores and by producing chemical signals to activate other genes to produce defensive compounds.

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(*Z,E,E*)-DODECATRIEN-1-ol: A MINOR COMPONENT OF TRAIL PHEROMONE OF TERMITE, *Coptotermes formosanus* SHIRAKI

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Abstract—In the course of the elucidation of the primary structure of an isolated trail pheromone from *C. formosanus*, a minor component that had the same molecular weight as the major trail pheromone, (*Z,Z,E*)-3,6,8-dodecatrien-1-ol [(*Z,Z,E*)-DTE-OH], was detected in the mass chromatogram of *m/z* 180 of capillary GC-MS. The mass spectrum of the minor component showed a prominent pattern of dodecatrien-1-ol. Chemical analysis demonstrated that the complete structure was (*Z,E,E*)-DTE-OH. Furthermore, capillary GC-MS-HR-SIM analysis indicated that the component existed only in the workers of *Coptotermes formosanus* Shiraki and was not present in workers of *Reticulitermes speratus* (Kolbe). This minor component may be a species-specific factor of *C. formosanus*, although this was not suggested by a two-choice bioassay.

Key Words—Subterranean termite, *Coptotermes formosanus*, *Reticulitermes speratus*, Rhinotermitidae, Isoptera, trail pheromonal minor component, geometrical isomer, (*Z,E,E*)-3,6,8-dodecatrien-1-ol, capillary GC-MS-HR-SIM.

INTRODUCTION

Recent investigations demonstrated that the trail pheromones produced by two Japanese rhinotermitids, *Reticulitermes speratus* (Kolbe) and *Coptotermes formosanus* Shiraki (Yamaoka et al., 1987; Tokoro et al., 1989, 1991, 1992) had identical chemical structures. This compound was identical to

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(*Z*,*Z*,*E*)-3,6,8-dodecatrien-1-ol [(*Z*,*Z*,*E*)-DTE-OH], which was previously obtained from fungus-infected wood (Matsumura et al., 1968, 1969) and later identified as the trail pheromone of *R. virginicus* (Tai et al., 1969). However, Howard et al. (1976) suggested that termite species should produce specific trail pheromones, as demonstrated by the ability of four rhinotermitid species to recognize extracts containing their own trail pheromones. This result suggests that species specificity could be associated with chemical variation in trail pheromones. Previous workers suggested that chemical variation in trail pheromone may result in species specificity and that small quantities of chemical analogs may act in a multicomponent way conferring specificity (Kaib et al., 1982; Traniello, 1982; Rucie, 1987).

In the course of the isolation and identification of a trail pheromone from *C. formosanus*, a small amount of an unidentified material, which was not identical to (*Z*,*Z*,*E*)-DTE-OH but showed the trail-following activity, was obtained as described previously (Tokoro et al., 1992). Capillary gas chromatography–mass spectrometer (CGC-MS) analysis of the material suggested that it was an isomer of (*Z*,*Z*,*E*)-DTE-OH. Therefore, this material, a minor component of the trail pheromone, may impart specificity to *C. formosanus*.

Capillary GC-MS high-resolution selected-ion-monitoring (CGC-MS-HR-SIM) analysis is a recent and unique detection technique. This highly sensitive and highly selective analysis can essentially facilitate the quick identification of a compound whose molecular weight is known.

It was necessary to determine whether the minor component was actually biosynthesized by termites (in sternal glands) or whether the trail pheromone (*Z*,*Z*,*E*)-DTE-OH was isomerized into the minor component by the extraction procedure. Highly sensitive and selective analysis (CGC-MS-HR-SIM) can facilitate the detection of minor component before isomerization occurs. As the molecular weights of the minor component and trail pheromone are known (Tokoro et al., 1992), these compounds were detectable by means of CGC-MS-HR-SIM analysis.

The purpose of this experiment was to identify this compound and to determine whether it is a minor component of the trail pheromone of *C. formosanus* and *R. speratus* by using CGC-MS-HR-SIM. In addition, the species-specific function of the minor component was examined in a choice bioassay using the two termite species.

METHODS AND MATERIALS

Test Termites. Termites of *R. speratus* were obtained from small colonies at the campus of Kyoto University in Uji, Japan. Individual termites were removed from wood for extraction. The termites were reared at 26 ± 2°C and

60 ± 5% relative humidity for one month until use. *C. formosanus* were collected in Wakayama, Japan, and reared at the Wood Research Institute, Kyoto University, for about 10 years on Japanese red pine (*Pinus densiflora* Sieb. et Zucc.) at 28 ± 2°C and ca. 80% relative humidity. The test termites were undifferentiated pseudergates, older than third instar as determined by the size of the termite body (hereafter referred to as "workers"). Termites from which extracts were made were fed moistened filter paper for about five days to minimize food or nest odor contamination before extraction. The workers for bioassay were placed in a Petri dish with a moistened filter paper for 1 hr prior to bioassay.

Other worker termites (200 individuals) used for sternal gland excision were immobilized in a refrigerator at −20°C for 10 hr. Two hundred abdominal fifth sternites at which the sternal gland was positioned were carefully dissected from the bodies of worker termites under a binocular stereoscopic microscope. The dissected parts were extracted with *n*-hexane as sternal gland extract.

Chemicals. The authentic DTE-OH isomers were supplied by Dr. H. Yamamoto (Nagoya University); all other reagents were purchased from Nacalai Tesque Inc. (Kyoto, Japan).

Isolation and Identification of Minor Component. Workers (300,000) were extracted with *n*-hexane for three days. The minor component was isolated and identified as described previously for the major component of the trail pheromone (Tokoro et al., 1989, 1992). Subsequently, the isolated minor components were identified by means of capillary GC-MS and capillary GC-FTIR analysis combined with microscale chemical reactions (acetylation, partial reduction, ozonolysis) as described elsewhere (Yamaoka et al., 1987; Tokoro et al., 1992).

Authenticity of Minor Component by CGC-MS-HR-SIM Analysis. Three analytical samples were prepared as follows. For sample 1, 500 whole body workers were extracted in *n*-hexane for 20 hr, after which the excess solvent was evaporated with nitrogen gas. This extract was redissolved in 30 μl *n*-hexane and purified by silica-gel pipet flash column chromatography with EtOAc/Hx eluants. For sample 2, 50 whole body workers were extracted with diethyl ether for 5 min, and excess solvent was evaporated with nitrogen gas. The extract was redissolved in 5 μl *n*-hexane. For sample 3, one whole body worker was extracted with *n*-hexane for 40 min, and excess solvent was evaporated with nitrogen gas. The extract was redissolved in μl *n*-hexane. Three samples each were prepared for both termite species. One microliter of each sample was used for each analysis. All samples were subjected to CGC-MS-HR-SIM immediately following preparation.

These samples were first analyzed by capillary GC and subsequently analyzed by CGC-MS-HR-SIM. The capillary GC-MS apparatus was a Hewlett Packard (HP) model 5890 gas chromatograph combined with a model M-80B mass spectrometer (Hitachi, Ibaraki, Japan), equipped with a model 0101 on-line

data system. A fused-silica WCOT capillary column (25 m × 0.25 mm ID, liquid phase PEG-HT; GL Science Ltd. Tokyo, Japan) was used with this system. The analytical conditions were as follows: The ion source temperature was 180°C, and ionization energy was 70 eV. Samples were dissolved in 1 μl of *n*-hexane injected with a Grob-type splitless injector. The oven temperature was increased from 60 to 210°C at a rate of 30°C/min for a 5-min period, and the injection temperature was 210°C. The resolution was approximately 6000 units, and the SIM monitor was set at *m/z* 180.1513, the precise molecular weight of DTE-OH.

Bioassay Method. In order to determine the threshold response levels for trail-following and species specificity of trail pheromone, choice tests (Y-tests) were conducted.

A modification of the Howard et al. (1976) choice bioassay was used to examine the preferential difference between the two rhinotermitid termites in trail-following activity of some substances (Figure 1). One microliter each of a dissolved sample was streaked from the junction to the distal end of the branched line (1.5 cm long) and stem (3 cm long). Another 1 μl was streaked from the junction to the end of basal point of the Y-shaped pencil guideline (ca. 1.5 cm long and the angle formed by the branches is 45°). One microliter of another dissolved sample was streaked along another branch (1.5 cm long) and the same stem of the Y-shaped pencil guideline drawn on the clay-coated paper with a 5-μl micropipet. The same was done for the other branched and basal lines with the other dissolved sample. After evaporation of the solvent (at least 10 sec), a plastic cylinder (1 × 1.5 cm ID) was placed on the paper at a side of the test arena. One opening of the cylinder directed the termite toward the test arena. A worker termite was then introduced into the cylinder, and a red-colored Petri

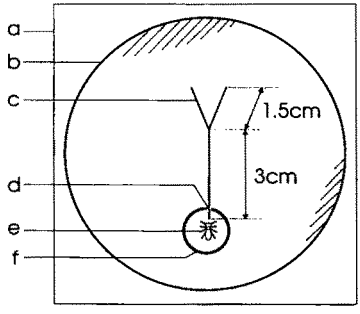

FIG. 1. Scheme of modified choice bioassay: (a) fine-quality clay-coated paper, (b) Petri dish lid (.5 × 9 cm), (c) pencil guideline, (d) opening, (e) test termite, (f) plastic cylinder.

dish lid (1 × 5.7 cm ID) was placed above it in order to minimize the influence of air movements and light.

When a worker termite succeeded in moving along the sample streaked Y-shaped line within 1 min, it was considered that a "basic activity" was induced. When the termite completed a trail-following and reached one distal end of the branched part of a Y-shaped line or deviated from the trail at least 2 cm, the termite was removed from the test stage immediately. Ten replicates of Y-shaped lines were prepared, and 10 termites (one at a time) were tested on each line. The numbers of test worker termites that selected either of the branched parts were recorded. A given sample was considered to have elicited a threshold trail-following response if more than 50% of 100 test termites showed a basic activity. Twofold dilution series of each sample were employed to determine the trail-following activity of the test materials. Bioassays were carried out at approximately 26 ± 2°C and 60 ± 5% relative humidity under the fluorescent lighting to estimate the trail-following activity. No termite was used for more than one bioassay. All the data were analyzed by a chi-square statistical analysis using the Yates correction for continuity (Yates, 1934).

RESULTS AND DISCUSSION

Isolation and Identification of Minor Component. We estimated that the mean amount of the minor substance extracted from each individual worker termite was approximately 5 pg on the basis of GC analysis.

The capillary GC-MS data of the minor component (Figure 2A) and of the authentic DTE-OH isomers (Figure 2B) indicated that the two samples were similar. The peaks (scan numbers 333 and 346) had similar retention times, and the mass spectra showed a prominent molecular ion at *m/z* 180 ($M^+ \cdot$). A series of diagnostic ions at *m/z* 91, 105, 119, 133 (C_nH_{2n-7}) were also found, as reported previously (Tokoro et al., 1992), suggesting that the minor component was an isomer of DTE-OH.

The partially hydrogenated products of the minor component were analyzed by capillary GC-MS. Seven peaks were obtained from each compound resulting from the partial hydrogenation procedure. These were identified as *n*-dodecanol, (*Z*)-3-dodecen-1-ol, (*E*)-6-dodecen-1-ol + (*E*)-8-dodecen-1-ol, (*Z*,*E*)-3,8-dodecadien-1-ol, (*Z*,*E*)-3,6-dodecadien-1-ol, (*E*,*E*)-6,8-dodecadien-1-ol, and (*Z*,*E*,*E*)-3,6,8-dodecatrien-1-ol [(*Z*,*E*,*E*)-DTE-OH]. Results of ozonolysis and capillary GC-FTIR analysis upheld these results. It was concluded that the minor component was identical to (*Z*,*E*,*E*)-DTE-OH.

Capillary GC and Capillary GC-MS-HR-SIM Analyses of Whole-Body Extracts. Results of capillary GC analyses of the whole-body extract (sample 1) and that of authentic DTE-OH isomers are shown in Figures 3A and 4B,

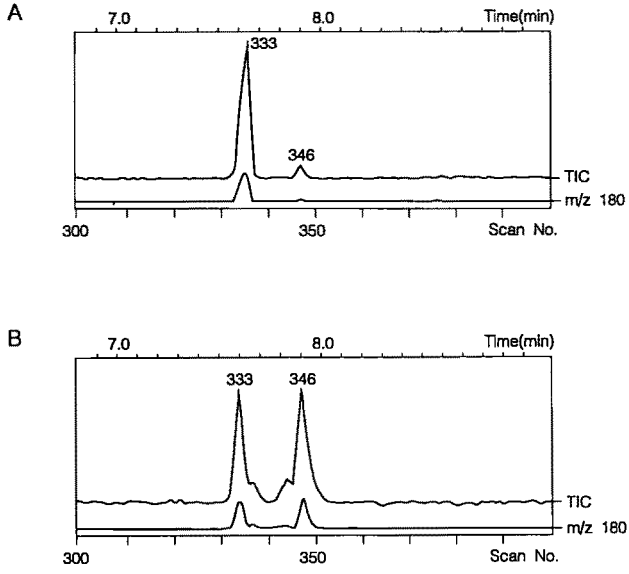

FIG. 2. Total ion chromatogram and mass chromatogram (*m/z* 180) of the trail pheromone and the minor component isolated from *C. formosanus* and authentic (*Z,E,E*)-DTE-OH. (A) Trail pheromone (scan no. 333) and the minor component (scan No. 346); (B) authentic DTE-OH isomers: scan No. 333, (*Z,Z,E*)-DTE-OH; scan No. 346, (*Z,E,E*)-DTE-OH.

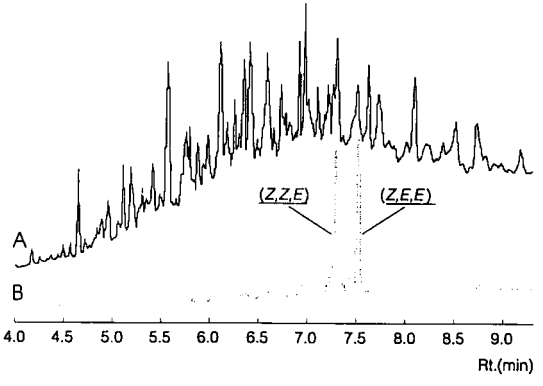

FIG. 3. Capillary GC data of the whole body extracts from *C. formosanus* (A) and authentic DTE-OH isomers (B).

respectively. The chromatogram in Figure 3A shows many peaks consisting of the components of the extracts. However, because of the large number of peaks, it is impossible to distinguish between the target components and the impurities.

Results of capillary GC-MS-HR-SIM analyses of the minor component and (*Z*,*E*,*E*)-DTE-OH demonstrated that the DTE-OH isomers (i.e., trail pheromone isomers) could be detected by HR-SIM at *m*/*z* 180.1513 and that both could be isolated from impurities. Two samples (2 and 3) from both termite species exhibited several impurity peaks due to diethyl ether solvent impurities (R_f 6.2: sample 2) or the background impurity from the column liquid phase (sample 3). In the case of *C. formosanus* (Figure 4A), all test samples exhibited the prominent (*Z*,*Z*,*E*)-DTE-OH peaks. Samples 1 and 2 also exhibited the clear peaks

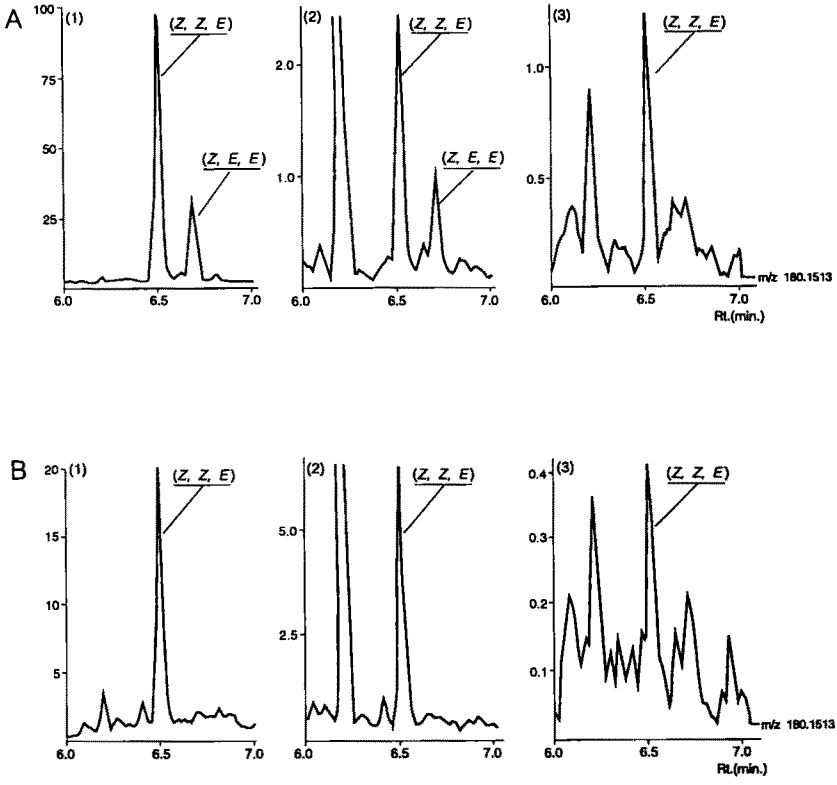

FIG. 4. Capillary GC-MS-HR-SIM data of the whole-body extracts from *C. formosanus* (A) and *R. speratus* (B). HR-SIM chromatograms at *m*/*z* 181.1513 (WE = worker equivalent). Sample 1: 16.7 WE/μl; sample 2: 10 WE/μl; sample 3: 1 WE/μl.

of (*Z*,*E*,*E*)-DTE-OH, which were each equivalent to 35% (w/w) of the quantity of (*Z*,*Z*,*E*)-DTE-OH.

All test samples from *R. speratus* (Figure 4B), also exhibited the prominent (*Z*,*Z*,*E*)-DTE-OH peaks. Thus, in these two rhinotermitid termites, the minor component, (*Z*,*E*,*E*)-DTE-OH, may be produced only by *C. formosanus*. As the extraction procedure was the same for the two species, these results suggest that the minor component was not a product of isomerization of the trail pheromone (*Z*,*Z*,*E*)-DTE-OH.

In the CGC-MS-HR-SIM analyses, the amounts of detected trail pheromone and minor component from a worker for each termite species was determined with an external standard technique. (*Z*,*Z*,*E*)-DTE-OH was extracted in ca. 15 ng, 142 pg, and 46 pg quantities in samples 1, 2, and 3, respectively, for *C. formosanus*. The minor component, (*Z*,*E*,*E*)-DTE-OH, amounted to 525 pg and 50 pg in samples 1 and 2, respectively. For *R. speratus*, the extract of (*Z*,*Z*,*E*)-DTE-OH consisted of 313 pg, 301 pg, and 32 pg in samples 1, 2, and 3, respectively. As a result, the minor component, (*Z*,*E*,*E*)-DTE-OH, was apparently extracted from the workers of *C. formosanus* in concentrations equivalent to 35% (w/w) of (*Z*,*Z*,*E*)-DTE-OH. Thus, the CGC-MS-HR-SIM analysis is very effective for the detection of pheromone isomers, and it allows detection with high sensitivity and high selectivity from crude lipid extracts.

Results of Y Test Using Sternal Gland Extract and Authentic DTE-OH. In order to determine species specificity, Howard et al. (1976) first employed a choice bioassay technique. Preliminary trials that employed the choice bioassay technique of Howard et al. supported that termites showed a statistically equal response to the two chemicals of the same activity level generating 50/50 distribution. However, it was noticed during the bioassay that a group of termites undertook tandem running, and some termites seemed to disturb the natural behavior of the others in the test arena. Therefore, a single worker termite at a time was tested instead of a group of 15 termites to rule out group effects. In addition, the shorter branched lines were considered to result in the evenness of the streaked quantity of test chemicals along the trail line.

Results obtained in the Y tests are shown in Tables 1–10. As expected, two samples that had the same trail-following activity level generally induced a 50/50 distribution. The results suggested the applicability of the bioassay technique to examine the species specificity using two termite species. Each threshold quantity, which could induce the basic activity in more than 50% of test workers, of the samples was estimated as follows in the case of *R. speratus*: (*Z*,*Z*,*E*)-DTE-OH 100 fg, (*Z*,*E*,*E*)-DTE-OH 50 pg/1.5-cm trail, *R. speratus* sternal gland extract (R.s.-SGE) 10^{-4} WE/1.5-cm-trail, *C. formosanus* sternal gland extract (C.f.-SGE 5×10^{-5} WE/1.5-cm trail. In the case of *C. formosanus* workers: (*Z*,*Z*,*E*)-DTE-OH 100 fg, (*Z*,*E*,*E*)-DTE-OH 25 pg/1.5-cm trail, R.s.-SGE 10^{-4} WE/1.5 cm-trail, C.f.-SGE 5×10^{-5} WE/1.5-cm trail. Since

TABLE 1. DISTRIBUTION OF TERMITES IN Y TEST WHEN SEVERAL OF THE SAME (*Z, Z, E*)-DTE-OH CONCENTRATIONS OR (*Z, E, E,*)-DTE-OH CONCENTRATIONS ARE APPLIED TO *R. speratus* WORKERS

Test samples (weight/1.5 cm)	Number of termites choosing			Chi-square[a]
	Left		Right	
(*Z,Z,E*)-DTE-OH				
50 ng	8	(72)[b]	20	4.32
5 ng	46	(13)	41	0.18
500 pg	54	(0)	46	0.49
50 pg	48	(0)	52	0.09
5 pg	41	(6)	53	1.29
500 fg	54	(6)	40	1.80
250 fg	38	(12)	50	1.38
100 fg	46	(10)	44	0.00
50 fg	18	(66)	16	0.03
25 fg	15	(82)	3	6.72
5 fg	0	(100)	0	
(*Z,E,E*)-DTE-OH				
50 ng	41	(2)	57	2.30
5 ng	48	(1)	51	0.04
500 pg	39	(4)	57	3.01
50 pg	41	(9)	50	0.70
25 pg	21	(65)	14	1.03
10 pg	8	(88)	6	0.07
5 pg	14	(78)	6	2.45
500 fg	1	(94)	5	1.50

[a] Chi-square analysis made on the hypothesis that if the two-sample trail is identical, a 1 : 1 distribution will result, and the value of chi-square will be less than 3.84 at the 95% level of significance.
[b] Number of termites deviating.

the active threshold level of (*Z,E,E*)-DTE-OH was at least 100 times higher than that of (*Z,Z,E*)-DTE-OH (Tables 1 and 2), a behavioral effect of the component to the termite might be insignificant by itself. It is necessary to show whether the (*Z,E,E*)-DTE-OH and (*Z,Z,E*)-DTE-OH synergistically induce workers of *C. formosanus* to species-specific trail-following behavior.

Two kinds of solutions of (*Z,Z,E*)-DTE-OH were used for the Y tests. Solution A was a hexane solution of (*Z,Z,E*)-DTE-OH as an artificial trail pheromone of *R. speratus*, and solution B was a mixture of (*Z,Z,E*)-DTE-OH and (*Z,E,E*)-DTE-OH [35% (w/w) of DTE-OH] as an artificial trail pheromone of *C. formosanus*.

Results of the Y test with solutions A and B are shown in Tables 5 and 6. In all cases, the observed preference of termites corresponded well to the

TABLE 2. DISTRIBUTION OF TERMITES IN Y TEST WHEN SEVERAL OF THE SAME (*Z, Z, E*)-DTE-OH CONCENTRATIONS OR (*Z, E, E*)-DTE-OH CONCENTRATIONS ARE APPLIED TO *C. formosanus* WORKERS[a]

Test samples (weight/1.5 cm)	Number of termites choosing			Chi-square
	Left		Right	
(Z,Z,E)-DTE-OH				
50 ng	11	(83)	6	0.94
5 ng	39	(30)	31	0.70
500 pg	55	(2)	43	0.23
50 pg	42	(3)	55	1.48
5 pg	46	(2)	52	0.26
500 fg	46	(7)	39	0.42
250 fg	47	(0)	33	2.11
100 fg	48	(22)	30	3.71
50 fg	24	(58)	18	0.60
25 fg	0	(96)	4	2.25
5 fg	0	(100)	0	
(Z,E,E)-DTE-OH				
50 ng	54	(2)	57	2.30
5 ng	44	(1)	51	0.04
500 pg	58	(3)	39	3.34
50 pg	56	(6)	38	3.07
25 pg	40	(28)	32	0.68
10 pg	5	(92)	3	0.13
5 pg	2	(96)	2	0.25
500 fg	1	(99)	0	0.00

[a] See footnotes to Table 1.

50/50 distribution at the 95% level of significance. These results indicated that *R. speratus* workers could not distinguish between solutions A and B. Contrary to expectation, *C. formosanus* could not discern the two solutions either. Thus, the minor component (*Z,E,E*)-DTE-OH could not elicit a specific trail-following behavior in both *C. formosanus* and *R. speratus*.

Y tests were concurrently conducted with the sternal gland extracts (as natural pheromone) of the two species to examine whether a termite species could distinguish its own extracts from other extracts. As demonstrated in Tables 3 and 4, threshold trail-following levels of sternal gland extract from *C. formosanus* were two times higher than those from *R. speratus*, regardless of termite species. Therefore, test concentrations were selected based on the trail-following activities. In other wards, the concentrations that succeeded in inducing the same level of trail-following behavior to both termite species were streaked along the Y shaped line in choice bioassay.

TABLE 3. DISTRIBUTIONS OF TERMITES IN Y TEST WHEN SEVERAL OF THE SAME STERNAL GLAND EXTRACTS ARE APPLIED TO *R. speratus* WORKERS

Test samples (WE[a]/1.5 cm)	Number of termites choosing			Chi-square[b]
	Left		Right	
R.s-SGE[c]				
0.1	44	(6)[d]	50	0.27
0.01	46	(2)	52	0.26
0.005	50	(0)	50	0.01
0.001	42	(0)	58	1.40
0.0005	53	(8)	39	1.84
0.00025	48	(11)	41	0.40
0.0001	44	(13)	43	0.00
0.00005	18	(54)	28	1.76
0.000025	12	(72)	18	0.83
0.0000125	0	(96)	4	2.25
0.000005	0	(100)	0	
C.f-SGE[e]				
0.1	50	(1)	49	0.00
0.01	52	(2)	46	0.26
0.005	59	(0)	41	2.89
0.001	44	(6)	50	0.27
0.0005	52	(0)	48	0.09
0.00025	43	(3)	54	1.03
0.0001	40	(6)	54	1.80
0.00005	28	(49)	23	0.31
0.000025	12	(76)	12	0.04
0.0000125	2	(98)	0	0.50
0.000005	0	(100)	0	

[a] Worker equivalent.
[b] Chi-square analysis made on the hypothesis that if the two-sample trail is identical, a 1 : 1 distribution will result, and the value of chi-square will be less than 3.84 at the 95% level of significance.
[c] Sternal gland extract of *R. speratus*.
[d] Number of termites deviating is in parentheses.
[e] Sternal gland extract of C. formosanus.

Results of the Y test with interspecific sternal gland extract are shown in Tables 7 and 8. When the samples with the same trail-following activity were tested, any termite showed nonselective behavior to the extracts from both species. Termites could not recognize their own sternal gland extract trail under such test conditions but were rather more sensitive to quantitative differences. These results are different from the data of the previous report by Howard et al. (1976), who worked on *R. virginicus*, *R. flavipes*, *R. tibialis*, and *C. formosanus* and concluded that trail pheromones were species specific. This dif-

TABLE 4. DISTRIBUTIONS OF TERMITES IN Y TEST WHEN SEVERAL OF THE SAME STERNAL GLAND EXTRACTS ARE APPLIED TO *C. formosanus* WORKERS[a]

Test samples (WE/1.5 cm)	Number of termites choosing			Chi-square
	Left		Right	
R.s-SGE				
0.1	43	(8)	49	0.27
0.01	47	(5)	48	0.00
0.005	44	(9)	47	0.04
0.001	45	(8)	47	0.01
0.0005	50	(2)	48	0.01
0.00025	48	(11)	41	0.40
0.0001	39	(18)	43	0.11
0.00005	21	(52)	27	0.52
0.000025	12	(78)	10	0.05
0.0000125	9	(87)	4	1.23
0.000005	4	(95)	1	
C.f.-SGE				
0.1	51	(1)	48	0.04
0.01	48	(4)	48	0.01
0.005	48	(6)	46	0.01
0.001	45	(8)	47	0.01
0.0005	46	(10)	44	0.01
0.00025	44	(9)	47	0.04
0.0001	40	(8)	52	1.32
0.00005	33	(34)	33	0.02
0.000025	24	(57)	19	0.37
0.0000125	14	(68)	18	0.28
0.000005	11	(75)	14	0.16

[a] See footnotes to Table 3.

TABLE 5. Y TEST OF SOLUTION A [(*Z,Z,E*)-DTE-OH] TRAIL VERSUS SOLUTION B [(*Z,Z,E*)-DTE-OH + (*Z,E,E*)-DTE-OH] TRAIL FOR *R. speratus* WORKERS

Quantity of (*Z,Z,E*)-DTE-OH (weight/1.5 cm)	Number of termites choosing			Chi-square[b]
	Solution A		Solution B[a]	
5 ng	32	(20)	48	2.81
500 pg	56	(4)	40	2.34
50 pg	47	(1)	52	0.16
5 pg	45	(1)	54	0.65
500 fg	48	(10)	42	0.28
100 fg	27	(32)	41	2.49

[a] Chi-square analysis made on the hypothesis that if the two-sample trail is identical, a 1 : 1 distribution will result, and the value of chi-square will be less than 3.84 at the 95% level of significance.
[b] (*Z,E,E*)-DTE-OH was added to solution A at the rate of 35% of (*Z,Z,E*)-DTE-OH quantity.
[c] Number of termites deviating.

TABLE 6. Y TEST OF SOLUTION A [(*Z*,*Z*,*E*)-DTE-OH) TRAIL VERSUS SOLUTION B [DTE-OH + (*Z*,*E*,*E*)-DTE-OH] TRAIL FOR *C. formosanus* WORKERS[a]

Quantity of (*Z*,*Z*,*E*)-DTE-OH (weight/1.5 cm)	Number of termites choosing			Chi-square
	Solution A		Solution B	
5 ng	30	(30)	40	1.16
500 pg	49	(7)	44	0.17
50 pg	44	(7)	49	0.17
5 pg	40	(6)	54	1.80
500 fg	36	(8)	55	3.56
100 fg	24	(36)	38	2.73

[a]See footnotes to Table 3.

TABLE 7. Y TEST OF STERNAL GLAND EXTRACT FROM *R. speratus* VERSUS THAT FROM *C. formosanus* USING *R. speratus* WORKERS

Test samples (WE[a] 1.5 cm)		Number of termites choosing			
(a) R.s.-SGE[c]	(b) C.f.-SGE[d]	a		b	Chi-square[b]
0.02	0.01	40	(0)[e]	60	3.61
0.002	0.001	38	(5)	57	3.41
0.0002	0.0001	43	(11)	46	0.04
0.0001	0.00005	42	(3)	55	1.48
0.03	0.01	57	(1)	42	1.98
0.003	0.001	76	(0)	24	26.01
0.0003	0.0001	57	(3)	39	3.01
0.01	0.01	12	(5)	83	51.58
0.001	0.001	30	(5)	65	12.17
0.0001	0.0001	16	(8)	76	37.84
0.01	0.02	13	(1)	86	52.36
0.001	0.002	12	(1)	87	55.31
0.0001	0.0002	10	(1)	89	61.45

[a]Worker equivalent.
[b]Chi-square analysis made on the hypothesis that if the two-sample trail is identical, a 1 : 1 distribution will result, and the value of chi-square will be less than 3.84 at the 95% level of significance.
[c]Sternal gland extract of *R. speratus*.
[d]Sternal gland extract of *C. formosanus*.
[e]Number of termites deviating is in parentheses.

TABLE 8. Y TEST OF STERNAL GLAND EXTRACT FROM *R. speratus* VERSUS THAT FROM *C. formosanus* USING *C. formosanus* WORKERS[a]

Test samples (WE/1.5 cm)		Number of termites choosing			
(a) R.s.-SGE	(b) C.f.-SGE	a		b	Chi-square
0.02	0.01	39	(6)	55	2.69
0.002	0.001	40	(2)	58	2.95
0.0002	0.0001	43	(10)	47	0.16
0.0001	0.00005	27	(30)	43	3.21
0.03	0.01	68	(6)	26	17.88
0.003	0.001	62	(2)	36	6.38
0.0003	0.0001	62	(11)	27	12.99
0.01	0.01	22	(10)	68	22.50
0.001	0.001	26	(4)	70	19.26
0.0001	0.0001	6	(6)	88	69.80
0.01	0.02	7	(3)	90	69.32
0.001	0.002	15	(4)	81	44.01
0.0001	0.0002	10	(6)	84	56.69

[a] See footnotes to Table 7.

ference may be due to the different test termites. However, it is of great interest to point out that termites always chose the line with a sample of two to three times higher trail-following activity (Tables 9 and 10). Such sensitive preference might be directly related to orientation activity, as demonstrated with *R. hesperus* by Grace et al. (1988). We also found similar results as the artificial trail studies conducted by Howard et al. (1976) with these two termite species.

Although (*Z,E,E*)-DTE-OH did not induce species-specific trail-following behavior in either species in this bioassay, the minor component may possibly impart a species-specific mechanism to the *C. formosanus* trail pheromone. Possibly, this bioassay condition was unsuitable for distinguishing between pheromone trails of the two species. On the other hand, it is possible that this minor component is only a by-product of the biosynthesis of (*Z,Z,E*)-DTE-OH. It is necessary to determine specific and colonial or seasonal variety of the quantitative ratio of (*Z,Z,E*)-DTE-OH and (*Z,E,E*)-DTE-OH to all rhinotermitid termites.

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TABLE 9. DISTRIBUTION OF TERMITES IN Y TEST WHEN SEVERAL OF THE SAME STERNAL GLAND EXTRACTS ARE APPLIED TO *R. speratus* WORKERS

Test samples (WE[a]/1.5 cm)	Number of termites choosing			Chi-square[b]
R.s.-SGE[c]	×2[d]		×1[d]	
0.1	71	(1)[e]	28	17.82
0.01	80	(2)	18	37.97
0.005	92	(0)	8	68.89
0.001	97	(0)	3	86.49
0.0005	90	(6)	4	76.86
0.0001	69	(12)	19	27.28
0.00005	40	(55)	5	25.69
0.000025	14	(83)	3	5.88
R.s.-SGE	×3		×1	
0.1	94	(3)	3	83.51
0.01	94	(0)	6	75.69
0.001	100	(0)	0	98.01
0.0001	96	(4)	0	94.01
C.f. SGE[f]	×2		×1	
0.1	83	(1)	16	44.00
0.01	80	(2)	18	37.97
0.005	92	(0)	8	68.89
0.001	85	(2)	13	51.44
0.0005	95	(0)	5	79.21
0.0001	82	(2)	16	8.00
0.00005	87	(5)	8	64.04
0.000025	32	(62)	6	16.45
C.f.-SGE	×3		×1	
0.1	95	(2)	3	84.50
0.01	94	(1)	5	78.22
0.001	98	(0)	2	90.25
0.0001	89	(2)	9	63.68

[a] Worker equivalent.
[b] Chi-square analysis made on the hypothesis that if the two-sample trail is identical, a 1 : 1 distribution will result, and the value of chi-square will be less than 3.84 at the 95% level of significance.
[c] Sternal gland extract of *R. speratus*.
[d] Number means a multiple of test concentration.
[e] Quantity of applied sample per trial.
[f] Sternal gland extract of *C. formosanus*.

TABLE 10. DISTRIBUTION OF TERMITES IN Y TEST WHEN SEVERAL OF THE SAME STERNAL GLAND EXTRACTS ARE APPLIED TO *C. formosanus* WORKERS[a]

Test samples (WE/1.5 cm)	Number of termites choosing			Chi-square
R.s.-SGE	×2		×1	
0.1	68	(6)	26	17.88
0.01	65	(4)	31	11.34
0.005	82	(5)	13	48.67
0.001	58	(4)	38	3.76
0.0005	74	(9)	17	34.46
0.0001	64	(18)	18	24.70
0.00005	56	(18)	26	10.26
0.000025	22	(74)	4	11.12
R.s.-SGE	×3		×1	
0.1	83	(7)	10	55.74
0.01	79	(3)	18	37.11
0.001	80	(9)	11	50.81
0.0001	84	(10)	6	65.88
C.f-SGE	×2		×1	
0.1	78	(6)	16	39.59
0.01	71	(2)	27	18.87
0.005	60	(5)	35	6.06
0.001	70	(3)	27	18.19
0.0005	66	(15)	19	24.89
0.0001	58	(20)	22	8.00
0.00005	57	(18)	25	11.72
0.000025	62	(22)	16	25.96
C.f-SGE	×3		×1	
0.1	94	(2)	4	80.83
0.01	74	(8)	18	32.88
0.001	84	(4)	12	52.51
0.0001	76	(18)	6	58.06

[a]See footnotes to Table 9.

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Book Review

Sawfly Life History Adaptations to Woody Plants. Michael Wagner and Kenneth F. Raffa (eds.) San Diego, California: Academic Press, 1993. $84.00 (cloth), 581 pp., ISBN 0-12-730030-9.

Forest health issues around the world are varied and often complex. Biotic and abiotic stresses, singly or in combination, affect the ability of forests to perform a myriad of ecological functions and, at the same time, meet an array of human demands. Insects play a dominant role as disturbance agents in the normal functioning of managed and unmanaged forest ecosystems. The tenthredinoid sawflies include major pests of broad-leaved and needle-bearing trees worldwide. They are especially important from an economic perspective in North America, because this continent harbors many introduced species. In this book, 33 of the world's most knowledgeable sawfly researchers and several outstanding insect ecologists review and discuss the life history adaptations that have allowed species in the families Pergidae, Argidae, Cimbicidae, Diprionidae, and Tenthredinidae to become major pests of trees and shrubs.

The material is presented in 20 wellorganized chapters arranged into five related sections. Part I includes a concise review of systematics (D.R. Smith), and interesting pieces about the diversity of life histories (G. Knerer), sex ratio variation and its effects on population dynamics (T.P. Craig and S. Mopper), an intriguing assessment of feeding strategies (W. Heitland and H. Pschorn-Walcher), and a comprehensive summary of pheromone biology and test methods (O. Anderbrant). Part II begins with a thoughtful discussion of the ways in which sawflies defuse host defenses (D.G. McCullough and M.R. Wagner), followed by interesting chapters on the effects of plant chemistry and phenology on sawfly behavior (C. Geri, J.P. Allais, and M.-A. Auger) and the role that host stress plays in sawfly outbreaks (S.C. Krause, K.F. Raffa, and M.R. Wagner). The section ends with a summary and discussion of the importance of biological traits to the systematics and population dynamics of nematine gall formers on willow (P.W. Price and H. Roininen). Two chapters comprise Part III: a good review of defense strategies of folivorous sawflies (S.G. Codella, Jr., and K.F. Raffa) and an interesting discussion of the tritrophic interactions between willow and gall-forming sawflies and their natural enemies (K.M. Clancy). Part IV reviews the influence of stand characteristics and site quality on population dynamics (J.D. McMillin and M.R. Wagner), the role played by plant development and architecture in regulating populations (K.D. Floate and

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R. DeClerck-Floate), inducible resistance of woody plants (P. Niemela and J. Tuomi), the biology and population dynamics of *Diprion pini* (A.A. Sharov), and the European pine sawfly and microbial interactions mediated by the host plant (K. Saikkonen and S. Neuvonen). These chapters provide good reviews of subject matter, some are thought provoking, others are quite speculative. Finally, Part V provides an interesting and objective review of outbreak causes (S. Larsson, C. Bjorkman, and N.A.C. Kidd) and a discussion of life history strategies and ecology of Australian pergids (J. Macdonald and C.P. Ohmart). The book ends with a discussion of the broad diversity of sawfly life histories, behavior, and population dynamics and the ways in which sawfly studies can contribute to our understanding of insect ecology (R.A. Haack and W.J. Mattson).

Chapters are well organized. A few contain some awkward sentences, which result from the frugal use of commas and (or) the understandable difficulty that some authors had with English. Each chapter is accompanied by its own list of references. Many of the 1662 total entries are redundant, and a single reference section would have saved space and would be easier to use. I found 19 typographical errors.

Readers may encounter difficulty, as I did, with narrative concerning the role of plant chemistry in sawfly population dynamics. Several authors readily acknowledge the conflicting results and diverse hypotheses contained in the literature and temper their conclusions accordingly. Others forge ahead with theory that, at best, is equivocal and highly speculative. One also senses that sawfly ecologists are struggling to elucidate a single theory of population dynamics, when the literature clearly suggests that variations in behavior, host–sawfly interactions, and the role of natural enemies indicate that a single theory is most unlikely. The processes that drive insect populations vary with habitat, species, and geography.

Overall, the book is very well done and should be valuable to an international assemblage of students and researchers. It is a must for those interested in sawflies, but it also will be enlightening for those emersed in the more broad discipline of insect ecology. From a more applied standpoint, I was pleased to encounter material germane to forest management. The editors are to be congratulated for compiling this thorough review of tenthredinoid sawfly–host plant interactions. In my view, the book is well worth the price.

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VOLATILE ORGANIC CHEMICALS OF A SHORE-DWELLING CYANOBACTERIAL MAT COMMUNITY

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Abstract—The main components of a cyanobacterial mat community of a hypersaline lake shore consist of edaphic, mat-forming strains (ecophenes), and littoral strains of *Oscillatoria animalis* Agardh and *O. subbrevis* Schmidle, other microorganisms associated with these cyanobacteria, several species of *Bembidion* (Carabidae: Coleoptera), and two halophytic flowering plants: *Puccinellia nuttalliana* (salt meadow grass) and *Salicornia europaea rubra* (samphire). The volatile organic compounds of this community are a blend of those emitted by each of these components such as the C_{17} alka(e)nes, geosmin, 2-methylisoborneol, β-cyclocitral, β-ionone, dimethyl sulfide, and dimethyl trisulfide of cyanobacteria and associated microorganisms; alcohols, esters, and aldehydes usually associated with flowering plants; and possibly some insect-derived esters, particularly isopropyl tetradecanoate. The dominant compounds were: C_{11}, C_{13}, C_{15}, and C_{17} alka(e)nes, methyl esters of C_{16} and $C_{18:2}$ acids, isopropyl tetradecanoate, heptanal, 3-octanone and 2-nonanone, the acyclic terpene linalool, and the alcohols 1-heptanol, 1-hexanol, 1-octanol, 3-hexen-1-ol, and 2-octen-1-ol. It is concluded that this community may be distinguished from related communities by its repertoire of volatile organic compounds.

Key Words—*Oscillatoria*, *Bembidion obtusidens*, *Salicornia*, *Puccinellia*, allelochemics, dominant volatiles, community volatile blend, cyanobacterial assemblages, edaphic cyanobacteria.

INTRODUCTION

Individual species of plants and animals, including microbial forms, can be characterized by the kinds and amounts of volatile organic compounds (VOCs) they emit into the environment; these "body odors" (Price, 1986) are distinct

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chemical signatures that can be just as important as morphological characters in taxonomic descriptions of organisms. Different patterns of VOCs are obtained from strains of *Anabaena* and *Nostoc* (cyanobacteria), for instance, with differences occurring in hydrocarbons, alcohols, aldehydes, and ketones (Möhren and Jüttner, 1983). Assemblages of species populations that live in prescribed areas (habitats) are called communities, and the blend of VOCs emanating from all members of such a group is a distinctive property of all communities that can be thought of in much the same way as species composition, species dominance, and species diversity. Some of these volatiles serve as chemical messages that mediate inter- and intraspecific interactions, particularly those implicated in the intricate relationships of members of community food webs. Studies of community VOCs are useful for a better understanding of community structure, which is concerned, among other things, with interactions, but they are also useful for providing clues to biochemical pathways of secondary product emissions of constituent populations (see Jüttner, 1988). However, the most immediate and practical use of these investigations will probably be for the characterization of communities based on VOC occurrence, relative abundance of VOCs (derived from patterns of gas chromatogram peaks), or on dominance (large GC peaks only); this kind of characterization is analogous to the use of terms such as species occurrence, species abundance, and species dominance in community descriptions.

Investigations of chemically mediated habitat recognition by insects led to this study of the volatile chemicals of an edaphic cyanobacterial mat community inhabiting a salt-encrusted beach on the north shore of Wells Lake, a hypersaline lake in Marsden, Saskatchewan. When wet, mat-forming strains of ecophenes (Drouet, 1980) of *Oscillatoria animalis* Agardh and *O. subbrevis* Schmidle, and other microorganisms (Evans, 1982) give the soil surface a greenish color but, upon drying, thin (0.1–0.3 cm) crusts develop, appearing as patchworks of dried polygonal flakes with curled edges; interspersed with the flakes are areas of salt (mostly sodium sulfate) incrustations beneath which the cyanobacterial layer continues to grow. Long winding desiccation cracks lace the habitat and shallow (10–15 cm) depressions that are found throughout the area form pools of cyanobacteria-rich waters. As these dry out, thick (0.5–1.5 cm) "pedicled" (Metting, 1991) polygonal crusts of *Oscillatoria* develop, in contrast to the thinner crusts of the soil. From spring to fall, a strong earthy "algal" odor continually pervades the habitat and on days with light breezes is perceptible some hundreds of meters downwind.

A narrow fringe of halophilic plants that are consistently associated with this kind of habitat occurs in the upper, drier part of the beach. These consist of tussocks of Nuttall's salt meadow grass, *Puccinellia nuttalliana* (Schultes) Hitchcock (Gramineae), and clumps of samphire, *Salicornia europaea rubra* A. Nels (Chenopodiaceae). An area of wild barley (*Hordeum jubatum* L.) -domi-

nated saline flats extends beyond these plants for approximately 500 m with other chenopodiacean plants (see Moss, 1983) such as oak-leaved goosefoot (*Chenopodium salinum* Standl.) and western sea blite (*Suaeda calceoformis* (Hook) Moq.) also present.

Aside from occasional shore birds, the most conspicuous animals in this community are small (3–6 mm) ground beetles (Carabidae), which, when disturbed, emerge from shrinkage cracks and from under crustal flakes in large numbers (sometimes $>100/m^2$) and run over the salt surface or take flight. *Bembidion obtusidens* Fall is the most common but others encountered include *B. henshawi* Hayward, *B. scudderi* LeConte, *B. insulatum* LeConte, *B. obtusangulum* LeConte, *B. salinarium* Casey, *B. graphicum* Casey, *B. diligens* Casey, *B. roosvetti* Pic, *B. bifossulatum* LeConte, and occasionally *B. versicolor* LeConte. Individuals of some of these species have been shown to respond behaviorally to volatiles associated with this community (Evans, 1982, 1988). Conspicuous insects also found in this habitat are species of other genera of carabids, along with staphylinid beetles, shore bugs (Saldidae), and muscid flies.

METHODS AND MATERIALS

The area of the habitat chosen for sampling was determined by observing *Bembidion* adults, which forage from the wet soil near the water to the drier parts of the beach occupied by samphire and salt meadow grass. Qualitative analyses of the volatiles were performed on the following "dry" samples collected over several years at different dates during the summer months when the dominant components of the community appeared to be flourishing: salt incrustations and underlying cyanobacterial layer, cyanobacterial flakes, pedicled cyanobacterial crusts, salt meadow grass, and samphire. Water from depressions and from shallow lagoons (littoral zone) extending into the habitat constituted the "wet" samples. Samples were not collected during the occasional season when the habitat was obviously disrupted by excessively wet or dry conditions or when it was blanketed in 1986 by wave-swept phytoplankton blooms deposited on the shore in thick carpets.

The *Oscillatoria* mat layer, flakes, and salt incrustations were collected by lightly scraping the surface of the soil, placing the loosened material into 500-ml canning jars and tamping it to reduce air spaces; care was taken to avoid disturbing the underlying black, anaerobic clay soil. Fragments (5–6 cm diameter) of the thick, pedicled *Oscillatoria* crusts from shallow depressions were also put into jars. Since samples kept frozen for several months were still viable when brought to room temperature, they were kept at $-30°C$ until needed; some cell disintegration must occur during this treatment, but it also occurs under natural conditions. Samphire plants were pulled out of the soil, which

was removed from the shallow roots, but, because of their extensive fibrous root systems, salt meadow grass samples were broken off at the soil surface. Both plant samples were placed in plastic bags and refrigerated until they were processed the following day.

The dry samples were purged with nitrogen by placing approximately 500 ml into two 5-cm-ID × 21-cm-long glass cylinders connected to each other by 55/50 ground glass joints. Each end of the connected tube tapered to an 8-mm-OD stem, one of which was connected with a Swagelok fitting to a high-purity nitrogen source and the other to a stainless steel cartridge containing approximately 350 mg of a solid adsorbent (20–35 mesh Tenax GC) for trapping the volatiles. After purging the sample for 3–4 hr at 100 ml/min, the cartridge was flushed with nitrogen for 20 min to drive off residual water and then desorbed by placing it in a nichrome-wire-heated glass tube. The temperature was increased from ambient to 240°C at 8°/min and kept there for 45 min. The desorbed volatiles were trapped in a liquid nitrogen-cooled 1-mm-ID capillary tube and dissolved in 20–30 μl double-distilled reagent grade acetone. The solutions were stored in microvials in a freezer until needed.

Water samples were collected in 1-liter brown glass bottles and refrigerated until processed. For nitrogen purging, about 1.5 liter were put into a 2-liter Erlenmeyer flask with a 40/50 ground-glass joint connected to a stopper with a tube through it attached to the nitrogen source on the top side and to a gas dispersion frit, for purging the water, on the bottom. The water was maintained at 60°C and stirred during the 2-hr purging. The nitrogen exited through a side arm that held the adsorbent cartridge, which was treated after purging in the manner described above.

Separations and identifications of the desorbed volatiles were performed on a Varian Vista 6000 gas chromatograph and a VG Analytical 70E mass spectrometer with a 30-m nonpolar DB-1 capillary column (35°C for 2 min then to 280°C at 7°/min) and a 30-m polar Supelcowax 10 column (35°C for 2 min then to 240°C at 7°/min). Headspace analyses of low-molecular-weight compounds of the dry *Oscillatoria* samples were performed using a 10-m, 0.32-mm-ID Chrompack PLOT column and a Hewlett Packard 5890 GC programmed for 90°C for 2 min to 200°C at 15°/min. Identification of the majority of compounds were confirmed with comparisons of the mass spectra and retention times of synthetic analogs. Compounds with overlapping or small GC peaks could not be identified.

RESULTS AND DISCUSSION

Some of the VOCs of the mat community (Tables 1 and 2) are typical of those found in aquatic cyanobacterial communities (Slater and Blok, 1983a, b; Jüttner, 1984a; Jüttner et al., 1986), and cyanobacterial cultures (Jüttner, 1988a).

TABLE 1. VOLATILE ORGANIC COMPOUNDS EXTRACTED FROM SALT INCRUSTATIONS, CRUSTAL FLAKES, AND MAT LAYER OF CYANOBACTERIAL MAT COMMUNITY, WELLS LAKE, MARSDEN, SASKATCHEWAN

Aliphatics
- 1-methyl-2-cyclohexen-2-ol
- 1-heptanol[a]
- 1-hepten-3-ol
- 1-nonanol
- 2-nonanol

- propanal
- butanal
- isobutanal
- pentanal
- isopentanal
- hexanal
- heptanal[a]
- octanal
- nonanal
- dodecanal
- tetradecanal
- hexadecanal

- unknown cyclic terpene ketone
- 3,5,5-trimethyl-2-cyclohexen-1-one
- 5-methyl-2-heptanone
- 6-methyl-2-heptanone
- 6-methyl-5-hepten-2-one
- 3-octanone[a]
- 6-octen-2-one
- 2-nonanone[a]

- hexyl formate
- isopropyl tetradecanoate[a]
- methyl hexadecanoate
- ethyl hexadecanoate

- octanoic acid
- nonanoic acid
- decanoic acid
- dodecanoic acid
- tetradecanoic acid

- hexane
- decane
- undecane
- dodecane
- tridecane
- tetradecane
- pentadecane[a]
- hexadecane
- heptadecane[a]
- octadecane

- 1-hexene
- 3,4,4-trimethyl-2-hexene
- 3,5,5-trimethyl-1-hexene
- 1-undecene[a]
- 2-dodecene
- 1-tridecene
- tridecene isomers
- 1-tetradecene
- tetradecene isomer
- 1-pentadecene
- pentadecene isomers
- 1-hexadecene
- hexadecene isomers
- 1-heptadecene[a]
- heptadecene isomers[a]
- heptadecadien isomer
- 1-octadecene

Acyclic terpene
- myrcene

Cyclic terpenes
- β-cyclocitral
- β-ionone
- α-ionone
- limonene
- caryophyllene
- *p*-cymene

Cyclic tertiary alcohols
- geosmin
- 2-methylisoborneol

Miscellaneous
- dimethyl sulfide
- dimethyl trisulfide
- *p*-dichlorobenzene
- sesquiterpene isomers (mol wt 204)

Heterocyclic aromatic
- 2-*n*-pentylfuran

[a] Dominant compounds.

TABLE 2. VOLATILE ORGANIC COMPOUNDS IN WATER AND THICK PEDICLED MATS OF CYANOBACTERIAL MAT COMMUNITY, WELLS LAKE, MARSDEN, SASKATCHEWAN

Water in shallow pools and lagoons	*Oscillatoria* mat in dried shallow depressions	
Aliphatics	Aliphatics	methyl hexadecanoate
1-decanol	1-heptanal	methyl octadecanoate
nonanal	unknown cyclic terpene ketone	9-methyl octadecenoate
decanal	2-nonanone[a]	
methyl tetradecanoate	3,5,5-trimethyl-2-cyclohexen-1-one	Cyclic terpenes
methyl pentadecanoate		β-cyclocitral
methyl hexadecanoate[a]		α-ionone
methyl 15-methylhexadecanoate	pentadecane[a]	β-ionone
ethyl hexadecanoate	undecane[a]	limonene
methyl octadecanoate	dodecane	caryophyllene
methyl 9-octadecanoate	hexadecane	*p*-cymene
ethyl octadecanoate	heptadecane[a]	
methyl 8,11-octadecadienoate[a]		Cyclic tertiary alcohol
isopropyl tetradecanoate	1-undecene[a]	geosmin
	tridecene isomers	
1-decene	1-pentadecene[a]	
1-undecene	hexadecene isomers	Miscellaneous
		dimethyl trisulfide
Cyclic aliphatic	hexadecadiene isomers	sesquiterpene isomers (3)
3,5,5-trimethyl-2-cycohexen-1-one	1-octadecene	

[a] Dominant compounds.

The high concentrations of C_{17} alka(e)nes are characteristic of cyanobacteria (Tsuchiya and Matsumoto, 1988), and so are the occurrences of geosmin and 2-methylisoborneol, two compounds that impart disagreeable odors to drinking water during cyanobacterial blooms (Izaguirre et al., 1982; Jüttner, 1987; Slater and Blok, 1983a) although they also occur in saline waters (Tabachek and Yurkowski, 1976). An unusual compound, isopropyl tetradecanoate, which may be anthropogenic in origin, is widely used in the cosmetic and medical industries, and has been reported as a component of the volatiles of a polluted forest atmosphere (Helmig et al., 1989) and as a contaminant of water treatment chemicals (Thompson and Karasek, 1987). I have also found this compound in substrate from other shore beetle/microbial mat communities in habitats bordering rivers, creeks, and freshwater lakes. It may be associated with insects of the mat community since it is produced by ants (Cavill and Houghton, 1974),

armyworms (Klein et al., 1990), philanthine wasps (McDaniel et al., 1992), and dermestid beetles (Franke and Levinson, 1979). Hexyl formate is an ester that does not seem to be associated with biological systems; Ford et al. (1988) include it among a group of synthetic compounds used as raw materials for the manufacture of fragrances. The two odiferous cyclic terpenes β-cyclocitral and β-ionone (Slater and Blok, 1983b; Jüttner, 1983, 1987) as well as dimethyl sulfide and dimethyl trisulfide (Jenkins et al., 1967; Jüttner, 1984b; Bechard and Rayburn, 1979) have also been implicated in causing malodors in reservoirs. Other cyanobacterial indicators are acids, aldehydes, 3-octanone, and the methylhepta(e)nones, although algae and actinomycetes in freshwaters also produce some of these compounds (Gerber, 1983; Jüttner et al., 1986).

However, in contrast to freshwater aquatic cyanobacteria, those of this community are adapted to live in highly saline water of the littoral zone of the lake and shallow pools and in the saline–alkali soil (Evans, 1986) of both the wetter lower part of the beach and the upper drier part, thus accounting for the other compounds shown in the tables. For example, the methyl and ethyl esters of C_{14}–C_{18} fatty acids found in the pools and in the littoral zone of the habitat and in the thick *Oscillatoria* mats (Table 2) are not usually associated with cyanobacteria but have been identified from marine algae (Sakagami et al., 1990, 1991; Shirane et al., 1989) and freshwater dinoflagellates (Robinson et al., 1987). The preponderance of aldehydes and alkenes, along with lesser amounts of acids, cyclic terpenes, and keto(e)nes seems to be unusual when compared to VOCs of other cyanobacterial and algal assemblages (Jüttner, 1981, 1983, 1988a; Slater and Blok, 1983a; Tsuchiya and Matsumoto, 1988). An unknown monoterpene (Tables 1 and 2) appears to be specifically associated with this community, as it occurs in very small amounts in all the dry samples. It has a mass fragmentation pattern of *m/z* 68 (100%), 96 (92), 152 (M^+, 55), 41 (20), 55 (10), 109 (10), 137 (5), 81 (5), and 124 (2). This pattern closely matches *m/z* values given for 2-isopropylidene-4,4-dimethylcyclopentanone, a synthetic reaction product (Brownbridge et al., 1976), and for the spectrum of 2,6,6-trimethyl-2-cyclohexene-1,4-dione, which is listed as a cyanobacterial VOC by Jüttner (1988a).

The samphire and salt meadow grass volatiles (Table 3) also contribute to the overall blend of compounds of this community and, in general, are typical of those produced by leaves, fruit, and flowers (Bauer et al., 1990) of many flowering plants. There is a large complement of aliphatic alcohols, plus esters and aldehydes not associated with *Oscillatoria*, including the commonly encountered C_6 leaf alcohol isomers, although the leaf aldehyde, *trans*-2-hexenal, was not found. Since the atmosphere around these plants is saturated with *Oscillatoria* volatiles, adsorption of some of the latter by the leaves may be expected, so compounds such as the C_{13}–C_{17} alkanes may actually originate in the crusts. Finally, the VOCs produced by the insect members of the mat community cannot

TABLE 3. VOLATILE ORGANIC COMPOUNDS OF HALOPHILIC VASCULAR PLANTS OF CYANOBACTERIAL MAT COMMUNITY, WELLS LAKE, MARSDEN, SASKATCHEWAN

Salicornia europaea rubra (samphire)		*Puccinellia nuttalliana* (salt meadow grass)	
Alphatics	pentadecane	Aliphatics	6-methyl-5-hepten-2-one
methylbutanol	tridecane[a]	3-hexen-1-ol isomers[a]	5-methylene-3-heptanone
1-heptanol[a]	heptadecane	2-hexen-1-ol isomers	6-octen-2-one
1-hexanol[a]		3-hepten-1-ol	2-octanone
(*Z*)-3-hexen-1-ol	1-dodecene	2-octen-1-ol[a]	3-octanone
1-octanol	(*E*)-6-methyl-3-undecene	7-octen-4-ol	
3-octanol	1-tridecene	*trans*-2-endecen-1-ol	*cis*-3-hexenyl acetate
2,7-dimethyl-1-octanol	2-tridecene	1-hexanol	ethyl decanoate
3,7-dimethyl-1,6-octadien-3-ol	1-heptadecene	1-heptanol	ethyl octanoate
7-octen-4-ol	heptadecene isomers	1-octanol	
1-nonanol	(*E*)-9-eicosene	1-nonanol	Cycloaliphatics
1-decanol		2-decanol	α-hydroxytoluene
	Cycloaliphatics	2-methyl-1-propanol	2-phenylethanol
heptanal	α-hydroxytoluene	3-methyl-1-butanol	
nonanal	2-phenylethanol		Acyclic terpenes
5-methylhexanal		2-heptenal	geranylacetone
benzaldehyde	Miscellaneous	hexanal	linalool
safranal	2-methoxy-3-isobutylpyrazine	nonanal	nerolidol
	3-(4-methyl-3-pentenyl)furan	decanal	*trans*-β-ocimene
5-methyl-2-heptanone		dodecanal	
5-methyl-3-heptanone	Acyclic terpene		Cyclic terpenes
6-methyl-5-hepten-2-one	linalool[a]	pentadecane	α-terpineol
3-octanone		tridecane	pinane
1-undecanone	Cyclic terpene		β-cyclocitral
	3-cyclohexene-1-carboxaldehyde	1-dodecene	β-ionone
ethyl 2-hydroxybenzoate	β-cyclocitral	9-methyl-1-undecene	
ethyl phenylacetate	pinane	heptadecene isomers	
ethyl benzoate			
methyl salicylate			
ethyl salicylate			

[a] Dominant compounds.

be overlooked. Some of the esters (particularly isopropyl tetradecanoate) may be associated with *Bembidion*, because of its high densities of individuals.

Because the VOCs of this community constitute a mixture of compounds emanating from a group of organisms living in a particular kind of habitat, the chances of a similar blend occurring in another community is remote, although some overlapping may occur. For instance, the C_{17} alka(e)nes still dominate the VOC spectrum of a cyanobacterial/reed canary grass (*Phalaris arundinaceae* L.) community on a sand beach of a freshwater lake (Calling Lake, Alberta), but in other aspects the spectrum is very different than that of the Wells Lake community (Evans, unpublished). This is to be expected since differences in VOC composition and concentration pattern emissions occur between even closely related taxa of organisms that produce them. The VOCs of strains of *Oscillatoria* adapted for life in lake water, in the littoral zone, or in beach soil differ to an extent that may account for ester production in water and acids in the soil (Tables 1 and 2). Some strains of *Microcystis* (cyanobacteria) can be distinguished by the amounts of β-cyclocitral and the kinds of alkyl sulfides they liberate (Jüttner, 1984b), and Jüttner and Schröder, (1982) have shown differences in VOC patterns (of selected compounds) of microbial communities inhabiting the landward and lakeward sides of reed (*Phragmites*) beds. Furthermore, VOC analysis of a sample of water taken during a cyanobacterial bloom in August and one taken in April during development of the spring phytoplankton had different compositions and different concentrations of cooccurring compounds (Jüttner et al., 1986).

It is well known that environmental factors influence the kinds and amounts of metabolites produced in axenic cyanobacterial cultures (Rippka et al., 1979) and VOCs may also be similarly affected. Nevertheless, the VOCs examined in this study were produced by a community under, presumably, optimum conditions, such that similar patterns of abundance for the dominant compounds occurred between seasons. This suggests that the VOC blend is a distinct community character that may be useful for comparison with other communities, particularly closely related ones such as those in other beach habitats, but it is unwieldy to use because so many compounds are involved. Alternatively, if only the dominant compounds (see Tables 1–3) are considered, an abbreviated and possibly more useful group of compounds is obtained, i.e.: C_{11}, C_{13}, C_{15}, and C_{17} alka(e)nes, methyl esters of C_{16} and $C_{18:2}$ acids, isopropyl tetradecanoate; heptanal; 3-octanone, 2-nonanone, the acyclic terpene linalool; and the alcohols 1-heptanol, 1-hexanol, 1-octanol, 3-hexen-1-ol, and 2-octen-1-ol. This chemical signature alone is probably enough to distinguish the cyanobacterial mat community of a saline lake shore from any other similar community, and its use is analogous to the widely used method of describing communities by their dominant species. The complete blend, however, is much more relevant, both chemically, to obtain a more inclusive account of the volatiles, and bio-

logically, if interactions between members of the community are considered. The compound represented by the smallest GC peak could be involved in a chemically mediated behavior of some organism, whereas the most dominant compound may only be a secondary metabolic product and have no allelochemic role at all.

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EVOLUTION OF BEHAVIORAL RESPONSES TO SEX PHEROMONE IN MUTANT LABORATORY COLONIES OF *Trichoplusia ni*

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Abstract—Male cabbage looper moths, *Trichoplusia ni*, from two colonies in which all females express an abnormal sex pheromone production phenotype were evaluated in a laboratory wind tunnel for upwind flight responses to the normal and abnormal sex pheromones. The abnormal sex pheromone blend consisted of 20 times as much (*Z*)-9-tetradecenyl acetate and 30-fold less (*Z*)-5-dodecenyl acetate compared to the normal pheromone blend. Initially, these males exhibited poor behavioral responses to the abnormal sex pheromone and maximum responses to the normal pheromone blend, indicating that there was no linkage between signal production and response. After 49 generations of laboratory rearing, males from the mutant colonies maintained good responses to the normal pheromone and increased their behavioral response to the abnormal sex pheromone to the same levels as for the normal pheromone. Over the same period, normal males maintained their preference for the normal pheromone. These results indicated that evolution had occurred in mutant colonies in favor of greater male responsiveness to the abnormal sex pheromone, resulting in the broadening of the response spectrum to pheromone blend ratios. This evolution presumably resulted from a mating advantage to those males that did not discriminate against mutant-type females in the mutant colonies.

Key Words—*Trichoplusia ni*, Lepidoptera, Noctuidae, sex pheromone, behavior, evolution, sexual selection.

INTRODUCTION

In moths, species-specificity of the communication channel is largely ensured by unique sex pheromone blends and corresponding behavioral response spec-

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ificities to those pheromone blends in males. In these pheromonal communication systems, it is thought that males are at a selective advantage if they respond maximally to the pheromone blend produced by the majority of female moths and females are at a selective advantage if they emit a pheromone blend corresponding to the peak sensitivity of males. In theory, this stabilizing selection would constrain the divergence of new pheromonal communication systems [discussed by Cardé and Baker (1984) and Phelan (1992)]. One possible way for signal–response systems to change is that signals and responses are genetically linked so that mutation in signal is coupled by corresponding changes in response (Alexander, 1962). The mutant-type moth that produces abnormal pheromone would be mated more frequently by mutant males, leading to rapid divergence of a new communication system and reproductive isolation. However, there is no evidence for genetic coupling between pheromonal signal and behavioral response in moths (Löfstedt, 1990; Phelan, 1992). In *Ostrinia nubilalis*, pheromone production and behavioral response to sex pheromone are regulated by separate loci, located on different chromosomes (Klun and Huettel, 1988; Löfstedt et al., 1989; Roelofs et al., 1987). In contrast to the genetic coupling or stabilizing selection theory, Phelan (1992) proposed asymmetric tracking as a way of evolution of sexual communication. In this framework, the pheromone signal of the limiting sex (female) will only be under weak selection. The response of males will be driven by stronger selection because females are the limiting sex. With these assumptions, males might be expected to track the limiting sex if the female's signal changes (Phelan, 1992).

In the cabbage looper moth, *Trichoplusia ni* (Hübner) (Lepidoptera: Noctuidae), an abnormal pheromone phenotype was found to be regulated by a single recessive gene (Haynes and Hunt, 1990). The abnormal pheromone blend is far less attractive than the normal pheromone blend to male *T. ni* in the field. In fact, mutant female *T. ni* attract male *Agrotis ipsilon* (Haynes and Hunt, 1990). The normal sex pheromone blend emitted by calling females of *T. ni* consists of one major component, (*Z*)-7-dodecenyl acetate (100) (Berger, 1966), and five minor components, dodecyl acetate (7.19), (*Z*)-5-dodecenyl acetate (9.35), 11-dodecenyl acetate (2.78), (*Z*)-7-tetradecenyl acetate (1.05), and (*Z*)-9-tetradecenyl acetate (0.46) (Bjostad et al., 1984). The abnormal pheromone blend emitted by mutant females differs from the normal blend in reduction in the emission rate of (*Z*)-7-dodecenyl acetate, near absence of (*Z*)-5-dodecenyl acetate, and an almost 20-fold increase in (*Z*)-9-tetradecenyl acetate (Haynes and Hunt, 1990). In this study, we report that the response specificity of mutant male *T. ni* was initially similar to normal males, but there was a gradual improvement in upwind flight responses to the abnormal sex pheromone blend over 49 generations.

METHODS AND MATERIALS

Insect Colony. A *T. ni* colony, originally from a field population in Riverside, California, had been reared in the laboratory without introduction of field moths for at least 15 years. An abnormal pheromone type moth was isolated from the colony, and a pure mutant colony was established (Haynes and Hunt, 1990). Both normal and mutant *T. ni* colonies were reared on a semisynthetic medium (Shorey and Hale, 1965). For the maintenance of each colony, about 80 pupae (about equal numbers of males and females) were used to initiate a mating group each generation. For behavioral experiments, males were separated from females as pupae. Emerged moths were removed daily and provided with 8% sucrose water. Pupae and moths from both normal and mutant colonies were kept in 3.8-liter paper cartons under 16:8 hr light–dark photoperiod and 27 ± 1°C.

Sex Pheromone Gland Extracts. Sex pheromone gland extracts were made from both normal and mutant female moths to be used as pheromone sources in wind-tunnel tests. The glands were excised from 3 to 4-day-old female moths during the first hour of the scotophase. Five glands were immersed in 0.20 ml methylene chloride for 24 hr. The methylene chloride extract was then filtered through an activated magnesium silicate column (Florisil No. F-5754, Sigma, St. Louis, Missouri) to remove alcohols such as (*Z*)-7-dodecenol, a behavioral antagonist of male *T. ni* moths. The filtered extracts from each type of colony were then combined and evaporated under a nitrogen gas stream to a final concentration of about 0.1 female equivalent (FE)/μl. Each combined extract contained pheromone from 40 females.

Wind-Tunnel Tests. Individual 3 to 4-day-old male moths were confined in 4-cm-diam. × 8-cm-high hardware cloth cages in the last hour of the photophase. They were tested in a wind tunnel between the fifth and seventh hours of the scotophase. The design of the wind tunnel was similar to that described by Miller and Roelofs (1978). The wind tunnel was illuminated with white and red light from above the tunnel ceiling to yield a light intensity of about 0.3 lux at plume height. The wind velocity was 50 cm/sec. Temperature ranged from 21 to 29°C and did not change in a systematic way over the course of the experiment.

Pheromone gland extract was applied to a 1-cm^2 piece of filter paper at a dose of 1 FE (10 μl of gland extract). The pheromone-laden filter paper was fixed to a downwind edge of a metal plate with a pin. The metal plate was then placed on a metal mesh surface 20 cm above the tunnel floor at the upwind end of the tunnel. Each moth was released on a metal mesh surface 20 cm above the tunnel floor and 1.5 m downwind of the pheromone source and was allowed about 1 min to initiate a response to a pheromone source. Males that initiated

upwind flight in the plume were allowed to continue to respond until they reached the pheromone source or terminated upwind flight. After 12 moths were tested, the treated filter paper was discarded. Gland extracts of normal and abnormal sex pheromones were tested alternately.

Mutant male moths were tested in the wind tunnel for behavioral responses to pheromone gland extracts from normal and mutant females at generation 10, 15, 16, and 20 after establishment of the pure mutant colony. At the twentieth generation, two mutant lines (referred as mutant colony A and mutant colony B hereafter) were established by setting up two groups of about 80 pupae randomly taken from the mutant colony. Procedures of colony maintenance were the same as stated above. Mutant males from the two newly established lines were then tested in the wind tunnel with pheromone gland extracts from normal and mutant females at generation 21, 22, 24, 26, 37, 44, 47, and 49. At generation 10 and 49 of the mutant colony, both normal and mutant male moths were tested in the wind tunnel for comparison. The numbers of males tested per generation for each pheromone blend from each colony ranged from 50 to 90. The G test (Sokal and Rohlf, 1981) was performed to compare source contact rates for the normal and abnormal pheromone sources.

RESULTS AND DISCUSSION

Initially, both normal and mutant males showed similar percentages of upwind flight responses to normal and abnormal blends (Table 1). While there was some expected variation in the source contact rate among generations, in

TABLE 1. PERCENTAGE PHEROMONE SOURCE CONTACT OF MALE CABBAGE LOOPER MOTHS FROM NORMAL AND MUTANT COLONIES TO NORMAL AND ABNORMAL SEX PHEROMONES

	Source contact rate (%)				
	10th generation[a] response			49th generation[b] response	
Colony	Normal pheromone	Abnormal pheromone	Colony	Normal pheromone	Abnormal pheromone
Normal	67.8[c]	32.2	Normal	76.3[c]	53.8
Mutant	50.0[c]	21.0	Mutant A	76.3	75.0
			Mutant B	86.3	88.8

[a] The number of males tested for each blend from each colony was 90.
[b] The number of males tested for each blend from each colony was 80.
[c] The difference between normal blend and abnormal blend in source contact rate was significant, G test, $\alpha = 0.05$ (Sokal and Rohlf, 1981).

general the behavioral responses to the abnormal blend increased over the 49 generations that mutant colonies had been maintained in isolation from the parent colony (Figure 1). The difference in source contact rate of mutant males responding to the normal and abnormal pheromone blends was no longer significant at the 37th generation. In contrast, normal male *T. ni* maintained greater responsiveness to the normal pheromone blend (Table 1).

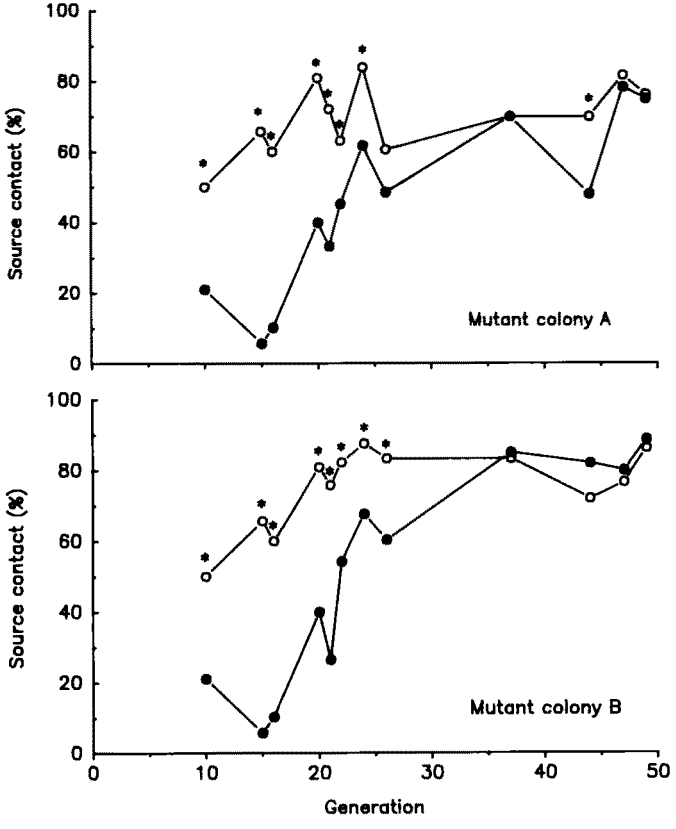

FIG. 1. Source contact rate of mutant male cabbage looper moths in response to sex pheromone gland extracts from normal and mutant female cabbage looper moths over generations. A pure mutant colony was split into mutant colony A and mutant colony B at the 20th generation. Data collected before the 20th generation were used for both mutant colony A and mutant colony B. Open circles: response to normal pheromone blend. Filled circles: response to abnormal pheromone blend. *: Source contact rate for the normal pheromone blend was significantly higher than that for the abnormal pheromone blend, G test, $\alpha = 0.05$ (Sokal and Rohlf, 1981).

The initial lack of correlation between the female pheromone blend and the male behavioral response in the mutant colony indicated that these characteristics are not genetically linked. Similarly, Todd et al. (1992) in an electrophysiological study found that normal and mutant male *T. ni* had similar frequencies of sensory neurons for each pheromone component. These results are consistent with studies of other moths in which no linkage has been observed between pheromonal signal and behavioral response (Collins and Cardé, 1980; Collins et al., 1990; Klun and Huettel, 1988; Löfstedt, 1990; Roelofs et al., 1987).

The fact that male *T. ni* from the mutant colonies had maintained a high source contact rate to the normal blend as the response to the mutant blend improved indicates the spectrum of the behavioral response to sex pheromone blend ratios was broadened by the selection that occurred in the mating colonies. Similarly, in the pink bollworm moth, *Pectinophora gossypiella*, male moths showed an improved response to a pheromone blend with a higher proportion of (*Z*,*E*)-7,11-hexadecadienyl acetate, while still showing a maximum response to the normal pheromone blend [44:56 (*Z*,*E*)–(*Z*,*Z*)-7,11-hexadecadienyl acetate] as artificial selection had elevated the proportion of the *Z*,*E*-isomer of the pheromone blend (Collins and Cardé, 1989). While these studies were conducted under laboratory conditions, it is possible that even in the natural context, the evolution of a new specificity of behavioral responses to a different pheromone blend will be preceded by broadening of the response spectrum as part of the speciation process.

A related proposal based on studies with Hawaiian *Drosophila* suggests that founder effects and sexual selection play an important role in the speciation process (Kaneshiro, 1980). One step in this process may be the relaxation of sexual selection following a founder event (colonization of a new habitat) because of small population size. This relaxation of sexual selection was hypothesized to be responsible for the asymmetry in isolation between "parental populations" and "derived populations." Derived populations were less selective. There are a few parallels between Kaneshiro's hypothesis and the results with the mutant cabbage looper moth. Once the mutant colony was founded and isolated from the parent colony, the males' response specificity broadened. This created an asymmetry, with males from the parent colony continuing to be selective based on pheromone blends, while the derived mutant colonies became less selective. In the study with European species of small ermine moths, Löfstedt et al. (1991) suggested that the derived species lost pheromone components and that initially males of the derived species may have continued to be responsive to the more complex ancestral pheromone blend. The result would be a temporary asymmetry in matings between the ancestral and derived population until selection against hybrid matings had an impact. This parallels our discovery in the mutant colonies of *T. ni* with the exception that the pheromone blend in the mutant

colonies is not simpler than the normal pheromone blend, but the male response spectrum is now broader.

While the observed changes in the males from mutant colonies occurred under laboratory conditions, the fact that a mutation in sex pheromone production in females preceded a gradual change in male responses in the mutant colonies seems to be consistent with the asymmetric tracking hypothesis proposed by Phelan (1992). Because females are often the limiting sex, males might be expected to track evolutionary changes in the female's blend. The converse should not be as likely. After mutant colonies were isolated, selection has favored a broadening of the males' behavioral response spectrum to pheromone blends. In contrast, the mutant pheromone blend has not changed (Liu and Haynes, unpublished data). We suggest that the evolution for the broadening of the behavioral response spectrum to sex pheromone resulted from differential mating success based on the ability of a few preadapted males to respond to the abnormal pheromone.

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ANALYSIS OF SECRETIONS FROM SCENT-PRODUCING GLANDS OF BRUSHTAIL POSSUM (*Trichosorus vulpecula* Kerr)

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Abstract—A characterization at a molecular level of the chemical composition of the secretions of the two pairs of paracloacal glands and of the sternal sebum of the brushtail possum (*Trichosorus vulpecula* Kerr) has been undertaken with a view to evaluating the potential of volatile "pheromone" components as species-specific attractants for use in novel baiting systems. Particular attention has been given to the respective fatty acid fractions produced by chemical hydrolysis, since these are believed to be the products of postemission microbial degradation (fermentation) of the secretions. In all instances, the highly complex distribution of the constituents present in these organic components of the secretions were shown to be virtually identical in adult males and females. A unique suite of low-molecular-weight branched-chain carboxylic acids has been shown to be produced by chemical degradation of the holocrine (oil-secreting) gland secretion. This odor signature is suggested to function as a unique "scenting-the-habitat" pheromone that might act as an attractant to all members of the species.

Key Words—Paracloacal glands, fatty acids, mammalian pheromones, possum, *Trichosorus vulpecula* Kerr.

INTRODUCTION

The herbivorous brushtail possum (*Trichosorus vulpecula* Kerr) was introduced to New Zealand from Australia late in the 19th century, primarily with a view to starting a fur and skin industry. In the absence of diseases and predators the possum population proliferated to the point where it is now widespread through-

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out the country. Agricultural authorities now consider the possum to be a major pest in native forest and horticultural areas and, most particularly, in pastoral areas where it is the known vector for the transmission of bovine Tb to cattle and deer.

Present strategies designed to manage populations of the possum have focused principally upon the use of the poison sodium monofluoroacetate (Tenate). Arguments against the undeniably cost-effective use of this poison center around the lack of species-specificity, there being apparently deleterious effects upon both endemic and exotic wildlife (Morgan, 1982). Ecologists also recognize that a significant proportion of the possum population is poison-shy, that is, the animals can detect the presence of the poison in a bait.

With the purpose of seeking methods of circumventing either or both of these phenomena, we have undertaken a study of the chemical compositions of secretions of several glands of the possum with the ultimate expectation of developing a synthetic "pheromone"-based attractant for incorporation into the baiting system.

There have been many studies devoted to the identification and use of pheromones in insect mating disruption, and these studies have led to the development of successful population suppression strategies (Kydonieus and Beroza, 1982, Ridgway et al., 1990). However, studies that have addressed the nature and function of chemical signals in mammalian systems have revealed them to be much more complex by comparison (Albone, 1984). A few reports of the use of animal odors in animal population control studies have been published. For the greater part, these address the use of kairomones as "predator" (carnivore) odors in herbivore pest control (Sullivan and Crump, 1984; Sullivan et al., 1988, 1990a,b; Müller-Schwarz, 1990; Boag and Mlotkiewicz, 1991) and demonstrate the obvious potential that such repellency strategies have in certain situations. On the other hand, the use of mammalian pheromones as conspecific attractants in field situations has, to our knowledge, only recently been evaluated with mustelids and shown to have some potential as a species-specific (bait) attractant (Clapperton and Woolhouse, 1991).

Because the nature of the possum problem predicates the utilization of an approach based upon attractancy, we have undertaken an investigation of the organic constituents of secretions from the two pairs of paracloacal glands that have been characterized as oil-secreting (holocrine) and as cell-secreting (apocrine) glands (Russell, 1987) and also from the sternum region, within which is a modified sebaceous gland (Tyndale-Biscoe, 1975). Explanations of possum behavior elicited by each of the three gland secretions have been suggested (Bolliger and Hardy, 1944; Bolliger and Whitten, 1948; Thomson and Pears, 1962; Tyndale-Biscoe, 1975) but direct evidence of the importance of scent(s) in the wild has not been established. Field observations indicate that the holocrine secretion is delivered in copious quantities when the possum is aroused

by handling, fighting, or other stimuli and that the cell-containing material is secreted more or less continuously in the urine and feces, but not in quantity when the animal is excited (Thomson and Pears, 1962). Males and females were observed to show a number of behavioral responses to the holocrine scent of males, in particular, that were interpreted as being important in defense. By virtue of its presence in the urine, the cell secretion was considered to be important in recognition of sex and in territorial marking. These authors also found that the sternum gland sebum elicited no marked responses from either males or females of the species when compared to the holocrine scent. However, more recent studies of an anecdotal nature indicate that this secretion is implicated in defining social status and marking (Morgan, personal communication). Bioassays of this secretion have also been shown to elicit pronounced olfactory investigation in other conspecifics (Biggins, 1979). In this latter work, all major lipid classes were shown by thin layer chromatography (TLC) to be represented in the paracloacal gland secretions, which were both more complex than those present in the sternal hair extracts. The putative presence of low-molecular-weight alcohols in the holocrine oil was suggested as the major class responsible for the musky odor, although no bioassays on these secretions were conducted. As the results of preliminary pen bioassays for conspecific attractancy of the ether-soluble materials recovered from each set of paracloacal glands of both males and females revealed no enhanced behavioral activity (Innes and Frampton, 1991), we elected to further pursue the possibility that a unique "odor signature" with potentially attractive characteristics might be generated after emission as a result of (aerobic) microbial degradation of the glandular secretions. The significance of such fermentation products in chemical recognition among conspecifics of other mammalian species has been addressed by a number of researchers (Gorman, 1976; Albone and Perry, 1976; Albone, 1983; Gorman and Trowbridge, 1989), and fatty acids have been shown to be predominant in the in situ volatiles generated anaerobically within the sacs of many carnivore species. Whereas microorganisms have been observed in the sacs of carnivores (Albone, 1984), none has apparently been observed within that of the possum (Cowan, personal communication).

Herein we describe a preliminary chemical characterization of each of the gland secretions and, more particularly, of the respective fatty acid profiles produced by saponification that are expected to reflect those generated by bacterial fermentation in the wild.

METHODS AND MATERIALS

Holocrine (oil-producing) and apocrine (cell-producing) glands were excised from freshly killed wild adult possums and immediately frozen prior to subsequent manipulation. The volumes of the contents of each member of the pair

of glands were virtually identical and were expressed after puncturing the glands and pooled (10–20 sets) into diethyl ether (ca. 50 ml). The ethereal suspension was stirred vigorously at ambient temperature until finely dispersed, the ether decanted, and the operation repeated. The combined ether phase was filtered and concentrated by slow distillation (<35°C); the final traces of solvent were then removed in a stream of argon to furnish the lipid fractions. Those lipids recovered from the holocrine glands of adult males and females were consistently ~30% by weight of the secretion and the ether-insoluble residues (obtained after lyophilization) were ~5% by weight. Similarly recoveries from the apocrine glands were ~5% lipid and ~10% residue. Sternum gland secretions were isolated from solutions in ether, as described above, and obtained as waxy solids by swabbing the pigmented chest/hair regions of the same animals. Swabbings from contiguous chest/hair areas were also taken and the lipids isolated similarly.

Saponification of each lipid specimen was carried out using 2.0 M potassium hydroxide in 50% aqueous ethanol, and the hydrolysis products were isolated by standard procedures. Fatty acids were esterified with boron trifluoride-methanol prior to chromatographic analysis on either an HP5890II GC equipped with a 25-m HP Ultra 2 capillary column operating in the split mode at either (1), 50° (1 min) then 5°/min to 280°C (3 min) or (2), 40° (1 min) then 8°/min to 340°C (36.5 min) prior to flame ionization (FID) or mass selective detection (MSD) and analysis. Tetratriacontane (R_t 32.4 min, system 2) was employed as an internal reference. TLC was performed on aluminum-backed silica plates (0.25 mm Merck) using standard solvent systems (Belitz and Grosch, 1986).

Synthesis of Carboxylic Acids. (±)-4-Methyl- and 5-methyl-hexanoic acids (caproic acids) were prepared by standard methods (Milburn and Truter, 1965) from *s*-butyl bromide and isobutyl bromide respectively, by sequential homologation firstly with cyanide ion and secondly with sodium diethylmalonate followed by hydrolytic decarboxylation. (±)-5-Methylheptanoic acid was prepared according to the general method via sequential two-carbon homologation of *s*-butylbromide, reported by Vogler and Chopard-dit-Jean (1960). 6-Methylheptanoic acid and (±)-6-methyl- and 7-methyl-octanoic acids respectively, were all prepared by the addition of the appropriate alkyl Grignard reagent to 2,3-dibromotetrahydropyran followed by a standard sequence of reduction and oxidation reactions (Crombie and Harper, 1950a,b). All of the above known carboxylic acids (see Figure 3 below) were purified by distillation under reduced pressure to give colorless liquids. The 300 MHz ^{1}H- and ^{13}C NMR data were entirely consistent with expectation. Samples of each were methyl-esterified as described above. Efforts are presently underway to establish unequivocally the stereochemistry of these anteiso acids.

RESULTS

Holocrine Gland Secretions. In an attempt to establish the presence or absence of volatile organic compounds in the heavy odorous lipid fraction, a headspace sampling technique employing Tenax GC (followed by solvent elution rather than thermal desorption) was applied to ca. 1-g samples of oil and the eluted "volatiles" subjected to GC (Olafsdottir et al., 1985). By this technique insignificant amounts were recovered and no identifications were made. TLC analysis of the lipid material with reference to representative lipid standards confirmed the presence of most neutral lipid classes with triacyl glycerides predominating and capillary GC of the whole oils from both adult male (Figure 1) and female animals revealed essentially the same complex profiles.

When compared qualitatively to a selection of lipid standards (e.g., milk fat, $C_{2:0}$–$C_{18:0}$ cholesterol esters), holocrine lipids are seen to be characterized by the occurrence of low-molecular-weight triacylglycerides and by the occurrence of two pairs of relatively abundant components with retention times in the range 6–10 min. Diazomethylation experiments revealed (Figure 2) these to be saturated fatty acids and GC-MS (GC conditions 1, Methods) confirmed molecular weights corresponding to C_7–C_9 acids. Moreover, analytical methanolysis of the whole lipid under conditions that are known to leave free fatty acids unaffected (Bannon et al., 1982) produced the same suite of methyl esters as did diazomethylation.

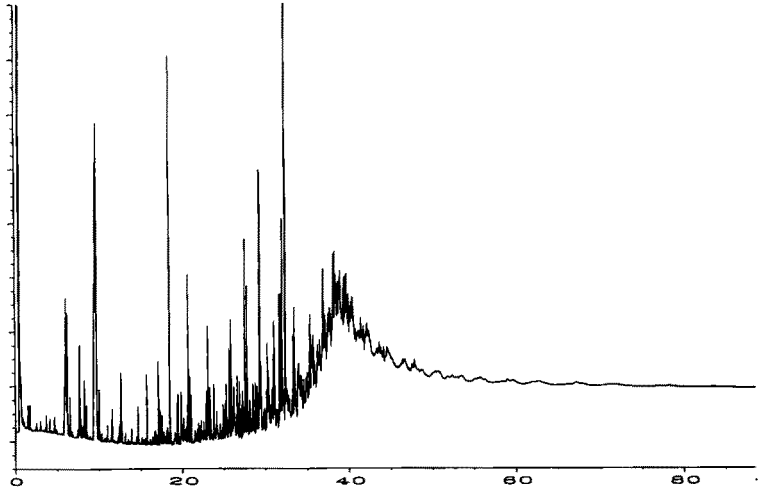

FIG. 1. Gas chromatogram (GC conditions 2, Methods) showing total neutral lipid profile from male (and female) holocrine anal glands.

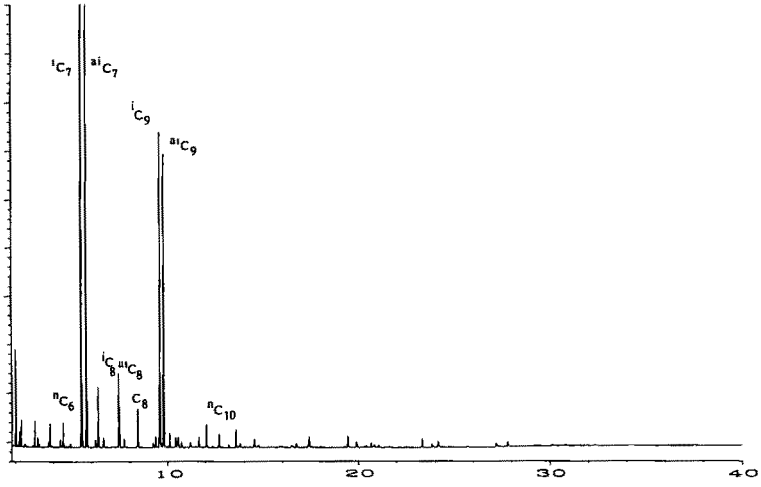

FIG. 2. Free fatty acid methyl ester profiles from diazomethylation of the male (and female) holocrine gland lipids.

Saponification and subsequent esterification of samples of both male and female holocrine lipids confirmed the above findings and revealed (Figure 3) also the presence of C_{14}, C_{16}, and C_{18} fatty acids derived from the structural lipid component of the secretion. Further confirmation of the fact that these low-molecular-weight components were branched-chain acids stems, firstly from the chromatographic behavior of the corresponding methyl esters with respect to a standard *n*-alkanoic acid methyl ester cocktail and secondly, from an examination of (GC) MS data, which revealed prominent fragmentation attributable to the loss of C_3H_7 for the more mobile iso series (denoted i in Figure 2) and to C_2H_5 losses for the anteiso series (denoted ai in Figure 2), with respect to that of ester methoxyl loss (Abrahamsson et al., 1963). The presence as minor constituents only of esters derived from *n*-hexanoic, *n*-octanoic, and *n*-decanoic acids was also established (by coelution). That these abundant components are in fact the C_7, C_8, and C_9 iso and anteiso branched-chain fatty acids was established unequivocally by synthesis and methyl ester coelution experiments. Each of these compounds was synthesized by published procedures (Figure 4); the anteiso acids were obtained as racemates.

Apocrine Gland Secretion. TLC analyses of the odorless lipid fractions recovered from both male and female apocrine glands show essentially the full suite of neutral lipid classes, in which the triacylglycerides predominate. Capillary GC of both lipid extracts are complex but very similar at this level of resolution and analysis. Unlike the holocrine secretion, the apocrine lipids con-

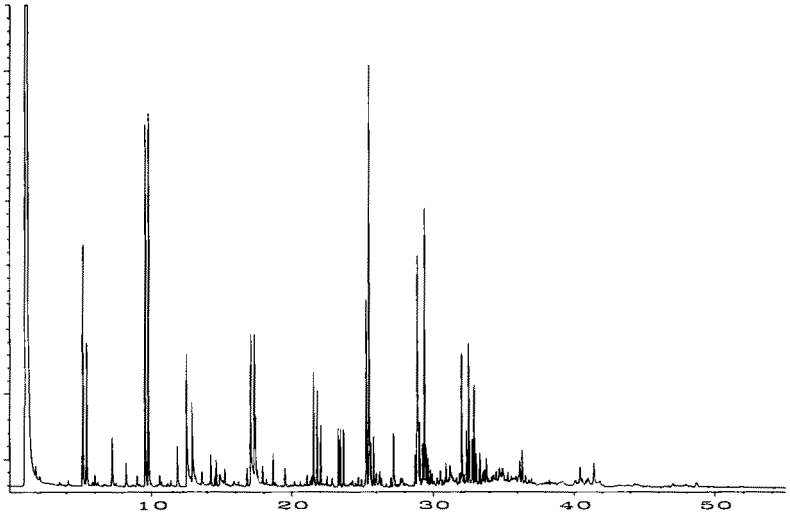

FIG. 3. Fatty acid methyl ester profile (GC conditions 2, Methods) from saponification of the male (and female) holocrine lipids.

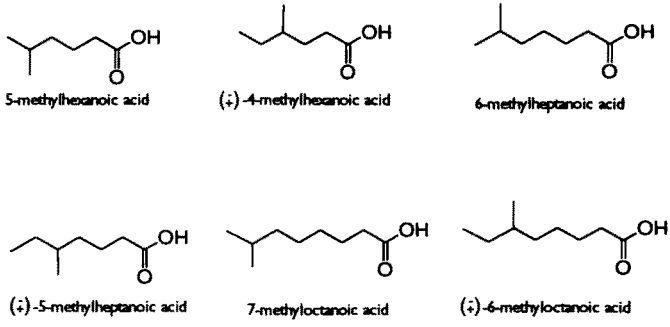

FIG. 4. Iso and anteiso aliphatic carboxylic acids shown to be present as "free fatty acid" and as acylglyceride in the male and female holocrine secretions. All have been authenticated by synthesis.

tain (Figure 5) larger amounts of regular triacylglycerides (R_t 40–75 min) typical of animal tissue fats (Davy et al., 1983).

In accord with expectation, saponification and esterification of the fatty acids recovered from both adult male and adult female apocrine lipids clearly showed (Figure 6) the suite of $C_{10:0}$, $C_{12:0}$, $C_{14:0}$, $C_{16:0}$, $C_{18:1\omega6}$, $C_{18:2\omega6}$, $C_{18:1\omega9}$, $C_{18:3\omega3}$, and $C_{18:0}$ acids in which $C_{16:0}$ and $C_{18:1\omega9}$ predominate.

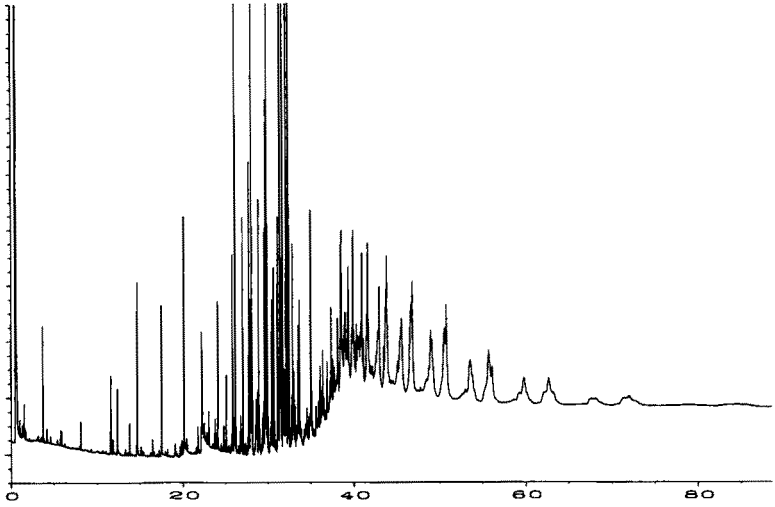

FIG. 5. Gas chromatogram (GC conditions 2, Methods) showing total neutral lipid profile from male (and female) apocrine glands.

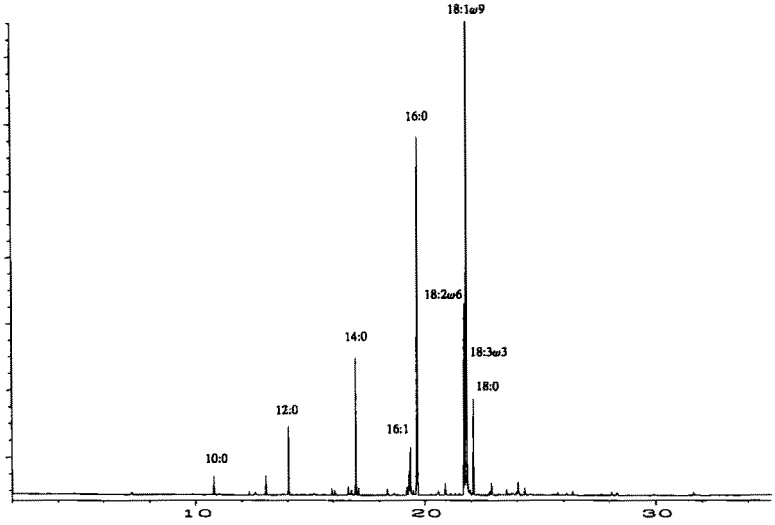

FIG. 6. Fatty Acid methyl ester profile (GC conditions 2, Methods) from saponification of the male (and female) apocrine gland lipids.

Sternum Lipids. An examination of the respective pairs of the waxy yellow sternum and contiguous-skin lipids recovered from both adult male (Figures 7 and 8) and adult female animals indicates little compositional variation in the relative abundances of the lipid components between the sexes and between those recovered from the easily recognized sternum and surrounding skin regions of each animal. TLC analysis suggests that the dominant lipid classes are those corresponding to the steryl/wax ester class; triacylglycerides are undetectable under these conditions. The predominant suite of components (R_t = 42–55 min) is believed to be representative of the wax ester (rather than the steryl ester) class. Authentic $C_{16:0}$, $C_{18:1}$, $C_{18:0}$ cholesteryl esters coelute with those species indicated with an asterisk in Figure 7.

Saponification and esterification of each of the pairs of lipids reveals, not surprisingly, virtually identical fatty acid profiles (Figure 9, for example). With respect to the whole sternum lipid profile (Figure 7), this finding suggests that the wax esters are perhaps representatives of a less common lipid class such as the diester group, that is based upon an α-hydroxyacid or an alkane-1,2-diol (Nicolaides, 1974; Albone, 1984). This is inferred also from a comparison of the gas chromatogram of the neutral lipids from orange roughy oil, a marine wax ester lipid class that is known to comprise regular linear esters derived from $C_{16:1}$–$C_{22:1}$ acids and $C_{18:1}$–$C_{24:1}$ alcohols and that elute under these conditions in the R_t = 35–41 min region (Buisson et al., 1982).

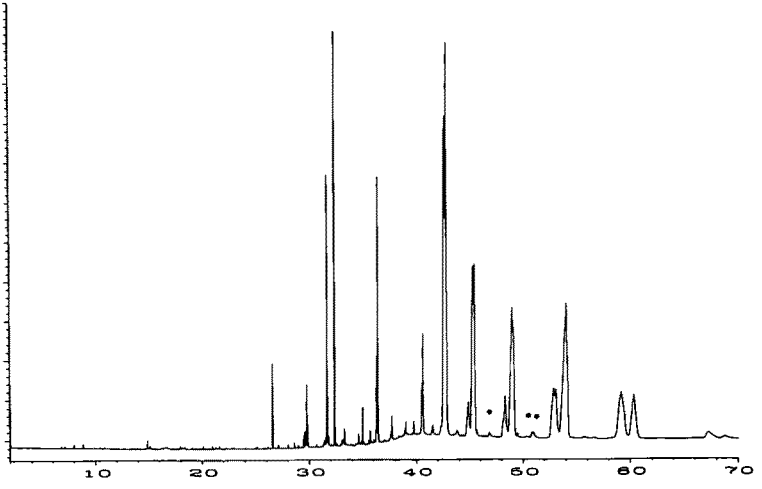

FIG. 7. Gas chromatogram (GC conditions 2, Methods) showing total lipid profile from male (and female) sternal integument (asterisks indicate R_t for cholesterol esters $C_{16:0}$, $C_{18:1}$, and $C_{18:0}$).

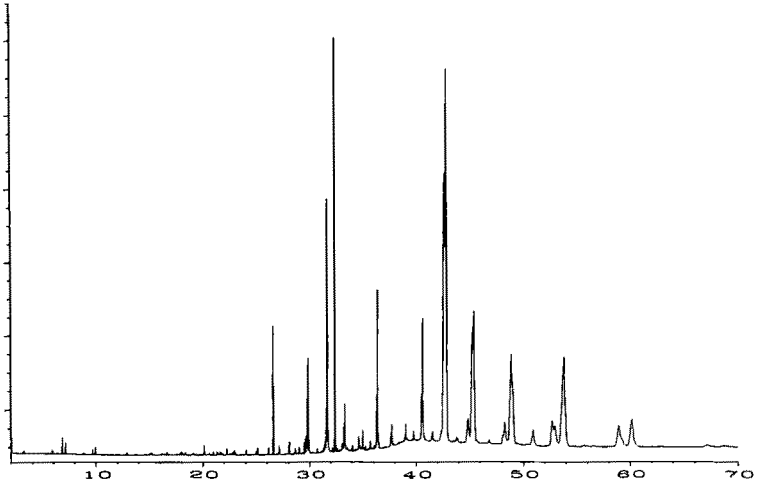

FIG. 8. Gas chromatogram (GC conditions 2, Methods) showing total lipid profile from male (and female) nonsternal skin regions.

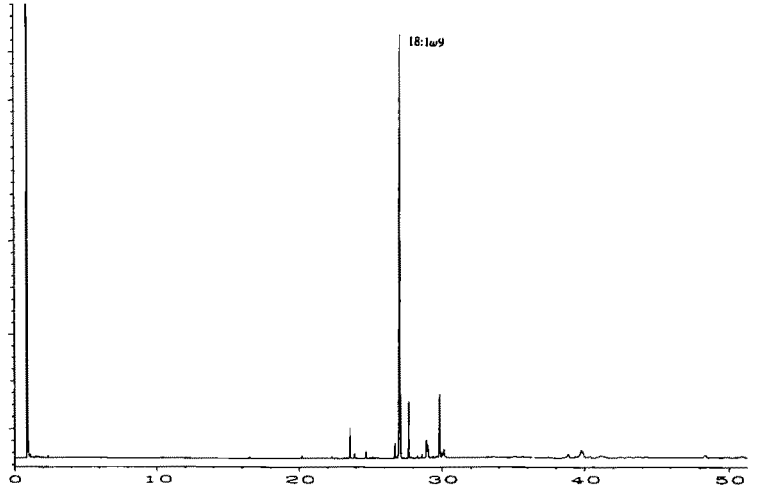

FIG. 9. Fatty acid methyl ester profile (GC conditions 2, Methods) from saponification of the male (and female) sternal and nonsternal lipids.

DISCUSSION

Our results confirm the presence of a diversity of lipids in the holocrine, apocrine, and sternum gland secretions, a finding that is not inconsistent with Christie's (1983) statement that lipids from different tissues of an animal can vary markedly in structure and that such differences reflect likely differences in function of the tissues.

No multivariate analysis has been undertaken in this initial attempt to delineate significant differences between adult male and adult female lipid secretions which are chemically extremely complex. However, the striking compositional similarities in distributions of components in each of the pairs of gland secretions and their respective fatty acids, at this level of analysis suggests that "odors" derived from these secretions do not play a significant role in specific communication between the sexes.

The presentation of whole ether-soluble gland materials, recovered from each of the holocrine (oil) and apocrine (cell) glands, to possums elicited no significant behavioral change or olfactory investigation (Innes and Frampton, 1991). Therefore, we reasoned that the chemical signal would more than likely be due not to an in situ volatile, but rather to a signal generated as a result of postemission bacterial degradation of one or another of the secretions. As fatty acids have been shown to be the principal odorous components produced by bacterial fermentation of gland secretions, we reasoned that a simple chemical saponification of the three possum gland secretions would be a reasonable mimic of this process.

Our findings of the existence of a predominant suite of low-molecular-weight branched-chain free fatty acids (Figure 2) in the copious holocrine secretions and in the hydrolysates of the same secretions recovered from both adult males and adult females can perhaps be taken as evidence that the odor profile described by this mixture is unique to the possum and that it might serve as a pheromone to regularly "scent the habitat" to aid colonization, for example. Moreover the presence of these fatty acids in the whole gland secretion (Figure 1) suggests that anaerobic microorganisms are indeed likely to be present within these sacs. The reported occurrence of fatty acids and of lipids containing them in this molecular weight range is unusual in mammalian secretions (Albone, 1984). To our knowledge there are only two other reports of the occurrence of branched-chain fatty acids in this range in marking fluids of other animals. The first of these describes a suite of 4-ethyl-substituted octanoic, decanoic, dodecanoic, and tetradecanoic acids as components of goat scent, the first member of which is believed to be responsible for the characteristic odor of the male goat (Sugiyama et al., 1981). In the marking fluid of the tiger, a suite comprising 14 low-molecular-weight saturated fatty acids was identified that included *n*-

and iso-pairs of hexanoic, heptanoic, octanoic and nonanoic acids (Sarkar and Brachmachary, 1991).

The fatty acid distributions in the much less copious apocrine (cell) gland secretions of adult males and adult females are again virtually identical (Figure 5) as are those derived from the sternal and nonsternal secretions of both sexes. All are characterized by the presence of fatty acids regularly associated with animal fats (Davey et al., 1983). The apocrine acids are comprised of a suite of these acids in the $C_{10:0}$–$C_{18:0}$ range, and the sternal secretion yields only $C_{18:1}$ as the predominant component. Based upon the volumes of the holocrine (oil) gland secretion and the obviously greater volatility of the fatty acid component derived from it, it is tempting to speculate that this secretion rather than the apocrine secretion, as suggested by Tyndale-Biscoe (1975), is used more regularly, perhaps in response to fright or alarm. This suggestion is also consistent with the observations of Thomson and Pears (1962).

A previous (and hitherto unpublished) study has demonstrated a behavioral response by adult male possums, manifested as olfactory investigation and scent-marking activity, to the representation of a highly polar lipid fraction derived from the (male) sternal sebum (Biggins, 1979). This so-called lipid fraction was said to be characteristically pigmented with a rufus coloration that was similar to the staining uniquely associated with the sternal hair in adult male possums. The work was not developed further, and no suggestion was advanced as to the lipid class to which the bioactive material(s) belonged. While we have not yet undertaken bioassays with sebum lipids, the fact that the profiles of sternal and nonsternal lipids from both males and females are identical suggests that the positive bioassay response observed by Biggins could be due to other components, which are unlikely to be free low-molecular-weight alcohols. Based upon the observed compositional similarities of the ether extracts of these secretions and of the fatty acids derived from them, it would appear that these constituents, either alone or in concert, are unlikely to be uniquely responsible for signaling social status unless they are associated perhaps with a discrimination between adults and juveniles.

It is recognized however that there is the probability that the whole secretion as discharged from whatever gland contains in its aqueous component proteinaceous (and possibly polysaccharide) material and enzymes that might effect the chemistry necessary to generate an odor signal. Previous studies with scent sacs of the otter, *Lutra lutra*, have indeed revealed the presence of both protein and mucopolysaccharide in addition to sebaceous gland lipid (Gorman et al., 1978).

Because the short-chain volatile fatty acids from the holocrine paracloacal glands are uncommon in the animal world and have a distinctive animal odor, it is possible that, when cocktailed, they may be utilized to attract possums and ultimately to be used in conjunction with poisoned bait in order to better control populations of possums within localized areas.

SUMMARY

1. The lipids from the sternum, apocrine, and holocrine glands of the brushtail possum are identical in both male and female animals. These lipids therefore appear to have no function in sexual attraction.

2. The lipid classes from each of the three glands are distinctly different.

3. The three glands can be easily differentiated by the profile of fatty acids obtained by hydrolysis of the total lipid fraction.

4. The volume of oil from the holocrine gland and the volatility of the short chain fatty acids in this secretion suggest that it may function as a source of pheromone by both male and female animals to perhaps "scent the habitat" and to signal alarm.

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SPATIAL DISTRIBUTION OF ODORS IN SIMULATED BENTHIC BOUNDARY LAYER FLOWS

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Abstract—Many animals orient to odor sources in aquatic habitats where different flows and substrates affect the hydrodynamics of benthic boundary layers. Since the dispersal of chemicals is due to the fluid mechanics of a particular environment, we quantified the changes in the fine structure of an odor plume under different hydrodynamic conditions in the benthic boundary layer of a laboratory flume. We sampled turbulent odor plumes at 10 Hz using a microchemical sensor (150 μm diameter) under two flow speeds: 3.8 and 14.4 cm/sec, and at 1, 8, 50 mm above the substrate. These distances above the substrate occur within different flow regions of the boundary layer and correlate with the location of crustacean chemosensory appendages within boundary layer flows. The high flow velocity exhibited a greater level of turbulence and had more discrete odor pulses than the low flow velocity. In general, odor signals showed a high level of temporal variation in fast flow at heights 1 and 8 mm above the substrate. In slow flow, temporal variation was maximal at 50 mm above the substrate, exhibiting more variance than the same height at the fast flow. These patterns of odor signals resulted in

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part from differences in the height above the substrate of the main axis of the odor plume at the two flow speeds. Our results imply that animals chemically orienting to an odor source will need to compensate for varying hydrodynamic properties of odor transport and dispersal. The method by which animals extract spatial information from odor plumes will need to account for changing flow conditions, or else it will not be equally efficient in extracting information about chemical spatial distributions.

Key Words—Odor plume, chemical orientation, chemoreception, turbulence, hydrodynamics, electrochemistry, benthic boundary layer, flume.

INTRODUCTION

Odor concentrations in plumes are heterogeneous when measured at fast temporal and small spatial scales (Murlis and Jones, 1981; Atema, 1985; Moore and Atema, 1988, 1991; Zimmer-Faust et al., 1988; Murlis et al., 1991; Moore et al., 1992). The patchy structure of odor plumes is due to turbulence produced by the mechanical forces acting on a moving fluid. The magnitude of concentration fluctuations is dependent on the interaction between the size of the turbulent eddies and the size of the odor plume. This size dependency implies that estimates of odor concentration are determined in part by the temporal and spatial sampling scale of the chemical measurements (Aylor, 1976; Aylor et al., 1976; Miksad and Kittredge, 1979). As a result of this turbulence, animals located downcurrent of an odor source will experience periods during which odor concentrations are well above or below the mean odor concentration and which exhibit unpredictable temporal variation. Thus, mean concentrations and time-averaged distributions (i.e., 5–10 min) may not be indicative of the information available for many macroscopic animals attempting to orient towards an odor source (Elkinton et al., 1984; Moore and Atema, 1988, 1991; Zimmer-Faust et al., 1988; Zimmer-Faust, 1989).

The physical forces governing odor dispersal will depend on the flow regime into which the chemical stimulus is introduced (for review see Westerberg, 1991). Odor transport is influenced significantly by the interaction between a moving fluid (air or water) and a solid surface, such as when air flows over the earth's surface or water flows over the sea floor. The interface between a stationary solid and a moving fluid is called a boundary layer and is a region through which fluid velocities steadily increase as one moves away from the solid surface.

The boundary layer is generally divided into three regions, each of which is characterized by a particular combination of physical forces affecting chemical signal transmission. The region immediately above the solid surface is called the bed layer and actually consists of two sublayers (Wright, 1989). The sublayer closest to the surface (diffusive sublayer) may be only micrometers thick and is

characterized by a near absence of flow. The dispersion of chemicals in this sublayer occurs largely by molecular diffusion. The sublayer further away (viscous sublayer) is characterized by quasilaminar flow, but is dominated by viscous forces tending to dampen turbulence (Wright, 1989). The second region, still further away from the surface, accounts for about 30% of the boundary layer and is called the buffer zone or log layer. This region derives its name from the fact that flow velocity through the log layer is a function of the logarithmic distance above the solid surface. In the log layer, both viscous and inertial forces are important in maintaining the pattern of flow. It is here that small, energetic turbulent eddies are generated when energy associated with the momentum of the advection component of flow is converted to energy associated with eddies. The final, most distant region of the boundary layer is called the outer layer or log-deficit layer. Here, flow is nearly independent of the frictional force imposed by the solid surface on the moving fluid and is characterized by the presence of relatively large turbulent eddies.

Boundary layer flows over any solid object or substrate are often characterized by determining frictional or shear velocity (u_*), and roughness Reynolds number (Re_*). Frictional velocity is a measure of the strength and correlation of turbulent fluctuations in velocity near the substratum. The magnitude of u_* is proportional to the magnitude of eddy diffusivity in a turbulent boundary layer (Fischer et al., 1979). As shear velocity increases, greater eddy diffusion will result in greater dilution and, hence, lower time-averaged concentrations of odorants suspended in the fluid. However, the increased momentum fluxes responsible for eddy generation will also increase the temporal and spatial variation in odor concentration as odorants become entrained in coherent eddy structures. The Re_* provides a means for describing the distance to which turbulent eddies penetrate the boundary layer. As Re_* increases, the effect of turbulence is felt closer to the solid surface. The onset of turbulence in the outer reaches of the boundary layer over a seabed occurs at Re_* between 3.5 and 6 (Nowell and Jumars, 1984; Denny, 1988). Boundary layer flow is fully turbulent (i.e., the turbulence extends all the way to the substrate) when $Re_* > 75$–100.

The turbulence properties of odor dispersal in boundary layers vary with physical environmental conditions, especially substrate type, flow speed, and kinematic viscosity of the fluid. Yet, terrestrial and aquatic animals show the remarkable ability to use chemical signals in orienting to odor sources under a variety of flow conditions (for review see Bell and Tobin, 1982). Many benthic aquatic animals (e.g., decapod crustaceans and fishes) sample chemical signals and orient to plumes using receptor organs located on different parts of the body (McLeese, 1973; Reeder and Ache, 1980; Johnsen and Teeter, 1980; Devine and Atema, 1982). These receptor organs, which are located at different heights above the substrate, should receive different patterns of chemical stimulation from an odor source.

Previous studies have shown that chemical dispersal processes create spatial patterns of odor that could serve as directional cues (Murlis and Jones, 1981; Moore and Atema, 1988, 1991; Zimmer-Faust et al., 1988; Murlis et al., 1991) and that chemoreceptor appendages and cells have temporal filter properties that could serve to further enhance spatial differences in odor plumes (Moore and Atema, 1988). In order to apply the results from these studies to different environments, it is necessary to correlate patterns in the spatial distribution of odors with the physical properties of the fluid environment. Our current study was designed to quantitatively describe aquatic odor plumes emanating from a biologically relevant source in two different flow regimes. Hydrodynamic measurements were made in benthic boundary layers at three different heights above the sediment bed, corresponding roughly to positions within the viscous sublayer, log layer, and log-deficit layer. The spatial and temporal sampling scale of our chemical measurements were matched to those scales associated with benthic crustacean chemoreception. Our study experimentally demonstrates the critical importance of benthic boundary layer hydrodynamics to the spatial distribution of chemical signals.

METHODS AND MATERIALS

Flume Design and Flow Regime Calculations

Experiments were conducted in steady flows and fully developed turbulent boundary layers within a single-channel, recirculating flume (10 × 0.75 × 0.15 m; length × width × height). The working section, a fixed drop box (1 × 0.45 × 0.15 m; Figure 1), was placed 7.5 m downstream of the entry section

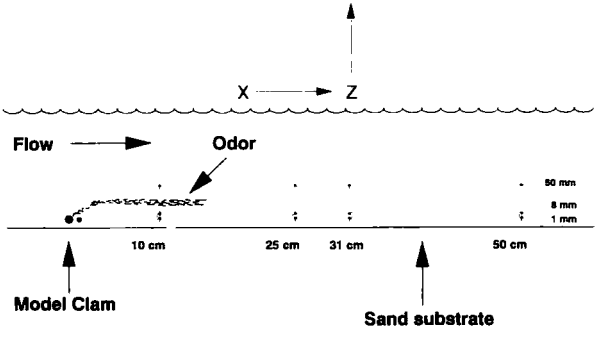

FIG. 1. Diagram showing the working section of the flume used in simulating flows of a benthic boundary layer. Flow was from left to right. Closed circle: model clam. Asterisks: sampling sites. The 75- and 100-cm sites are not shown.

and 1.5 m upstream of the exit weir. The drop box was filled with sand taken from local habitats and sieved to <1 mm to remove large particles (mean diameter of sand particles: 350 ± 10 μm; $N = 100$). The entire flume bed was carefully layered to a uniform depth of 0.5 cm with this material. The dimensions, placement of the working section, configuration of the upwelling section, and baffle arrangements were based on accepted principles of flume design (e.g., Nowell and Jumars, 1987) and ensured that flow through the test section was free from artifacts produced by improper flume architecture. Water exiting the flume was pumped through a 5-μm particle filter, activated charcoal bed, and a UV sterilization unit before being returned. Water salinity was 25 ppt and temperature was 25 ± 1 °C. Further details pertaining to the flume are presented in Weissburg and Zimmer-Faust (1993).

Hydrodynamic Measurements

The friction velocity (u_*) and roughness Reynolds number (Re_*) of water flowing through the working section of the flume were determined by measuring the vertical velocity gradient within the log layer. The vertical velocity gradient was characterized by determining the speed of neutrally buoyant particles injected at known heights above the substrate, a technique similar to that used by Ertman and Jumars (1988). Black styrene–divinylbenzene particles (350 μm diameter, specific gravity 1.03 g/ml; Bangs Laboratories, Carmel, Indiana) were injected isokinetically using a variable-speed syringe pump and microcapillary tubing (ID 1.1 mm, OD 1.4 mm) with the tube tip oriented parallel to the flow. The length of the capillary tube arm parallel to the flow was approximately 2 mm. The delivery tube was affixed to a three-dimensional micromanipulator that allowed precise spatial positioning of the tube. Velocity profiles were determined over at least five heights in the log layer, a necessary condition for accurate measurement of boundary shear velocities (Nowell and Jumars, 1984, 1987).

Particle trajectories were recorded on video tape for later motion analysis of velocity. The field of view was 2×2 cm and began 15 cm downstream from the tip of the delivery tube. At this distance ($50\times$ the length of the tube's projection into the flow), particles entering the field of view were free from the flow effects generated by the delivery tube. The velocity of 25 particles was determined on a frame-by-frame basis over 10–20 video frames. Thus, an average of 250–500 particle velocity realizations were determined for each height above the substrate. Full details on motion analysis of bead trajectories are described elsewhere (Weissburg and Zimmer-Faust, 1993). Velocity measurements based on particle trajectories and motion analysis were confirmed by making velocity determinations in some instances with a hot bead thermistor probe (LaBarbera and Vogel, 1976; as modified by M. Patterson and M.L. Judge).

The vertical velocity profiles provided data that allowed us to use the "Law of the Wall" in calculating shear velocity as:

$$U_z = \frac{u_*}{\kappa} \ln\left(\frac{z}{z_0}\right) \tag{1}$$

where U_z is the mean velocity at height z above the bed and κ is von Karman's constant (0.41). The hydraulic roughness length (z_0) was determined as the y intercept of the equation regressing log height above the substrate against the measured flow speed. Regression equations generated for repeated measures in slow (3.8 cm/sec) and fast (14.4 cm/sec) flows produced correlation coefficients, $r^2 \geq 0.993$. The z_0 values calculated from regressions differed by $\leq 11\%$ between trials, indicating the bed form remained uniform during the course of this study.

Roughness Reynolds number was determined as:

$$Re_* = \frac{u_* D}{\nu} \tag{2}$$

where D is the height of the roughness elements (e.g., the diameter of the sand grains forming the bed), and ν is the kinematic viscosity of the fluid (0.01 cm^2/sec). Based on the measured diameter of the sand grains, we used a value of 351 μm for D. We estimated the thickness of the viscous sublayer for each flow regime by reworking equation 2 and solving for D, setting $Re_* = 6$. Trials were conducted at two free-stream (U_∞) velocities, 3.8 cm/sec (slow flow) and 14.4 cm/sec (fast flow). A summary of the hydrodynamic measures for the two flow regimes is shown in Table 1.

Odor Plume Generation

To simulate a biologically relevant odor plume, a chemical tracer (dopamine) was introduced into the flow through the excurrent siphon of a model clam. The clam was designed using principles and procedures developed by

TABLE 1. SUMMARY OF HYDRODYNAMIC PARAMETERS FOR FLOW REGIMES USE IN EXPERIMENTS

	Fast flow (14.4 cm/sec)	Slow flow (3.8 cm/sec)
Roughness Reynolds number (Re_*)	3.56	1.26
Frictional velocity (μ_*)	1.22 cm/sec	0.36 cm/sec
Viscous sublayer thickness	0.042 cm[a]	0.207 cm[a]

[a]These values are only approximate estimates of the true layer thickness.

Monismith et al. (1990). The scale of our model bivalve (physical size and pumping rate) corresponds to a small hard clam, *Mercenaria mercenaria*, common in estuaries along the Atlantic and Gulf coasts of the United States. The model clam was created as a pair of tubes with their tips protruding vertically 5 mm above the substrate, simulating excurrent (3.1 mm ID) and incurrent (4.7 mm ID) siphons of a living animal. The excurrent tube was placed on the downstream side. Both the diameters and positions of the siphonal tubes were set, based on in situ observations and measurements of clams as part of another study (Weissburg and Zimmer-Faust, 1993).

The excurrent flow was supplied from a small, constant-head tank while the incurrent flow was taken by gravity feed from the flume (see methods section in Monismith et al., 1990). To ensure equality of the incurrent and excurrent flow rates, we continuously monitored these flows with inline flowmeters. Experiments were performed with a flow rate of 0.5 ml/sec through the siphon pair, with the Reynolds number of the siphon jet ≈ 170. At this Reynolds number, the jet is laminar at the point where it leaves the excurrent siphon (List, 1982; Monismith et al., 1990).

As a frame of reference, we define the spatial coordinates of the siphon as $x = y = 0$, $z = 0.5$ cm. The x dimension is the downstream axis and y dimension is the cross-stream axis. The z dimension is oriented vertically into the water column with $z = 0$ defined as the boundary between the bed and the water column. Chemical recordings were made at $y = 0$, $x = 10$, 25, 31, 50, 75, 100 cm. At each of these six sites, chemical measurements were repeated at z-axis heights of < 1, 8, and 50 mm. The heights corresponded roughly to positions within the viscous sublayer, log layer and log-deficit layer, respectively, as verified by theoretical calculations and repeated vertical profile measurements. Chemical recordings were made at 10 Hz for 3 min at each of the 18 different positions.

Electrochemical Microelectrodes

Since the introduction of microelectrochemical techniques to aquatic applications, it has become possible to quantify chemical distributions at very small spatial scales and with high temporal resolution (Moore et al., 1989). The spatial and temporal scales required in quantifying chemical stimulus distributions relevant to aquatic organisms can be estimated from behavioral and electrophysiological studies. For instance, most arthropods orient to chemical signals using sensory input from antennae or antennules (insects, Kennedy, 1986; crustaceans, Reeder and Ache, 1980; Devine and Atema, 1982). These appendages usually have small hairs or sensilla that are permeable to odors and contain the dendrites for the primary receptor cells (Ghiradella et al., 1968; Laverack, 1988). To match the spatial sampling area associated with a single sensillum (30 μm diam-

eter and 1000 μm length), we chose electrochemical electrodes with diameters of 100–150 μm. In contrast to spatial scales, the temporal sampling scales for chemoreceptor cells are not well understood and must be estimated from flicker fusion and receptor adaptation studies. Neurons, both peripheral and CNS, can give distinct responses to odor pulses as fast as 10 Hz in two moth species (Kaissling et al., 1987; Christensen and Hildebrand, 1988). Similar studies in the American lobster, *Homarus americanus* have found peripheral chemoreceptor cells that can follow 4-Hz pulses (Gomez et al., 1992) and begin to adapt in 500 msec (Voigt and Atema, 1990). From these studies, we can estimate a temporal sampling scale between 100 and 500 msec. In addition, frequency spectra of aquatic odor signals measured at sampling rates of 10, 25, and 200 Hz (Moore and Atema, 1988, 1991; Atema et al., 1991) have shown that most of the odor signal fluctuations lie below 10 Hz. Thus, a sampling rate of 10 Hz will provide enough resolution to capture signal features most relevant to aquatic chemoreceptors.

We used a single graphite–epoxy capillary electrode (Gerhardt et al., 1984) with a tip diameter of 150 μm. The sampling area is determined by the exposed carbon–epoxy surface area (Adams, 1969; Gerhardt et al., 1987). Recordings were made at a sampling rate of 10 Hz using the IVEC-5 (In Vivo Electrochemistry Computer System; Medical Systems Corp., Greenvale, New York). Each 100-msec epoch for the 10 Hz sampling rate is composed of a 50-msec epoch at $\pm$0.55 V (oxidation) followed by a 50 msec epoch at 0.0 V (reduction). The recording electrodes were sampled every 50 msec; analog-to-digital conversions of the samples occurred at 4 KHz, and data were averaged for the 50-msec time epoch. Further details of recording and digitizing are explained elsewhere (Moore et al., 1989).

Electrodes were calibrated in solutions of dopamine prepared in filtered seawater and exhibited excellent linearity over a concentration range of 0.5–100 μM (correlation coefficient; $r^2 > 0.96$). We used a source concentration of 2 mM dopamine (and 0.1 mM ascorbic acid as an antioxidant).

The effect of flow on concentration measured by a sensor was tested by placing an electrode in known concentration solutions at different flow velocities. The tip of the electrode was precisely positioned, then sealed in the center of tygon tubing (0.317 cm ID), which was connected to a calibrated syringe drive. The tubing was long enough (4 m) to ensure a fully developed boundary layer for flow before contact was made between an injected solution and the sensor tip. A 5 μM dopamine solution was driven past the sensor at six flow velocities ranging from 1.6 cm/sec to 10.1 cm/sec. Flow velocity at the sensor tip was calculated as 2$\times$ the mean averaged over the entire cross-sectional area of the tube, as required for flow in a cylindrical pipe. The concentration measured by the electrodes changes linearly with flow speed ($r^2 = 0.96$). The slope of this relationship for the GEC electrodes is 0.20. Thus, a 5 μm concentration in a

still solution is measured as 5.8 μM at 3.8 cm/sec and 7.8 μM at 14.4 cm/sec. This relationship is consistent with the performance of microchemical sensors, which behave as though they are in stirred solutions (Adams, 1969; Galus et al., 1982). This increase is due to greater chemical flux across the sensor per unit time and not due to any changes in the response properties of the electrode. For the sake of simplicity, we use the term "concentration" to describe the calibrated signal from the electrodes. A more accurate description in flowing systems may be "molecular encounters per unit time." In this way, the electrodes and chemoreceptor cells in a flowing environment measure chemical signals in an identical manner.

Flow Visualization

To visualize the behavior of the plume generated by the model clam, we mixed a dilute solution of fluorescein (0.01% by weight) into the stimulus fluid. At these concentrations, the dye was neutrally buoyant. We used a halogen slit light source to illuminate the dye as the jet was ejected from the excurrent siphon. Images of the excurrent plumes were video-recorded from the side of the flume for 1 min at a series of positions at known distances from the origin, providing a visual record of the plume extending 100 cm downstream. The field of view was either 5 $\times$ 5 cm ($<$10 cm downstream from source) or 15 $\times$ 15 cm ($>$10 cm downstream). After each recording sequence, vertical and horizontal scales were filmed for later determinations of the spatial coordinates of the plume and for transformation to coordinates established above. Images of the plume were replayed through a motion analysis system in order to determine the trajectory of the plume. The center of mass was estimated visually along the x axis at 2.5-mm intervals for the first 10 cm, and thereafter at 5-cm intervals. At each position along the x axis, average plume position was generated by using the center of mass coordinates on 30 alternate video frames.

Data and Statistical Analysis and Definition of Terms

All measurements were converted from computer to micromolar concentrations of dopamine using the calibration factor determined for the electrode in static solutions. Odor profile parameters were extracted from the calibrated odor plume signals based on the definitions set forth in Moore and Atema (1991). While many statistical measures and signal analysis procedures could be used to analyze the signals presented here, we chose those parameters and analysis procedures that are more relevant to neuronal detection and coding of chemical signals. Computer simulations (Moore and Atema, 1988; Moore et al., 1992) and electrophysiological studies (Gomez et al., 1992; Borroni and Atema, 1988, 1989) have demonstrated that stimulus parameters such as pulse slope, height, length, and off time are important for determining the temporal response char-

acteristics of primary chemoreceptor cells. In addition, statistical measures taken over the whole recording period, such as mean level of concentration over 3 min may be poor indicators of the information available to animals making decisions in seconds based on sensory information integrated over milliseconds (Elkinton et al., 1984). Thus, the analysis we use is especially relevant to the biological detection of chemical signals by chemoreceptor cells. The definitions of plume parameters (based on Moore and Atema, 1991) used for our analysis are reviewed here in brief.

Odor Profile: a plot of concentration versus time that shows the concentration fluctuations at a single sampling site (Figures 2 and 3).

Odor Burst: within an odor profile there are periods with and without odor. Periods during which the odor concentration is detectable by the sensor are called odor bursts. A burst ends when the concentration drops below detectable levels (Figure 2).

Odor Pulse: within an odor burst, there may be multiple peaks and valleys of odor concentration (asterisks in Figure 2). Each of these peaks is considered a separate odor pulse only when the valley between the pulses falls to a value that is below 30% of the first pulse height. Odor pulses can be characterized by *pulse height*, highest concentration obtained during the pulse; *pulse length*,

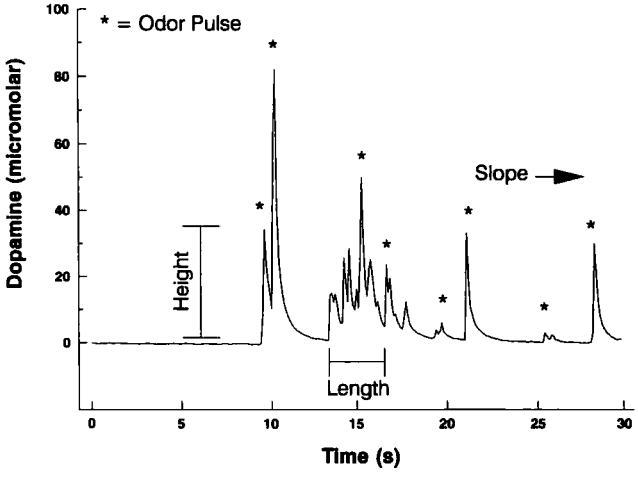

FIG. 2. Thirty-second temporal profile of dopamine concentrations at the slow flow velocity (3.8 cm/sec) demonstrating pulse parameters analyzed. Pulse height was measured against background level and pulse length was time from beginning to ending of odor pulse. Pulse slope was maximum slope on the rising side of the peak. Sampling site was located at $x = 31$ cm, $z = 50$ mm.

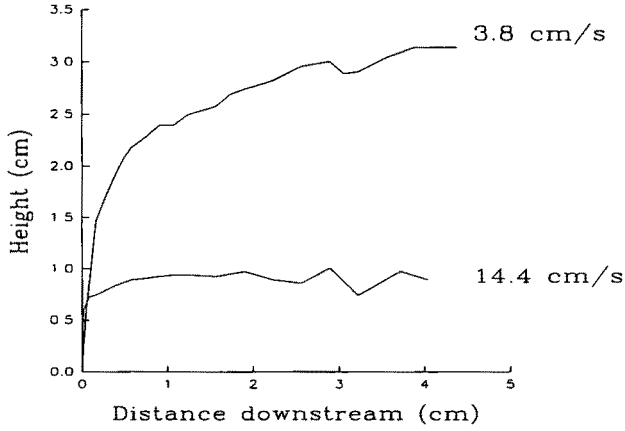

FIG. 3. Center of mass calculations showing differences in odor plume height at the two flow speeds (3.8 and 14.4 cm/sec). At the slower flow speed, the odor plume climbed to a higher level than at the faster flow speed during the first 5 cm downstream. Details on center of mass calculations are given in the text.

duration of pulse; and *pulse onset slope*, maximum value of rising slope of pulse.

Based on Moore and Atema (1988, 1991) further statistical analysis used pulse height and pulse slope as potential indicators of directional information. Differences in mean values for pulse height and slope were tested using an a posteriori statistic: Tukey test after an ANOVA (Zar, 1984). For spatial representation of probability distributions, pulse height (μM) and pulse slope (μM/sec) were grouped in five log-step bins (0.1–0.9, 1–9, 10–90, 100–900, 1000–9000). Probability distributions of pulse height and slope were calculated dividing the number of occurrences within a log-step bin by the total number of pulses encountered at that particular sampling site. A χ^2 analysis (SPSS, crosstabs, SPSS Inc., Chicago, Illinois) of the categorized probability distributions was used to examine the effect and interaction of flow speed, height above substrate, and distance downstream on the probability distributions of pulse height and slope.

RESULTS

Qualitative Description of Odor Plumes

The trajectories of the odor plumes were clearly different at the two free stream velocities (Figure 3). The plume at 3.8 cm/sec rose to greater height and bent parallel to the substrate at a greater distance downstream than the plume

at 14.4 cm/sec. The plume at 3.8 cm/sec showed evidence of turbulent boundary layer flow, generating ring vortices within the first 2 cm of the origin. Although eddies occasionally contacted the substrate, the plume never reattached to the bottom, and the center of mass continued to rise as it traveled downstream. The plume at 14.4 cm/sec exhibited greater effects of turbulence than the jet at 3.8 cm/sec. At 14.4 cm/sec, the plume trajectory was unsteady, oscillating vertically in response to turbulent fluctuations in the flow. This was often associated with the rapid generation of large-scale coherent structures that were subsequently shed, generally within 5 cm of the origin. The plume reattached to the bottom within the first 5 cm and maintained contact at least 100 cm downstream. Although the effects of turbulence presumably caused dispersion and elevation of the plume's center of mass, the greater range of eddy sizes caused an increase in dilution of the dye stream. This rendered the top edge of the plume difficult to see and produced an apparent leveling off of the plume trajectory as it traveled downstream.

Although the odor source was constantly emitting dopamine, odor profiles were heterogeneous in time (Figure 4). Odor profiles were characterized by both odor bursts and pulses. In the present study, bursts frequently lasted from 5 to 10 sec (Figure 2, 13–18 sec), while pulses lasted between 0.5 and 6 sec (Figure 2, 21–22 sec). Peak odor concentrations during the pulse were diluted about 20 times as compared to the initial source concentration; i.e., from 2 mM to 100 μM.

Effect of Flow Regime on Plume Parameters

Number of Pulses. The faster flow velocity had a greater number of pulses at most of the sites measured (14 of 18 sites) than the slow flow speed (Figure 5). This is consistent with greater turbulence generated at 14.4 cm/sec (indicated by the higher friction velocity). Only the z = 50 mm, x = 10, 25, 31, and 100 cm sites at the slow velocity had more pulses than the corresponding fast velocity sites. At all of the z = 1 and 8 mm sites, the fast velocity exhibited larger numbers of pulses than the corresponding sites at the slow velocity.

Within the fact velocity, all of the z = 8 mm sites consistently showed a greater number of pulses than either the z = 1 or 50 mm sites. (Figure 5A, gray bars). This ranged from 90 (z = 8, x = 25) to 157 pulses (z = 8, x = 10). There was no consistent association of the number of pulses versus downstream distance from the odor source within the z = 1, and 8 mm sites. The z = 50 mm depth showed an increase in the number of pulses as distance from the odor source increased. This trend was consistent except for the x = 100 cm site, which showed fewer pulses than the previous (x = 75 cm) site.

For the slow flow velocity, the z = 50 mm distance showed a greater number of pulses than the z = 1 and 8 mm sites (except the x = 75 cm site;

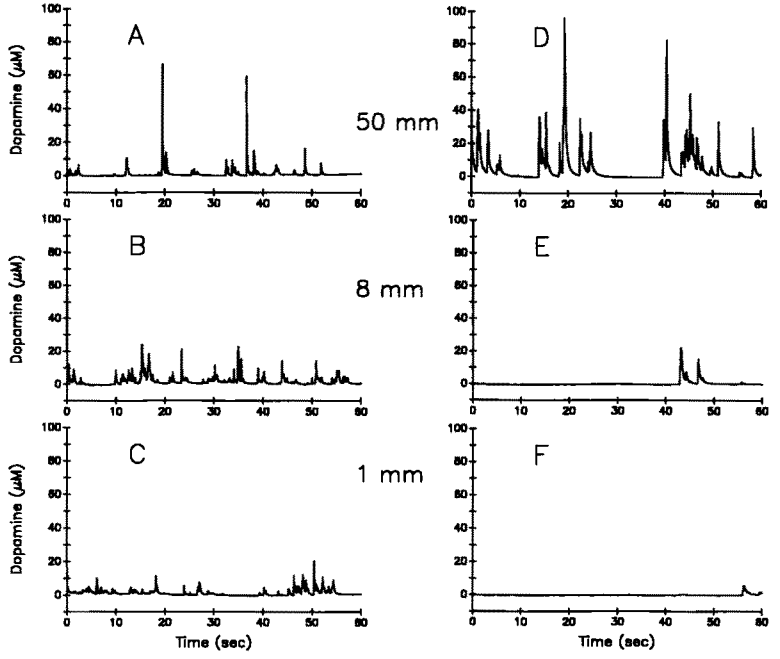

FIG. 4. One-minute dopamine concentration profiles recorded at the $x = 31$ cm cross-sectional plane. Profiles are from the fast flow velocity (14.4 cm/sec): $z = 50$ mm (A), $z = 8$ mm (B), $z = 1$ mm (C) and slow flow velocity (3.8 cm/sec): $z = 50$ mm (D), $z = 8$ mm (E), $z = 1$ mm (F). One-minute segments were chosen to show the most intense period of odor fluctuations in the 3-minute record.

Figure 5B, open bars). None of these sites had a consistent relationship between number of pulses and downstream distance from the odor source.

Pulse Height and Slope. At the fast flow speed the odor plume had a lower center of mass, which is reflected in higher mean pulse heights at 8 and 1 mm as compared to 50 mm (Figure 6). The mean pulse height was significantly higher ($P < 0.01$) at the 10-cm site than at the other distances at both the 8- and 1-mm height above from the substrate. The mean pulse height decreased with increasing distance from the odor source, although this change was not significant. It was only when the plume traveled 30 cm downstream that it dispersed vertically enough so that relatively large pulse heights are encountered at the 50 mm height. The maximum mean pulse height was reached at 30 cm downstream from the odor source, and this pulse height was significantly higher than only the 10-, 75-, and 100-cm sites ($P < 0.01$; Figure 6A). Once the

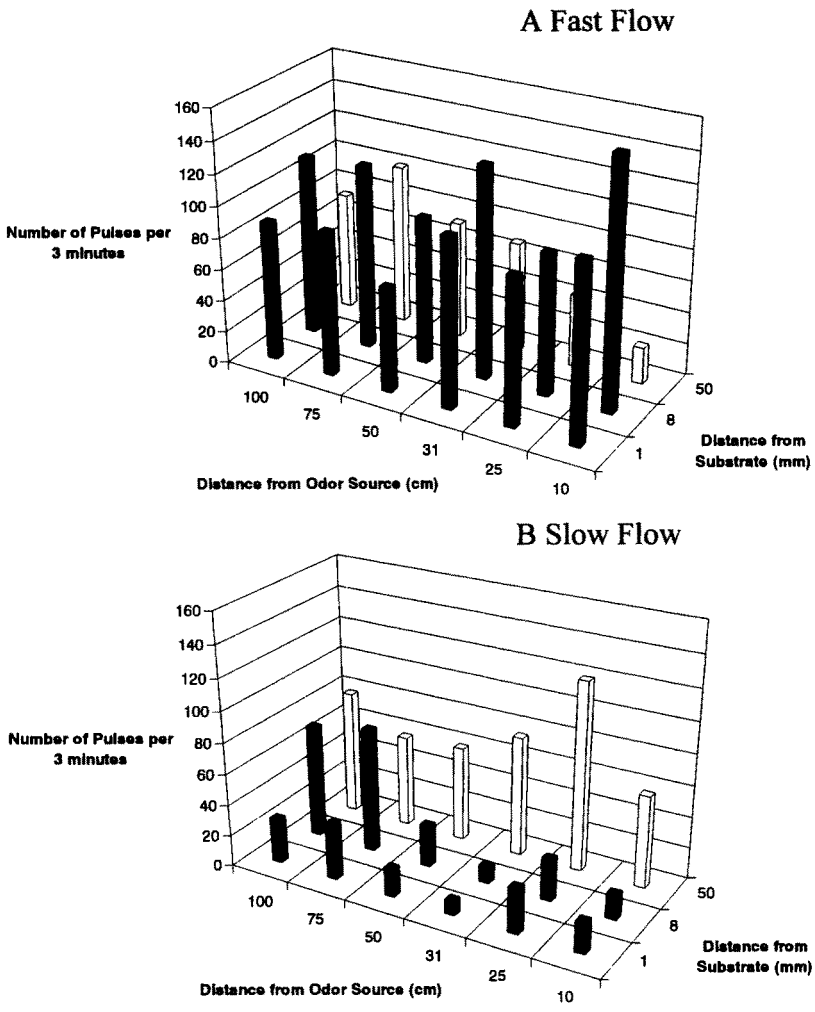

FIG. 5. Number of pulses within the 3-min odor profiles for the fast flow (A) and slow flow (B) conditions. Shaded bars represent distance up from substrate: 50 mm (open), 8 mm (gray), 1 mm (black).

maximum was reached, the pulse heights decreased with increasing distance downstream.

The distribution of pulse heights at the slow speed showed a different spatial pattern than those seen at the fast speed (Figure 6D, E, and F), since the plume heads parallel to the substrate rather high in the water column. The largest pulse

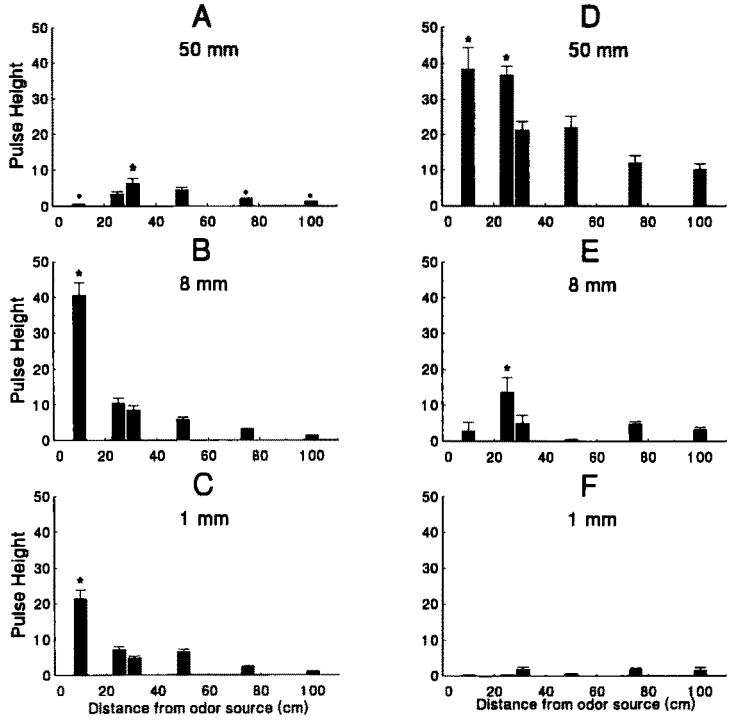

FIG. 6. Mean (±SEM) pulse height (in μM values) taken over the 3-min recording period for the fast flow (A, z = 50 mm: B, z = 8 mm: C, z = 1 mm) and the slow flow (D, z = 50 mm: E, z = 8 mm: F, z = 1 mm) conditions. Asterisks represent pulse heights that are significantly different from all means within any one graph, except A where an asterisked mean is different only from means with solid circles above them. Sample sizes for means are the same as in Figure 5.

heights were measured at the highest distance (50 mm) up from the substrate (Figure 6D). At this location, the pulse heights at both the x = 10 and 25 cm sites were significantly larger than at any other downstream site (Tukey-Kramer, $P < 0.01$). Although the pulse heights decreased with increasing distance from the odor source, this decrease is less than that measured at the higher flow velocity. The lower level of turbulence seen at the slow (versus the fast) flow velocity (see Table 1) had a lesser effect on dispersing and mixing the odor plume as it traveled downstream. Conversely, the high level of turbulence at the high flow velocity served to break up the larger patches of odor into smaller and more discrete odor pulses. This resulted in lower mean pulse height within the main axis of the odor plume at the slow flow as compared to the fast flow

(compare Figure 6B with 6D, sites 25, 30, 50, 75, and 100 cm). The spatial distribution of mean pulse slopes were very similar to the distributions seen for mean pulse height and thus were not shown.

Although the pulse heights were higher at the 50-mm sampling site and at the slow flow, the overall slopes compared to their heights were lower than for the pulses encountered at the faster flow. Higher slopes may just be a consequence of higher pulse height when sampling over a constant time interval. The slope-to-height ratio (Table 2) is an indication of the steepness of the onset of odor within a pulse and is independent of the absolute pulse height. This parameter allows for comparison of onset slopes of pulses of different heights (Figure 6A and D). It becomes evident when comparing the slope-to-height ratios for the fast and slow flows that the odor pulses within the fast flow are more discrete and tightly packed. Most of the odor pulses encountered in the fast flow had higher ratios than the corresponding sampling sites in the slow flow (Tukey-Kramer, $P < 0.01$; all sites were significant except for $z = 50$, $x = 25$, 100; $z = 8$, $x = 25$, 50; $z = 1$, $x = 10$). This is particularly evident at the 50-mm sampling height. While the slow flow has considerably higher mean pulse heights (sometimes 20 times larger, compare Figure 6D and 6A), the fast flow has larger slope-to-height ratios.

The probability distributions of pulse slopes from the fast flow velocity (14.4 cm/sec) are seen in Figure 7. All three heights from the substrate showed similar distributions far from the odor source, i.e., 75 and 100 cm. Differences in distributions increased near the odor source, i.e., 10 and 25 cm (χ^2 analysis, $P < 0.001$). The most consistent change with distance was seen in the probability distribution at the $z = 1$ mm height (χ^2 analysis, $P < 0.001$). Far away from the source (100 cm), most of the pulse slopes (80%) occurred in the 1–9

TABLE 2. MEAN (±SEM) SLOPE-TO-HEIGHT RATIO AS FUNCTIONS OF FLOW SPEED AND DISTANCE DOWNSTEAM

Height (mm) above substrate	Downstream distance					
	10 cm	25 cm	31 cm	50 cm	75 cm	100 cm
Fast Flow						
50	6.5(0.5)	6.3(0.4)	7.4(0.3)	7.7(0.3)	6.9(0.2)	7.2(0.3)
8	6.7(0.2)	5.6(0.3)	6.2(0.2)	5.6(0.2)	5.5(0.2)	6.1(0.2)
<1	5.5(0.2)	5.9(0.3)	6.7(0.3)	5.4(0.2)	5.8(0.2)	6.1(0.3)
Slow flow						
50	4.8(0.4)	6.2(0.2)	5.6(0.3)	6.5(0.3)	7.0(0.4)	7.3(0.4)
8	5.6(0.6)	5.7(0.6)	4.8(0.7)	5.5(0.4)	6.0(0.4)	4.5(0.3)
<1	6.0(0.4)	5.0(0.2)	3.0(0.7)	5.0(0.5)	4.3(0.3)	5.1(0.5)

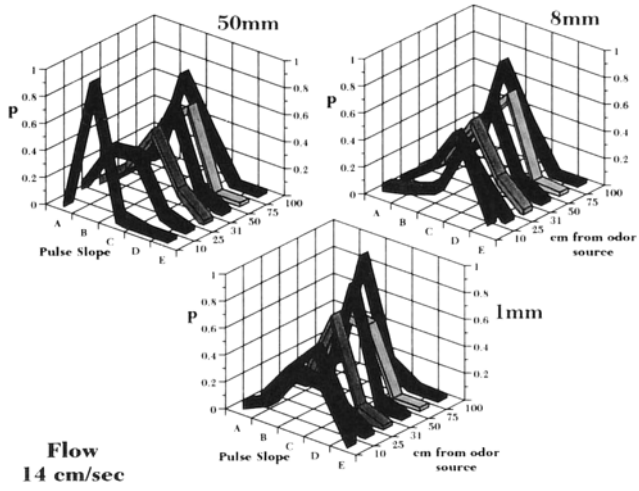

FIG. 7. Probability distributions of pulse slope taken in the fast flow (14.4 cm/sec) at the 50-, 8-, and 1-mm distances from the substrate. Probability values were calculated for five different bin sizes: (A) 0.1–0.9, (B) 1–9, (C) 10–90, (D) 100–900, (E) 1000–9000 μM/sec.

μM/sec bin. This range of possible pulse slopes gradually increased to the 10 cm site. Both the 50 and 8 mm heights showed this same trend although not as dramatically.

Each of the probability distributions at the slow speed (Figure 8) were different from the corresponding distributions at the fast speed (Figure 7; χ^2 analysis, $P < 0.005$). The highest pulse slopes were seen in the probability distributions at $z = 50$ mm height (Figure 8). The $z = 8$ mm height contained a mixture of pulse slope values and had no consistent change in distributions with distance from odor source. The $z = 1$ mm height had shallow slopes at all distances except at the $x = 25$ cm. None of these distributions had any association with downstream distance such as that seen at the fast flow speed.

The probability distributions for pulse height at 14.4 cm/sec showed no consistent trend with distance at any of the heights along the z axis (Figure 9). All three heights ($z = 1$, 8, 50 mm) had similar distributions at all downstream distances from the odor source except at the $x = 10$ cm, where at the $z = 50$ mm site, almost all of the pulse heights were 0.1–0.9 μM (χ^2 analysis, $P < 0.001$). In comparison, at the $z = 8$ and 1 mm sites, the majority of the pulse heights were in the 10–90 μM range.

The probability distributions for pulse height at the slow flow speed were shifted to the lower pulse heights at the $z = 8$ and 1 mm heights (Figure 10;

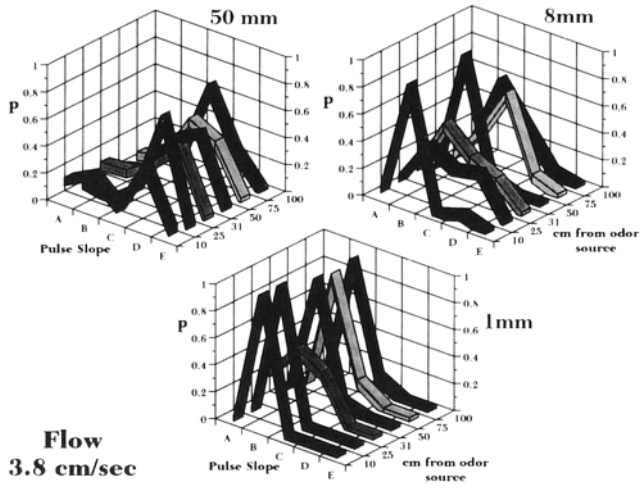

FIG. 8. Probability distributions of pulse slope taken in the slow flow (3.8 cm/sec) at the 50-, 8-, and 1-mm distances from the substrate. Probability values were calculated for five different bin sizes: (A) 0.1–0.9, (B) 1–9, (C) 10–90, (D) 100–900, (E) 1000–9000 μM/sec.

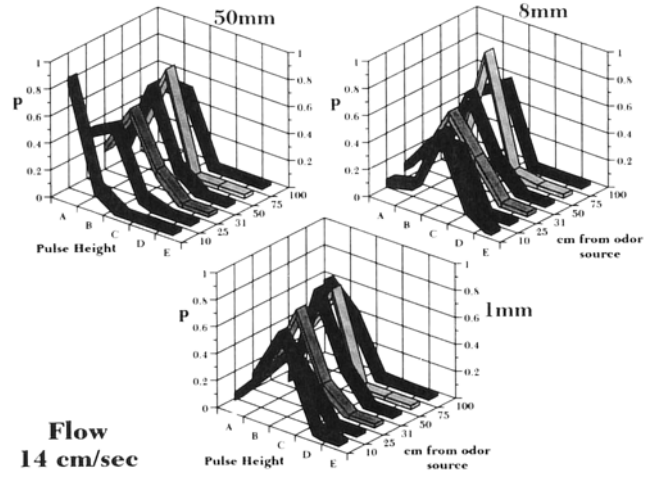

FIG. 9. Probability distributions of pulse height taken in the fast flow (14.4 cm/sec) at the 50-, 8-, and 1-mm distances from the substrates. Probability values were calculated for five different bin sizes: (A) 0.1–0.9, (B) 1–9, (C) 10–90, (D) 100–900, (E) 1000–9000 μM.

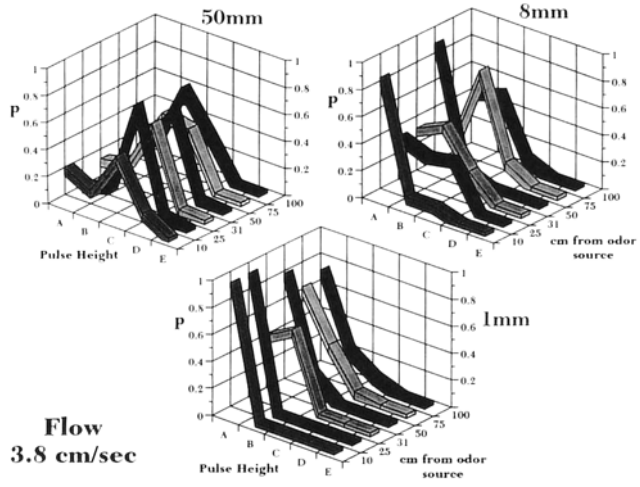

FIG. 10. Probability distributions of pulse height taken in the slow flow (3.8 cm/sec) at the 50-, 8-, and 1-mm distances from the substrates. Probability values were calculated for five different bin sizes: (A) 0.1–0.9, (B) 1–9, (C) 10–90, (D) 100–900, (E) 1000–9000 μM.

χ^2 analysis, $P < 0.001$). The majority of pulses had heights of 1–9 and 0.1–0.9 μM. The distributions for the $z = 50$ mm height (slow flow) were higher than those for the fast flow. Most of the pulses in this plane had heights of 10–90 μM.

DISCUSSION

These studies demonstrate three major consequences of different flow velocities in the distribution of chemicals. First, slower flow velocities allow the initial energy of the model clam excurrent to carry the dopamine farther into the water column before the carrier flow begins to transport the chemical downcurrent. As a result of this, the main axis of the plume will be located higher in the water column at slower flow velocities than at faster velocities (Figure 3). The main axis of an odor plume contains the most odor pulses and exhibits the largest fluctuations in concentration (compare Figure 4D with E and F; Figure 5). If animals are relying on the main axis of the plume as the major source of directional information during orientation, they must switch sampling and/or search strategies depending upon the flow conditions.

Secondly, there is a general relationship between plume microstructure and the magnitude and degree of penetrance of turbulence into the boundary layer.

Increased turbulence serves both to disperse odors and to distribute odor molecules into smaller and more discrete patches (Figure 6). Conversely, less turbulent flows result in greater average odor concentrations and more uniform distribution of odor further downstream from the source (Figure 6). Thus, within the main axis of the plume, average pulse height tended to show larger decreases at the high flow velocity (U_∞ = 14.4 cm/sec) than at the low flow velocity (U_∞ = 3.8 cm/sec). The plumes at the high flow velocity also exhibited a greater number of pulses and higher pulse slopes relative to the plume generated at the slower flow (Figure 5; Table 2). The effect of increasing u_* and Re_*, and thus boundary layer turbulence, is particularly clear when examining the distribution of pulse slopes and heights close to the substrate. At high flow, there is an increase in boundary layer turbulence close to the substrate which entrains odorants in eddy structures, transporting them closer to the bed (Table 1). This results in a greater number of discrete odor patches and therefore higher odor concentrations and faster onset slopes, within 1 mm of the bottom in faster flows.

Thirdly, increased turbulence generally resulted in a more predictable relationship between microscale plume structure and distance to the odor source. At 14.4 cm/sec plumes remained more patchy as they were advected downstream as compared to the slow flow velocity. These smaller and more discrete patches have higher concentration gradients between patch–nonpatch boundaries. Thus, in general, odor pulses have higher slope-to-height ratios at higher flow velocities. At the slower speed, odor patches take a longer time to move downcurrent. This increase in transport time is correlated with a smoothing of the concentration gradient at patch boundaries, resulting in decreased slope and slope-to-height values. Consequently, the distribution of pulse slopes, and to a lesser extent pulse heights, both tended to show a correlation with downstream distance from the odor source within all boundary layer regions at the higher flow speed. At the slow flow velocity, there was rarely any relationship between distance and plume microstructure. Due to the small degree of turbulent velocity fluctuations penetrating within the viscous sublayer, only pulse slopes within the log-deficit layer (i.e., $z \approx 50$ mm) show any correlation with distance to the odor source, and even here the relationship is not very robust. Thus, turbulence not only alters plume microstructure, but also the spatial information content potentially contained within regions of the boundary layer.

Turbulence creates large-scale eddies that transfer their energy to successively smaller eddies until the energy is dissipated as heat. This cascade of eddies (Kolmogorov scale) has a lower size limit below which molecular diffusion is the predominant transport mechanism. The spatial range of eddies is important because it is the interaction of the eddy size and plume size that contributes to the size and length of concentration fluctuations within the odor plume (Aylor, 1976; Aylor et al., 1976; Miksad and Kittredge, 1979). When

the plume or odor patch diameter is smaller than the smallest eddies, the eddies cause the plume/patch to meander as a whole. As the plume/patch expands to match the scale of eddies present, the plume/patch is broken into smaller more discrete patches of odor. The final stage of plume growth occurs when the plume/patch expands to sizes larger than the largest eddies. At this point, eddies begin to redistribute the odor within single patches and being to homogenize the odor between patches. Other factors, such as shear, may also play a role in smoothing patch–nonpatch boundaries (Tennekes and Lumley, 1972). Higher levels of turbulence will have smaller minimum eddy sizes and, thus, smaller and more discrete odor patches. Lower levels of turbulence will not have as many small eddies, and odor patches will tend to be larger and greater in concentration (Figures 4 and 6). The theoretical minimum eddy size (η) can be calculated from equation 3 (Tennekes and Lumley, 1972):

$$\eta = (\kappa Z \nu^3 / u_*^3)^{1/4} \tag{3}$$

This yields a minimum eddy size of 809 μm for 3.8 cm/sec and 323 μm for 14.4 cm/sec. The smaller theoretical minimum eddy size at the higher flow corresponds to the differences in the size and number of small odor patches seen between the two flow rates. The minimum concentration fluctuation (η_c) can then be determined from equation 4:

$$\eta_c = \eta (D/\nu)^{1/2} \tag{4}$$

where $D = 10^{-5}$ cm^2/sec (molecular diffusion coefficient for dopamine in seawater). Applying equation 4, we find that $\eta_c \approx 25$ μm for 3.8 cm/sec and 10 μm for 14.4 cm/sec.

Previous studies have begun to explore microscale properties of chemical distributions within odor plumes. These properties may contain information valuable to animals and potentially guide their orientation towards distant sources of odor (Murlis and Jones, 1981; Moore and Atema, 1988, 1991; Zimmer-Faust et al., 1988; Murlis et al., 1991). These studies have concentrated on pulse height, slope, length, and intermittency as possible sources of directional information. Our current study has found that the spatial distribution of these parameters is highly dependent upon the magnitude of turbulence present, which can be effected by hydrodynamic forces. Thus, any spatial information contained within the plume structure will vary in different flows. Pulse slope decreased consistently with downstream distance at 14.4 cm/sec, and could potentially give orienting animals directional information within a plume. Since the most consistent spatial information is located in different heights above the substrate in different hydrodynamic conditions, animals using these parameters for directional information during orientation may modify their sampling, searching, and/or locomotor strategies to fit the appropriate flow conditions. In future behavioral studies, it will have to be determined if changes in locomotor output represent

a switch in sampling strategies or the same strategy where different sensory input alters locomotory output.

Two recent behavioral studies on benthic decapod crustaceans illustrate these ideas (Scholz and Atema, 1991; Weissburg and Zimmer-Faust, 1993). These studies both used animals normally found within benthic boundary layer conditions and that rely heavily on chemical signals for a number of different behaviors. In both of these studies, there was a linkage between locomotory performance, chemical signal structure, and hydrodynamic conditions. These studies could indicate a changing of the overall search strategy of the animal. Conversely, the change in locomotor output may only represent the response to the different spatial distribution of chemicals rather than a change in search behavior. These changes in behavior have been shown for terrestrial insects (Baker et al., 1984) and are thought to be controlled by both the local wind direction and presence or absence of pheromone (David et. al., 1982).

Our results have demonstrated that in different turbulent flow regimes chemical signal distribution will change considerably near a solid surface. It has been demonstrated that the morphology of appendages influences the hydrodynamics of flow around and through them (Rubenstein and Koehl, 1977; Cheer and Koehl, 1987), and this in turn will affect the structure of chemical signals near receptor cells (Moore et al., 1991; Gleeson et al., 1993). Our present study shows that chemosensory appendages located within the viscous sublayer will receive slow and near-continuous signals, whereas appendages higher up in the flow will receive faster and more variable signals. The morphology of a chemosensory appendage and the sampling behavior may be correlated with the hydrodynamics of the flow regime in which they are normally located. Thus, the morphological influence on fluid flow and sampling behaviors of different appendages may be adapted to the different types of chemical signals in their relative microenvironments (Moore et al., 1991).

In summary, higher turbulence results in smaller and more discrete odor patches that arrive more frequently. These patches have very high slopes associated with the patch–nonpatch boundary. Conversely, lower turbulence results in large, slowly rising, high concentration odor patches that occur less frequently. Aquatic animals living in benthic environments therefore may have specific search strategies or locomotory patterns maximizing their ability to navigate towards a source of odor. These strategies would need to be sensitive to different hydrodynamic regimes and changing microscale stimulus conditions.

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FIELD EVALUATION OF SYNTHETIC COMPOUNDS MEDIATING OVIPOSITION IN *Culex* MOSQUITOES (Diptera: Culicidae)

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Abstract—3-Methylindole (skatole), a compound shown to be an oviposition attractant/stimulant for *Culex quinquefasciatus* in laboratory tests, was evaluated against natural populations of mosquitoes in the field. In experiments using paired black plastic tubs that contained water treated with an attractant solution containing a mixture of 3-methylindole, 4-methylphenol, 4-ethylphenol, phenol, and indole or that contained only tap water, the attractant-baited traps received significantly more *Cx. quinquefasciatus* egg rafts than did the untreated tap water. Gravid female traps that were baited with attractant solution collected significantly more *Cx. quinquefasciatus* females than did traps containing only tap water. There was no significant difference in number of *Cx. quinquefasciatus* collected in the traps baited with the attractant solution compared to the traps that contained water with 3-methylindole alone, indicating that 3-methylindole was solely responsible for the attraction. In replicated experiments conducted in experimental ponds, ponds that were treated with 3-methylindole received significantly more *Cx. quinquefasciatus*, *Cx. tarsalis*, and *Cx. stigmatosoma* egg rafts than did untreated ponds. Experimental ponds treated with 3-methylindole at two levels (0.12 and 0.6 mg/liter) were equally attractive to ovipositing *Cx. quinquefasciatus*. This is the first record of 3-methylindole showing attractancy/stimulation to ovipositing *Cx. stigmatosoma* and *Cx. tarsalis* under field or laboratory conditions.

Key Words—Diptera, Culicidae, *Culex quinquefasciatus*, *Culex stigmatosoma*, *Culex tarsalis* oviposition behavior, attractants, oviposition attractant, oviposition stimulant.

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INTRODUCTION

The selection of an oviposition site is an important part of the behavioral repertoire of mosquitoes, and the correct choice is crucial to the survival of progeny. The attractiveness of these sites to ovipositing mosquitoes is dependent upon a number of physical and chemical factors. Previous studies have shown that various chemical compounds may serve as oviposition attractants/stimulants for *Culex quinquefasciatus* Say, a mosquito species that breeds in a wide variety of polluted water habitats. Gjullin (1961) showed that water containing wood creosote was attractive to ovipositing *Cx. quinquefasciatus*, and Ikeshoji (1975) identified the attractants as phenols. Millar et al. (1992) isolated a five-component mixture (phenol, 4-methylphenol, 4-ethylphenol, indole, and 3-methylindole) from an infusion of Bermuda grass reported to be attractive/stimulatory to *Cx. quinquefasciatus* (Reiter 1983). The mixture was shown to be highly attractive/stimulatory to ovipositing *Cx. quinquefasciatus* in laboratory bioassays, with 3-methylindole (skatole) being the most stimulatory component (Millar et al., 1992). In the laboratory, 3-methylindole, a tryptophan degradation product, was shown to stimulate oviposition in gravid females at concentrations as low as 10 parts per trillion (Millar et al., 1992). The attractiveness of 3-methylindole to ovipositing *Culex* has been corroborated by Mordue et al. (1992). Further laboratory experiments showed that the chemical attractant mixture (containing all five constituents) acted synergistically with visual cues (Beehler et al. 1993). As none of these compounds had been tested in the field as attractants/stimulants for *Culex*, our objective in the work reported here was to assess the biological activity of these compounds to ovipositing *Culex* mosquitoes under field conditions.

METHODS AND MATERIALS

The first experiment to assess the effectiveness of an attractant solution in the field was conducted on the premises of the Orange County Vector Control District (OCVCD) in Garden Grove, California during August 1991. Three pairs of black plastic tubs (38 × 28 × 19 cm) were placed along a transect for a total of four nights. Each pair of tubs was separated from the others by approximately 25 m, and the tubs in each pair were placed 3 m apart. Each pair contained either 8 liters of tap water or the attractant solution made up of 8 liters of tap water containing a mixture of 4-methylphenol (100 mg), 4-ethylphenol (8 mg), phenol (19 mg), indole (1 mg), and 3-methylindole (6 mg) in 2 ml ethanol. The chemical components of this solution were present in the same ratio as those isolated by Millar et al. (1992) from Bermuda grass infusions. For four consecutive nights, the egg rafts deposited in the tubs were counted and removed. Subsamples of the rafts were collected, then returned to the lab-

oratory and reared individually to the L4 stage when they were identified to species. The experiment was repeated three times. Egg raft counts were transformed to square roots [$\sqrt{x}$, an appropriate transformation when the mean is proportional to the variance (Snedecor and Cochran, 1980)], and comparisons of the number of rafts recovered from treated tubs to the number recovered from controls were conducted using paired t tests.

The second experiment was also conducted at the OCVCD, using three pairs of gravid female traps (Reiter, 1983) baited with tap water alone or water and the attractant solution. The gravid female traps consisted of black plastic tubs on which were mounted a plastic tube containing a fan that pulled up mosquitoes as they approached and forced ovipositing mosquitoes into a collecting bag. Traps were set along a transect as described above. In each pair, one trap contained the attractant solution and the other contained only tap water. The traps were placed 2 hr before sunset and collected mosquitoes all night. Adult mosquitoes were taken from the traps the following morning and frozen until identification. Traps were set one night each week from September 29 to November 4, 1992. The numbers of adult *Cx. quinquefasciatus* collected in treatment traps were transformed to square roots ($\sqrt{x}$) and were compared to transformed controls using multiple-regression methods (Box et al., 1978), which accounted for seasonality.

The third experiment also used gravid female traps to compare traps baited with the attractant solution to traps that contained 3-methylindole alone to determine if 3-methylindole alone was the primary attractive component in the solution. Traps were placed one night each week along a transect as described above from March 8 to April 13, 1993. One trap in the pair contained the attractant solution at the dose used previously, and the other trap contained tap water with 3-methylindole alone at the same concentration as found in the attractant solution. The numbers of female *Cx. quinquefasciatus* collected in the traps containing the solution were transformed to square roots ($\sqrt{x}$) and compared to the numbers of females collected in the 3-methylindole treated traps using multiple-regression methods.

In order to assess the attractiveness of 3-methylindole under more natural conditions, experiments were conducted at the University of California-Riverside Aquatic and Vector Control Research Facilities in Riverside and in Oasis, California, using experimental ponds breeding natural populations of *Culex*. On October 30, 1992, six wooden-sided experimental ponds (27 m^2) were flooded in Riverside to a mean depth of 35 cm using irrigation water. The water level was maintained using a float-value system. Three days after flooding, a transect (5.5 m × 14 cm) along the south and west rail of each pond was examined for the presence of *Culex* egg rafts indicating oviposition activity prior to treatment. Three randomly selected ponds were then treated with 3-methylindole (5 g/pond or 0.6 mg/liter pond water) in 100 ml of ethanol. Control ponds were treated

with an equal volume of ethanol alone. Treatments were made 1 hr before sunset using a randomized block design. The following morning the transect was again examined for the presence of *Culex* egg rafts. The number of egg rafts per transect was recorded and transformed to a square root ($\sqrt{x}$). Treated and untreated ponds were compared using ANOVA. Subsamples of egg rafts were returned to the laboratory and reared individually for species identification. The experiment was repeated using six ponds on November 11, 1992.

These field experiments were repeated in Oasis, California (a site 120 km away from Riverside), using 30-m^2 ponds that contained vegetated sides. Eight ponds were flooded with well water on March 26, 1993, and levels were maintained at a mean of 35 cm using float valves. Three days after flooding, transects were checked for the presence of *Culex* egg rafts. Four ponds were then treated at 11:00 AM with 3-methylindole (5 g/pond or 0.48 mg/liter) as above using a randomized block design and four ponds were treated with the solvent only. The following morning the transects were checked for the presence of *Culex* egg rafts. On April 2, 1993, this experiment was repeated using 3 pairs of experimental ponds.

The final two experiments were conducted to determine if lower doses of 3-methylindole would be attractive to ovipositing *Culex* in the field. On May 11, 1993, 12 experimental ponds in Riverside were flooded as described previously. Three days later, ponds were treated 0.5 h before sunset with 5 g (0.6 mg/liter) or 1 g (0.12 mg/liter) 3-methylindole in 100 ml ethanol (four ponds each). Four ponds were treated only with ethanol. Treatments were assigned using a randomized block design. The following morning, egg rafts from along a transect were counted as described previously, transformed to natural logs (ln + 1), and treatments were compared using ANOVA. On May 18, 1993, the experiment was repeated using a similar design. The randomized block design accounted for four recently repaired ponds that had one or more of the redwood sideboards replaced. Subsamples of egg rafts were again reared for identification.

RESULTS

In the first experiment testing the field efficacy of the five-component attractant solution, the tubs that contained the solution received significantly more egg rafts than did the tubs containing only tap water ($P < 0.05$; paired t, Table 1). The tubs containing the attractant solution received egg raft cumulative means of 5.3–8.0/trap, while tubs containing only tap water collected cumulative means of 0.6–2.3./trap for each four night period. Larvae reared from egg rafts sampled were identified as *Cx. quinquefasciatus*.

A similar trend was shown in collecting female *Culex* in gravid female

TABLE 1. EVALUATION OF 5-CHEMICAL SOLUTION FOR ATTRACTANCY/STIMULATION TO OVIPOSITING *Culex quinquefasciatus* IN THE FIELD

Date	Cummulative mean egg rafts/tub (± SE)[a]	
	Attractant solution	Control
August 5–9[b]	6.0 ± 0.3	0.7 ± 0.3[c]
August 16–20	8.0 ± 1.5	2.3 ± 0.3[d]
August 23–27	5.3 ± 0.7	0.6 ± 0.2[c]

[a]Tests were conducted in black plastic tubs (38 × 28 × 19 cm) containing 8 liters of tap water with or without the attractant solution. Attractant solution is described in Methods and Materials.
[b]The tubs remained in the field for four nights. Each tube in a pair was separated by 3 m and each pair of tubs was separated by 25 m.
[c]$P < 0.05$, t test.
[d]$P < 0.025$, t test.

traps. Gravid female traps containing attractant solution collected significantly more *Cx. quinquefasciatus* than did traps containing only tap water (Figure 1a). Multiple-regression analysis of the number of *Cx. quinquefasciatus* females captured over the six collecting dates in treated and untreated traps yielded the following model: $y = 4.4 + 2.6x_1 - 0.27x_2 (F = 16.6; df = 2.34; P < 0.001)$, where y is the square root of number of *Cx. quinquefasciatus* females, x_1 is the effect of the attractant mixture, and x_2 is the temporal effect. The R^2 of the regression model was 0.49. The positive coefficient (x_1) indicates that the attractant solution was significantly more attractive to *Cx. quinquefasciatus* than tap water in these field experiments. The negative coefficient (x_2) reflects the seasonal decrease in *Cx. quinquefasciatus* populations that was occurring while the study was being conducted. Both treated and untreated traps also collected some *Culex tarsalis* Coquillett females (< 1% of the total) and several midges (*Chironomus*).

In the experiment to determine if 3-methylindole was the primary attractive component in the attractant solution, multiple regression analysis showed no significant differences in attractancy between the five-component attractant solution and the solution containing tap water with 3-methylindole alone (Figure 1b). There was also no significant seasonal effect ($F = 0.24$; $df = 2.34$; $P > 0.15$).

In the Riverside and Oasis experiments (two at each site) to determine the attractiveness of 3-methylindole in seminatural ponds, rather than tubs, experimental ponds treated with 3-methylindole received significantly more *Culex* egg rafts than untreated ponds. Since some oviposition may have occurred before the start of each experiment, precounts were taken before treatment. These

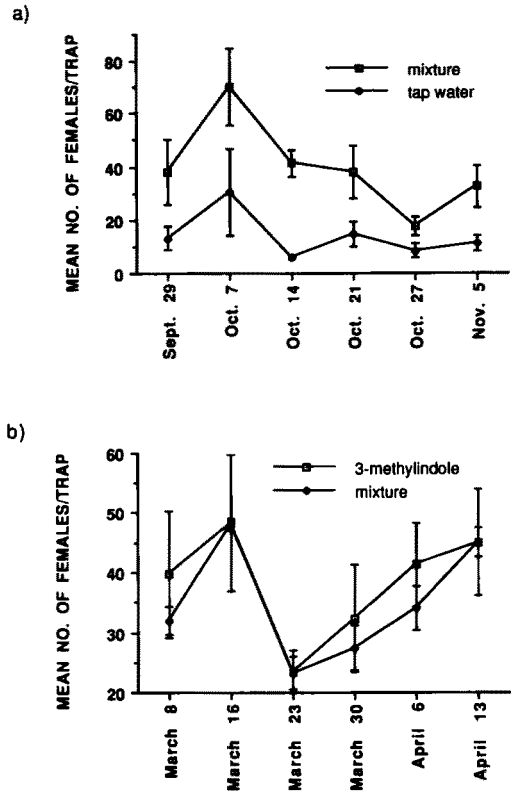

FIG. 1. (a) Mean number of *Cx. quinquefasciatus* females captured in gravid female traps containing either water treated with an oviposition attractant mixture containing 3-methylindole, phenol, 4-methylphenol, 4-ethylphenol, and indole, or trap water. (b) Mean number of *Cx. quinquefasciatus* females captured in traps containing the attractant mixture or 3-methylindole alone.

counts showed no egg rafts present along the transects sampled in the ponds before treatment in either Riverside experiment. Only a few *Culex* egg rafts (<5 total) were found in the pretreatment counts in both Oasis experiments. The number of rafts found in the precount along each transect before treatment was subtracted from the number of rafts counted postreatment to correct for egg rafts that were deposited before treatment. In both experiments in Riverside, 3-methylindole-treated ponds received significantly more egg rafts per transect than did untreated controls (Table 2). Approximately nine times as many *Culex* egg rafts were taken from transects in ponds treated with 3-methylindole. Sub-

samples of egg rafts collected were reared individually in the laboratory to the L4 stage and identified to be a mixture of *Cx. quinquefasciatus* (47%) and *Culex stigmatosoma* Dyar (53%).

In the two Oasis experiments, ponds treated with 3-methylindole also received significantly more *Culex* egg rafts than did untreated ponds (Table 2). Subsamples of egg rafts collected were reared individually to the L4 stage and were all identified to be *Cx. tarsalis*.

Two further experiments were conducted in Riverside to determine the effects of 3-methylindole dose on attractancy (Table 3). In both experiments there was no significant difference in attractancy between ponds treated with 0.12 mg/liter (1 g/pond) or 0.6 mg/liter (5 g/pond) 3-methylindole to ovipositing

TABLE 2. EFFECT OF 3-METHYLINDOLE AS AN ATTRACTANT FOR *Culex* OVIPOSITION BEHAVIOR IN SEMINATURAL PONDS

Location	Mean No. of rafts/transect (± SE)	
	3-Methylindole	Control
Riverside[a]	11.0 ± 3.5	1.3 ± 0.8[c]
Riverside[a]	7.3 ± 0.3	1.0 ± 0.3[d]
Oasis[b]	11.3 ± 1.5	0.5 ± 0.3[e]
Oasis[b]	5.0 ± 0.7	0.3 ± 0.2[d]

[a] Subsamples of larvae reared from egg rafts identified to be *Cx. quinquefasciatus* and *Cx. stigmatosoma*. 3-Methylindole concentration 0.6 mg/liter.
[b] Subsamples of egg rafts identified to be *Cx. tarsalis*. 3-Methylindole concentration 0.48 mg/liter.
[c] $P < 0.05$, ANOVA.
[d] $P < 0.01$, ANOVA.
[e] $P < 0.001$, ANOVA.

TABLE 3. COMPARSION OF TWO CONCENTRATIONS OF 3-METHYLINDOLE ON OVIPOSITION ACTIVITY OF *Cx. quinquefasciatus* IN RIVERSIDE EXPERIMENTAL PONDS

Date	Mean No. of rafts/transect (± SE)[a]		
	Control	3-Methylindole	
		1.5 mg/liter	6 mg/liter
May 14	0.3 ± 0.3a	8.0 ± 1.2b	13.3 ± 4.2b
May 18	5.3 ± 4.3a	13.7 ± 2.1ab	30.3 ± 11.2b

[a] Means in the same row followed by a different letter are significantly different (ANOVA-protected LSD, $P < 0.05$).

Culex (ANOVA-protected LSD, $P > 0.05$). The relationship between 3-methylindole and *Culex* oviposition was confounded by a strong block effect in the May 18 experiment (Table 4). More egg rafts were collected from all treatments in the block that contained the new redwood boards than were collected from all treatments in other blocks. Larvae reared from egg rafts sampled were all identified to be *Cx. quinquefasciatus*.

DISCUSSION

In some of the first studies of chemical cues influencing mosquito oviposition, Crumb (1924) reported that water-filled jars treated with 3-methylindole (at an unspecified concentration) did not receive *Cx. pipiens* Linn. egg rafts in field tests. In contrast, our experiments clearly show that 3-methylindole is an attractant for several *Culex* species and may also act as an oviposition stimulant. The first experiment, in which the five-component attractant solution was tested in black plastic tubs, demonstrated that the test solution was either attractive or stimulatory, or both, to *Cx. quinquefasciatus* under field conditions. Using this experimental design, it was not possible to distinguish the exact mechanism of action of the stimulatory material. Furthermore, in this experiment, it was necessary to leave the traps in place for four nights in order to collect a sufficient number of egg rafts for statistical analysis.

On the other hand, the second experiment, testing the effect of the five-component solution in gravid female traps, clearly demonstrated that the solution is an attractant, as many more mosquitoes were caught in the baited versus the control traps. Because the trap collects responding mosquitoes by suction, before they are able to contact and sample the water in the trap, the compounds must be perceived by olfaction in order to have obtained a response.

It was also noteworthy that the gravid female traps captured considerably

TABLE 4. ANOVA TABLE (ln + 1 transformed data) FOR EFFECT OF 3-METHYLINDOLE AND NEW REDWOOD SIDING ON OVIPOSITION OF *Cx. quinquefasciatus* IN RIVERSIDE EXPERIMENTAL PONDS (MAY 18, 1993)

Source	*df*	Mean squares	*F*	*P*
3-Methylindole	2	4.89	20.3	0.004
New redwood siding[a]	1	2.88	12.0	0.018
Interaction[b]	2	1.101	4.6	0.074
Error	6	0.241		

[a] Side boards replaced by new redwood planks before flooding.
[b] Interaction between 3-methylindole and redwood siding.

greater numbers of *Cx. quinquefasciatus* than the oviposition tubs received egg rafts. These comparatively large trap captures also occurred at a time when the *Cx. quinquefasciatus* densities (based on light trap catches by the OCVCD) were lower than when the oviposition tubs had been deployed. This difference can be explained in several ways. First, the oviposition tubs do not measure the number of mosquitoes that were attracted to the tubs; instead, they measure the number of mosquitoes that actually oviposited. Thus, mosquitoes may have been attracted to the tub in relatively large numbers, but relatively few of them may have actually oviposited. The gravid female traps, on the other hand, measure and catch a larger proportion of the *Culex* females that are attracted to the trap, because as the mosquitoes approach, they are sucked into the trap by the fan.

Second, the fan in the gravid female trap disperses a far larger plume of attractant than is dispersed passively by diffusion from the attractant mixture in the tubs. Consequently, many more mosquitoes may intercept volatile stimuli from this larger plume and be attracted, making for a more efficient trapping method than the oviposition tubs.

In the third experiment, gravid female traps were used to compare the attractiveness of 3-methylindole alone versus the 3-methylindole in combination with the four other components originally identified from an active fraction of a Bermuda grass infusion extract. As was found in laboratory studies (Millar et al., 1992), 3-methylindole was determined to be the key attractive component, as there was no difference in the attractiveness of the two solutions.

In further experiments, ponds treated with 3-methylindole attracted not only *Cx. quinquefasciatus*, but also *Cx. stigmatosoma* and *Cx. tarsalis*. These results are significant for several reasons. First, to our knowledge this is the first pure compound (as opposed to a complex mixture such as an organic infusion) to act as an oviposition attractant under field conditions for the latter two species. Gjullin (1961) showed that beechwood creosote and *N*-butyl-*N*-ethyl-*o*-veratrylamine were attractive to ovipositing *Cx. quinquefasciatus* in the laboratory. In later studies, Gjullin and Johnsen (1965) showed seven compounds to be attractive to ovipositing *Cx. quinquefasciatus*, but these compounds had little effect on the oviposition behavior of *Cx. tarsalis*. None of these compounds has ever been tested under field conditions for attractancy to various *Culex* species.

Second, these results represent the first demonstration of a habitat-derived semiochemical that may be useful in mosquito monitoring programs. Such a compound could be a valuable tool in arbovirus surveillance programs, because it could be formulated and deployed in standardized and reproducible doses. On the other hand, organic infusions are impossible to standardize due to rapid changes in microbial and chemical content over time. Furthermore, infusions made with slightly different ingredients or under slightly different conditions differ markedly from each other (Beehler, unpublished data) making them difficult to standardize.

The data from the dose–response experiment in Riverside were confounded by a strong block effect. This effect was shown to be caused by some recent repairs to several of the ponds where one or more of the redwood sideboards had been replaced. These newly repaired ponds received significantly more oviposition than did the ponds containing older planks. It was observed during the course of the experiment that the water in the ponds with the new planks was darker than the water in the other ponds. Either the darkened color or chemicals that leeched into the water from the new boards may have acted as additional cues to the ovipositing mosquitoes. It is known that color acts synergistically with chemicals in influencing *Culex* oviposition (Beehler et al., 1993; Isoe et al., 1993). It has also been shown that infusions of wood (logs) are attractive to gravid *Culex* mosquitoes (Gjullin et al., 1965). Despite the strong block effect, 3-methylindole was shown to be attractive and/or stimulatory.

In summary, this study has shown that 3-methylindole mediates *Culex* oviposition under field conditions, with at least part of its mode of action being long-range attraction. The demonstrated field activity for several mosquito species suggests that 3-methylindole may be a useful tool for mosquito surveillance programs and may eventually be used in control programs that target specific oviposition attractant-treated habitats for treatment with insecticidal compounds.

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IDENTIFICATION OF THE ANTIBIOTIC PHOMALACTONE FROM THE ENTOMOPATHOGENIC FUNGUS *Hirsutella thompsonii* VAR. *synnematosa*[1]

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Abstract—Dichloromethane extracts of culture broth from three strains of the entomopathogenic fungus *Hirsutella thompsonii* var. *synnematosa* were toxic to two species of tephritid fruit fly and inhibited conidial germination in vitro in several other entomopathogenic fungi including *Beauveria bassiana*, *Tolypocladium* spp., and *Metarhizium anisopliae*. A major metabolite, toxic to apple maggot, *Rhagoletis pomonella*, and inhibitory to conidial germination in *B. bassiana*, was isolated and identified as the antibiotic (+)-phomalactone, 6-(1-propenyl)-5,-6-dihydro-5-hydroxypyran-2-one. This is the first biologically active compound of low molecular weight isolated from the genus *Hirsutella*.

Key Words—*Beauveria*, *Ceratitis capitata*, Medfly, conidial germination inhibitor, entomopathogenic fungus, fungal toxin, *Hirsutella*, *Metarhizium*, mycotoxin, phomalactone, *Rhagoletis pomonella*, Diptera, Tephritidae.

INTRODUCTION

Fungi of the genus *Hirsutella* infect a variety of arthropods and nematodes (Minter and Brady, 1980) including several important agricultural pests, most notably the citrus rust mite (McCoy and Couch, 1978; Samson et al., 1980). Consequently, these fungi have been viewed with interest as potential biological agents (McCoy and Couch, 1978; Minter et al., 1983). Toxigenic strains are

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particularly attractive to those interested in exploiting these fungi for biological control because they offer the potential for rapidly killing their hosts. Although no toxic metabolites have yet been identified from *Hirsutella*, several reports of a toxigenic *Hirsutella* strain have surfaced recently. Vey et al. (1993) reported insecticidal effects of crude broth filtrates from *Hirsutella thompsonii* var. *thompsonii*. Mazet et al. (1992) reported the isolation of two proteins, hirsutellin A and B, from *Hirsutella thompsonii* var. *thompsonii*, that are toxic to *Galleria mellonella* larvae and cytotoxic to *Plutella xylostella* cells in vitro. McCoy et al. (1992) reported toxicity of *Hirsutella* extracts to aphids and mites and of hirsutellin A to mosquitoes and mites.

In an ongoing screen for insecticidal metabolites of entomopathogenic fungi, we discovered that dichloromethane extracts of culture broth from several strains of *Hirsutella thompsonii* var. *synnematosa* were toxic to apple maggot flies, *Rhagoletis pomonella* (Wash). To evaluate the potential for toxic interactions among beneficial entomopathogenic fungi, we routinely check insecticidally active extracts for antibiotic activity against other entomopathogenic species. By this route we discovered that insecticidal extracts of *H. thompsonii* var. *synnematosa* also inhibited conidial germination in several unrelated entomopathogenic fungal species. Herein we report the isolation and identification of (+)-phomalactone, 6-(1-propenyl)-5,-6-dihydro-5-hydroxypyran-2-one, and document its toxicity to apple maggot and its inhibition of conidial germination in *Beauveria bassiana*.

METHODS AND MATERIALS

Growth of Fungi. Three strains of *H. thompsonii* var. *synnematosa* deposited in the USDA-ARS Entomopathogenic Fungi Collection (ARSEF), were investigated for secondary metabolite production. ARSEF #256 (=CBS 450.78) was originally isolated from an eriophyid mite pest of coconut, *Eriophyes guerreronis* Keifer, from Jamaica, ARSEF #257 (=CBS 451.78) was isolated from *E. guerreronis* from Zimbabwe (Samson et al., 1980), and ARSEF #3323 was isolated from a tenuipalpid mite pest of pineapple, *Dolichotetranychus floridanus* Banks, from Costa Rica.

Cryopreserved mycelial material was microcultured on slants or plates of Sabouraud's dextrose agar supplemented with 1% yeast extract (SDAY). Mycelial plugs or conidia from these microcultures were used to inoculate 100-ml batches of a liquid medium composed of dextrose (20 mg/ml), yeast extract (5 mg/ml), bactopeptone (0.5 mg/ml), KH_2PO_4 (1.5 mg/ml), $MgSO_4$ (0.5 mg/ml), and $CaCl_2$ (0.01 mg/ml) (McCoy et al., 1972). These were grown on an orbital shaker at 22 $\pm$ 1°C (160 rpm; 2.5 cm throw) exposed to natural light from a window for 7–14 days and were then used to inoculate 1-liter batches of the

same medium in 4-liter Fernbach flasks. These were then grown under the same conditions as the primary liquid culture until harvesting (>21 days). Fungi usually grew as small pellets.

Extraction. The broth was filtered through four layers of cheesecloth and the filtrate extracted with three portions of dichloromethane. We describe details of the extraction procedure elsewhere (Krasnoff and Gupta, 1991). The organic layer was concentrated under vacuum to a reddish-brown oily residue (yield for 7.2 l ARSEF #256: 193 mg; 2 l ARSEF #257: 125 mg; 3.8 l ARSEF #3323: 252 mg).

Isolation and Identification of Phomalactone. A portion of the crude extract (ARSEF #256: 193 mg; ARSEF #257: 111 mg; ARSEF #3323: 240 mg) was dissolved in dichloromethane, applied to a silica gel column (35 g Baker 40-μm silica) and flash-chromatographed by elution with 100% dichloromethane (800 ml) and then 750 ml each of 0.2%, 1%, 2%, 5%, 20%, and 50% methanol in dichloromethane.

A portion of the active fraction from the silica gel column (eluted with dichloromethane: methanol 99:1) (ARSEF #256: 94.2 mg; ARSEF #257: 75.2 mg; ARSEF #3323: 186.4 mg) was dissolved in methanol and preparatively chromatographed on one of two HPLC systems. The first system [Phenomenex reverse-phase C-18 column: 250 × 10 mm, 5μm IB-SIL; mobile phase: acetonitrile: water (25:75); flow rate: 3.3 ml/min; detection by UV absorption at λ = 225 nm] separated the major component (retention time: 9.3 min) from several minor contaminants, with one of these (retention time: 10.5 min) eluting on the tail of the major component. Use of this system required peak-shaving to purify the major component. The second system [Varian Micro Pak SI-10 column, 500 × 8 mm; mobile phase: hexane–isopropanol (85:15); flow rate: 3.3 ml/min; detection by UV absorption at λ = 228 nm] afforded baseline separation of the major component (retention time: 16.1 min) from a contaminant (retention time: 15.0 min) and an accurate estimate of the proportion of the total integrated peak area of the chromatogram accounted for by the major component (82%).

Spectrometric analyses were performed on the major component from the active silica gel fraction purified by HPLC. High-resolution CI mass spectra were obtained from the Mass Spectrometry Laboratory Illinois Mass Spectral facility on a 70-VSE mass spectrometer. Low-resolution GC-MS analyses in the EI mode were performed on a Hewlett-Packard 5890 GC linked to a 5970 mass selective detector. Samples were injected in the splitless mode on a 30-m × 0.22-mm DB-5 (J & W Scientific) GC column held at an initial oven temperature of 60°C for 4 min and temperature programmed to 270°C at 10°/min. Helium was used as the carrier gas at a linear flow rate of 1 ml/min.

IR spectra were obtained on an HP5890 GC linked to a HP 5965A FTIR.

GC conditions were identical to those described above except for the column diameter (0.32 mm).

Optical rotation was measured on a Perkin-Elmer 241 polarimeter at $T = 25°C$ using a 10-cm cell. UV spectra were obtained on a Beckmann DU-600 instrument.

NMR spectra were obtained in deuterated chloroform, and residual peaks from undeuterated solvent were used as internal reference. Assignments were confirmed using data from different 1-D and 2-D experiments. ^1H-^1H COSY and DEPT experiments were carried out on a Varian XL-400 NMR spectrometer (400 MHz resolution for proton and 100 MHz for carbon). Heteronuclear multiple quantum coherence (HMQC) and heteronuclear multiple bond correlation (HMBC) experiments were performed on a Varian Unity-500 system (500 MHz for proton and 125 MHz for carbon).

Insect Bioassay. Apple maggot pupae were obtained from a laboratory colony maintained at the New York State Agricultural Experiment Station in Geneva, New York. Pupae were kept at 20 ± 1°C under a 16:8 hr light–dark photoperiod. Upon eclosion adults were segregated to provide insects of known age. Adults were provided with water, a vitamin mixture (U.S. Biochemical Corp. #23431), sucrose crystals, and a 4:1 casein–salt mixture (U.S. Biochemical Corp. Salt Mixture No. 2). Mediterranean fruit flies, *Ceratitis capitata* (Weidemann), were obtained from a colony maintained at the USDA Tropical Fruit and Vegetable Research Laboratory in Honolulu, Hawaii.

Test material was formulated in a stock solution of 4.75% ethanol, 10% sucrose, and 0.25% L-leucine in distilled water with 5 drops/100 ml of yellow food coloring (McCormick yellow) added. Controls consisted of the stock solution alone.

Flies were held under colony conditions but deprived of food and water for a fixed period before the test was administered (24 hr for apple maggot flies, 16 hr for Mediterranean fruit fly). Just before being exposed to the formulated extracts, flies were confined in groups of five (for preliminary screening) or 10 (for dose–response tests) in small tubular cages (10 cm × 28 mm ID) fabricated from 50 ml polyethylene centrifuge tubes with the pointed end cut off and covered with nylon mesh. The Styrofoam racks the tubes were packaged in were modified to hold the tubes for the assay. Twelve wells of the 25 in each rack were lined with paraffin. A 200-μl Eppendorf-type plastic pipet tip (total volume ≈ 350 μl) was heat-sealed at the small opening and was positioned in the center of each paraffin-lined well to serve as a reservoir for the formulated extract. A narrow triangular strip of filter paper (Whatman No. 1) was cut to fit from ≈5 mm above the top to the bottom of the sealed pipet tip. The formulated extract (350 μl) was then pipetted into a sealed tip with the filter paper triangle in place. The cut-off tip of a cotton swab was then placed on top of and down into the reservoir to form an absorbent plug that made contact with the filter paper. This

prevented flies from getting stuck in the tube and served as a landing and feeding surface. Cages containing flies were inverted in the racks with the threaded end down. Four cages were set up for each treatment. Cages were held in an environmental chamber under colony conditions. Cotton plugs soaked in a 10% sucrose solution were placed on the mesh tops of the cases 16 hr after the initiation of a test to prevent mortality due to starvation or dehydration. The plugs were replenished with the solution periodically to maintain a constant supply of sugar and water for the duration of a test. Mortality was assessed every 24 hr. Flies unable to right themselves were scored as dead.

Conidial Inhibition Bioassay. Conidia were harvested from SDAY cultures of strains taken from the ARSEF collection by suspending in sterile 0.1% Tween or sterile deionized water. Suspensions were adjusted in concentration to ca. 1×10^6 spores/ml. Extracts were dissolved in acetone and applied to the surface of a 35-mm SDAY plate. For preliminary tests and bioassays of fractions, a 100-μl aliquot was applied to the surface of the agar and allowed to spread. For dose–response studies of pure material, a 12.5-mm-diameter $\times$ 10-mm-long polypropylene cylinder was set into the agar as it cooled. This formed a smaller enclosed area that could be completely and evenly covered by a 30-μl aliquot of solution. In either case the solvent was allowed to evaporate for 30 min. At that time a 5-μl aliquot of a $\approx 1 \times 10^6$ spores/ml suspension ($\approx$5000 spores) was applied to the center of the plate and allowed to dry for 5 mins. The plate was then covered, sealed with Parafilm, and held in an environmental chamber under a 14:10 hr light–dark photoperiod at a fixed temperature (20, 22, or 27°C).

An assay was scored when 90–100% of the untreated control conidia had germinated. Percent germination was scored for a random sample of 100 spores using an inverted compound microscope at 150×. Percent inhibition was computed as 100 − % germination. Dose–response data for inhibition were corrected using Abbott's formula (Abbott, 1925), and the I_{50} (the dosage inducing 50% inhibition of germination) was estimated using the probit model (Finney, 1971).

RESULTS

Three isolates of *Hirsutella thompsonii* var. *synnematosa* (ARSEF #256, #257, and #3323) produced phomalactone (Figure 1) as a major component of the dichloromethane extract of culture broth. During screening for insecticidal activity of fungal metabolites, these extracts showed toxicity to *Rhagoletis pomonella* (apple maggot) and *Ceratitis capitata* (Mediterranean fruit fly) adults at concentrations of 1000 ppm or 2000 ppm (45–75% mortality at 48 hr). Testing for antifungal activity showed that these extracts inhibited conidial germination in isolates of several entomopathogenic fungal species, *Beauveria bassiana*

(ARSEF #152), *Metarhizium anisopliae* (ARSEF #1095), *Tolypocladium geodes* (ARSEF #2684), and *T. cylindrosporum* (ARSEF #962) (Table 1). No self-inhibition was detected (Table 1).

Bioassay-guided fractionation of the crude extract by flash chromatography on silica gel followed by preparative HPLC afforded a single compound, active as a conidial germination inhibitor of *Beauveria bassiana*, that was identified

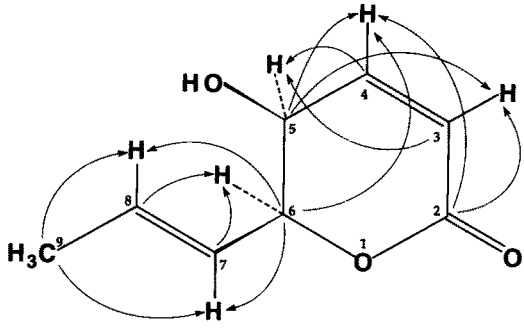

FIG. 1. Structure of (+)-phomalactone showing heteronuclear multiple bond correlation (HMBC) connectivities.

TABLE 1. INHIBITION IN VITRO OF CONIDIAL GERMINATION IN ENTOMOPATHOGENIC FUNGI BY CRUDE EXTRACTS OF THREE STRAINS OF *Hirsutella thompsonii* VAR. *synnematosa* TESTED AT 5 MG/ML[a]

		Extract tested			
ARSEF	Target species	256	3323	257	Thymol
152	*Beauveria bassiana*	+	+	+	+
962	*Tolypocladium cylindrosporum*		+		
2684	*Tolypocladium geodes*	+	+		+
		42 hr	42 hr		42 hr
		20°C	20°C		20°C
1095	*Metarhizium anisopliae*	+			+
		34 hr			34 hr
3323	*Hirsutella thompsonii*		−		
			20°C		

[a]Thymol (5 mg/ml acetone) served as a positive control. + indicates 100% inhibition of germination at a time when 100% germination was observed in the control (acetone alone); − indicates 0% inhibition. Blank cells in table indicate combinations not tested. Unless otherwise noted test plates were held at 22°C and germination was scored 24 h after inoculation.

as (+)-phomalactone by analysis of spectral data and comparison of these with published data for (+)-phomalactone. Phomalactone: EI-MS: *m/e* (% base peak) 85 (5), 84 (100), 71 (50), 56 (31), 55 (61), 53 (7), 43 (7), 41 (14), 40 (4), 39 (23); GC-IR: 3624, 2931, 2364, 1766, 1374, 1238, 1075, 1036, 968, 825 cm^{-1}; UV: λ_{max}^{MeOH} 204 nm (ϵ 9801); optical rotation: $[\alpha]_D^{25} = +175.3°$ (*c* 0.42 $CHCl_3$). The high-resolution mass spectrum (CI, methane) showed a protonated molecular ion at *m/z* 155.0715 that corresponded to an elemental composition of $C_8H_{11}O_3$. In a proton COSY experiment, a single contiguous system of spin connectivities was observed. The methyl double-doublet at 1.77 ppm (H-9) showed connectivities to olefinic multiplets at 5.95 ppm (H-8) and 5.73 ppm (H-7, long range). The signal at 5.73 ppm (H-7) showed direct connectivity to the double-doublet at 4.77 ppm (H-6). The olefinic double-doublet at 6.96 ppm (H-4) showed connectivities to the proton at 4.15 ppm (H-5) and olefinic doublet at 6.05 ppm (H-3). The carbon NMR spectrum showed the presence of eight carbons that included one quaternary carbonyl, six methines, and one methyl carbon. All the protonated carbons could be correlated to proton resonances by an HMQC experiment. The assigned structure was further substantiated by an HMBC experiment in which several long-range carbon-proton connectivities were observed, as shown in Figure 1. The following are the NMR assignments for phomalactone: ^1H ($CDCl_3$): 1.77 (3H, dd, J = 6.1, 1.5 Hz $C\underline{H}_3$-9), 2.75 (1H, br s,O<u>H</u>), 4.15 (1H, dd, J = 5.4, 2.9 Hz, H-5), 4.77 (1H, dd, J = 7.1, 2.9 Hz, H-6), 5.73 (1H, m, J = 15.5, 7.1, 1.5 Hz, H-7), 5.95 (1H, m, H-8), 6.05 (1H, d, J = 9.8 Hz, H-3), and 6.96 (1H, dd, J = 9.8, 5.4 Hz, H-4). ^{13}C ($CDCl_3$): 17.95 (C-9), 63.02 (C-5), 81.45 (C-6), 122.46 (C-3), 124.01 (C-7), 132.85 (C-8), 144.96 (C-4), and 163.74 (C-2).

HPLC-purified phomalactone showed dose-dependent insecticidal activity against apple maggot flies (Table 2) as well as inhibitory activity against conidial germination in *B. bassiana* (ARSEF #152; I_{50} = 468 μg/ml; 95% confidence limits 429–580 μg/ml) (Figure 2). It also showed inhibitory activity against

TABLE 2. PERCENT MORTALITY AT 24-HOUR INTERVALS AFTER TREATMENT OF APPLE MAGGOT FLIES WITH (+)-PHOMALACTONE.

Dosage, μg/ml (*N*)	Time				
	24 hr	48 hr	72 hr	96 hr	120 hr
0 (40)	0.0	0.0	0.0	0.0	2.5
500 (41)	2.4	9.8	12.2	14.6	24.4
1000 (43)	4.7	11.6	20.9	27.9	44.2
2000 (40)	17.5	40.0	57.5	70.0	82.5

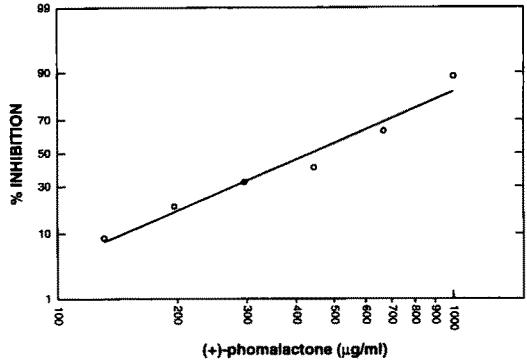

FIG. 2. Dose–response curve for inhibition by phomalactone of conidial germination in the fungus *Beauveria bassiana* (ARSEF #152). Each point represents the mean of duplicate samples of 100 conidia. Germination was scored 17 hr after inoculation on an SDAY plate treated with 30 µl of an acetone solution of phomalactone at one of the following concentrations: 1000, 667, 444, 296, 197, 131, and 0 µg/ml. Plates were held at 27°C. The 1000 µg/ml rate was equivalent to 245 ng/mm^2 surface area on the agar plate. Percent inhibition was corrected for control response (percentage of ungerminated spores at time of sampling) using Abbott's correction (Abbott, 1925) and plotted on a probability scale against dose plotted on a logarithmic scale (Finney, 1971). Probit equation: $y = 2.90x - 2.744$ where $x = \log_{10}$ dose and $y =$ probit mortality.

spores of *Metarhizium anisopliae* (ARSEF #1095 and #3864) (100% at 2000 µg/ml after 19 hr at 20°C). Under the same conditions phomalactone failed to inhibit germination of spores of two other entomopathogenic fungi of interest, *Verticillium lecanii* (ARSEF #2858) and *Paecilomyces fumosoroseus* (ARSEF #3322).

DISCUSSION

Phomalactone, 6-(1-propenyl)-5,6-dihydro-5-hydroxypyran-2-one, was first isolated from the plant-pathogenic fungus, *Nigrospora* sp. (Evans et al., 1969) and was subsequently isolated from another fungus, *Phoma minispora* (Yamamoto et al., 1970; Yamano et al., 1971). Inasmuch as these fungal genera are unrelated to each other, and neither is considered related to *Hirsutella*, it is likely that the production of phomalactone evolved independently in each of these fungi.

Several species of *Aspergillus* produce compounds structurally related to phomalactone. Asperlin (=asperline), an acetyl derivative of phomalactone with

an epoxide at the olefinic double bond in the propenyl side chain (see Figure 1), was originally isolated from *A. nidulans* (Argoudelis and Zieserl, 1966) and has subsequently been isolated from several other *Aspergilli, A. elegans, A. carneus* (Yamamoto et al., 1970), and *A. caespitosus* (Mizuba et al., 1975). The latter species also yielded acetylphomalactone (the acetyl derivative of phomalactone) as well as an epimer of asperlin (Mizuba et al., 1975). The absolute configurations at carbons 5 and 6 in naturally occurring phomalactone (Figure 1), acetylphomalactone, and asperlin are all the same (5S, 6S) (Evans et al., 1969; Mizuba et al., 1975), suggesting a common biosynthetic route. The absolute stereochemistry of the epoxide side chain of asperlin was only recently resolved (Ramesh and Franck, 1990).

Although Evans et al. (1969) did not report biological activity for phomalactone, Yamamoto et al. (1970) showed that the compound inhibited growth of a wide range of microorganisms including fungi, bacteria, and a protozoan. The related compounds isolated from *Aspergillus caespitosus* (see above) showed a similar spectrum of antimicrobial activity (Mizuba et al., 1975). Asperlin was also shown to have antitumor activity (Owen and Bhuyan, 1965). Prior to this report there has been no clear-cut demonstration of acute toxicity of phomalactone to a multicelled organism. Our assays indicate that phomalactone is mildly toxic per os to tephritid fruit flies. Further study of this compound against a broader spectrum of arthropods, especially the mite hosts of *Hirsutella thompsonii*, is warranted.

As mentioned above, some *Hirsutella* strains produce proteinaceous toxins, which, as yet, have not been fully characterized (Mazet et al., 1992). Ours is the first report of production by *Hirsutella* fungi of a low molecular weight compound with biological activity. Our findings, combined with those of other workers, indicate that toxigenicity may be widespread in the genus and ought to be investigated in greater depth both for the hope of discovering novel biologically active compounds and to gain a greater understanding of how these fungi function in nature and how they may be better exploited as biological control agents.

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TOLERANCE OF BITTER COMPOUNDS BY AN HERBIVORE, *Cavia Porcellus*

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Abstract—Many plant defensive chemicals are bitter to humans. Because of this taste characteristic, and because bitter compounds are often toxic, such substances, and the plants that contain them, are regarded as generally unpalatable to wildlife. These assumptions may be unwarranted. To test the hypothesis that herbivores are indifferent to 'bitter' tastants, we investigated the responsiveness of guinea pigs (*Cavia porcellus*) to denatonium benzoate, denatonium saccharide, limonene, L-phenylalanine, naringin, quebracho, quinine, Ro-Pel (a commercial animal repellent containing denatonium saccharide) and sucrose octaacetate. Only quinine and sucrose octaacetate slightly but significantly reduced feeding ($P < 0.05$). Our findings are inconsistent with the notion that herbivores generally avoid what humans describe as bitter tastes.

Key Words—Avoidance, bitter, *Cavia porcellus*, denatonium benzoate, denatonium saccharide, guinea pig, limonene, L-phenylalanine, naringin, quebracho, quinine, sucrose octaacetate.

INTRODUCTION

Many plants taste bitter to humans. Because of this taste characteristic, and because bitter compounds are often toxic, such substances, and the plants that

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contain them, are regarded as generally unpalatable to wildlife (Garcia and Hankins, 1975). Because of their plant diet, however, a priori rejection of what humans call bitter substances should be maladaptive for herbivores. Consistent with the view that herbivores tolerate so-called bitter tastants in food, Jacobs (1978) demonstrated that guinea pigs were essentially indifferent to quinine hydrochloride and sucrose octaacetate presented in two-bottle drinking tests. Jacobs et al., (1978) reasoned that the categorical exclusion of bitter forages by herbivores might result in the underexploitation of otherwise valuable and nutritious plants.

The present experiment was designed to elaborate Jacobs' earlier work. Although guinea pigs failed to avoid quinine and sucrose octaacetate, it is possible that the animals would respond to other putative bitter tastants. Accordingly, we evaluated the responsiveness of guinea pigs (*Cavia porcellus*) to nine bitter tastants chosen from seven structural classes (Belitz and Weizer, 1985; Maga, 1990): peptides (denatonium benzoate, denatonium saccharide); flavonoids (naringin); carbohydrates (sucrose octaacetate); alkaloids (quinine); terpenes (limonene); tannins (quebracho); and amino acids (L-phenylalanine). Except for sucrose octaacetate, denatonium benzoate, and denatonium saccharide, all of these tastants occur naturally in plants. A commercially available animal repellent (Ro-Pel), which contains denatonium saccharide as the active ingredient at a concentration of 0.065%, also was tested.

METHODS AND MATERIALS

Subjects. Eighteen experimentally naive adult guinea pigs were individually caged (50 × 50 × 30 cm) at 23°C, under a 12:12 hr light–dark cycle (light onset at 0700 hr). Water was freely available, and, prior to testing, Purina RP Guinea Pig Chow (Purina Mills, St. Louis, Missouri) was available ad libitum in cups with attached trays that caught spillage.

Chemicals. Naringin (NAR), CAS #10236-47-2), sucrose octaacetate (SOA, CAS #126-14-7), quinine monohydrochloride dihydrate (QUI, CAS #6119-47-7), limonene (LIM, CAS #5989-27-5), and L-phenylalanine (L-P, CAS #63-91-2) were obtained from Aldrich Chemical Company (Milwaukee, Wisconsin). Quebracho (QUE) was purchased from the Van Dyke Supply Company (Woonsocket, South Dakota). Denatonium benzoate (DB, CAS #3734-33-6), denatonium saccharide (DS, CAS #90823-03-84) and Ro-Pel[5] (ROP, commercial repellent) were donated by Atomergic Chemicals (Farmingdale, New York).

[5]Use of trade names in this manuscript is for the purpose of identification and does not indicate endorsement of commercial products by the U.S. Department of Agriculture.

For simplicity, we refer to these chemicals as bitter tastants throughout the remainder of the manuscript.

Stimulus Preparation. We chose to prepare stimuli in foods rather than solutions because bitter tastants are usually encountered in the former. Each chemical was dissolved in diethyl ether. The ether solutions were mixed with guinea pig pellets (250 ml of ether/250 g of feed) to produce 1.0% (mass/mass) tastant concentrations. This concentration was chosen because bitter tastants are typically avoided by omnivores at such high concentrations (i.e., $\geq 1.0\%$; Glendinning et al., 1990; Glendinning, 1992; Mason et al., 1985). Control pellets were prepared by mixing feed with ether alone. After mixing, the pellet samples were placed under a fume hood for 72 hr to ensure complete evaporation of the ether.

Procedure. Three weeks prior to the experiment, all animals were adapted to an 18 hr food-deprivation schedule that continued throughout the study. This deprivation period was implemented because a pilot study had indicated that deprivation was necessary to assure consistent, measurable consumption during the test period.

The adaptation period was followed immediately by four days of pretreatment. On each pretreatment day, 20 g of control pellets were presented at 0900 hr. At 1200 hr, the cups were removed from the cages, spillage was returned to the cups, and the weight of the cup contents was determined. Between 1200 and 1500 hr, animals were given free access to untreated pellets. Consumption during the measurement period was used to assign animals to nine pairs (i.e., those animals with the highest and lowest consumption were assigned to the first pair, those with the second highest and second lowest consumption were assigned to the second pair, and so forth.)

A four-day treatment period followed pretreatment. Treatment trials were similar to pretreatment tests, except that pairs of guinea pigs were given pellets adulterated with each of the bitter tastants during the 3-hr measurement period.

A three-day rest period, during which animals were given untreated pellets, immediately followed treatment. This rest period was followed by another four-days of pretreatment and four-days of treatment, as previously described. The cycle of pretreatment, treatment, and rest periods was repeated until each of the animals had been presented with all tastants in a counterbalanced design (i.e., each of the tastants was presented during each of the nine treatment periods).

Analysis. A two-way repeated measures analysis of variance (ANOVA) was used to assess consumption. The factors in this analysis were tastant (nine levels) and periods (pretreatment, treatment). Tukey tests (Winer, 1971, p. 201) were used to isolate significant differences among means subsequent to the omnibus procedure ($P < 0.05$).

RESULTS

There was a significant interaction between tastants and periods ($F = 4.6$; 8,136; $P < 0.0001$). Post-hoc tests revealed small but significant reductions in consumption of QUI and SOA pellets relative to pretreatment consumption (Figure 1). Otherwise there were no significant effects ($P > 0.25$).

DISCUSSION

Guinea pigs were generally indifferent to the bitter tastants evaluated in the present experiment. Only QUI and SOA moderately reduced feeding relative to pretreatment levels, and these reductions were small (23.9%, and 14.5% of pretreatment, respectively). Although animals were moderately food-deprived, these findings, together with the high bitter tastant concentrations tested, are consistent with the notion that herbivores either ignore or are insensitive to natural compounds that humans reject as bitter.

Indifference to bitterness probably does not reflect an overall inability to detect and avoid (or prefer) chemical stimuli in feed. Guinea pigs avoid citric acid, avidly consume at least some carbohydrate sweeteners, and show preferences for sodium chloride and sodium saccharin (Beauchamp and Mason, 1991; Jacobs, 1978).

Rather than insensitivity, indifference could reflect tolerance (i.e., guinea

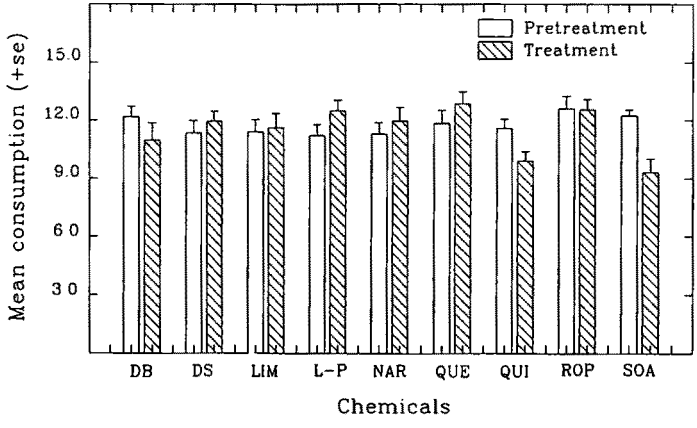

FIG. 1. Mean consumption by guinea pigs during pretreatment and treatment periods. Abbreviations: DB = denatonium benzoate; DS = denatonium saccharide; LIM = limonene; L-P = L-phenylalanine; NAR = naringin; QUE = quebracho; QUI = quinine; ROP = Ro-Pel; SOA = sucrose octaacetate.

pigs detect the tastants, but ignore them; Jacobs and Labows, 1979). Alternatively, it could be that guinea pigs perceive bitter tastants in terms of another taste quality. In either case, when SOA and QUI are paired with gastrointestinal distress, guinea pigs reliably acquire avoidance responses (Jacobs and Labows, 1979). Further, guinea pigs can learn to feed on those parts of bittersweet nightshade (*Solanum dulcamara*) that contain the lowest levels of toxicant (Jacobs and Labows, 1979). Other herbivores also exhibit this capability. For example, goats can learn to discriminate and avoid high tannin concentrations when foraging on blackbrush (*Coleogyne ramosissima*) (Provenza et al., 1990). These aversions are rapidly acquired within the first feeding bout (Provenza, et al., 1993).

More broadly, although herbivores generally tolerate bitter tastants, there is evidence that interspecific differences may exist. For example, guinea pigs tolerate QUE, but meadow voles (*Microtus pennsylvanicus*) avoid it. Cattle and guinea pigs respond similarly to SOA, yet guinea pigs are much more tolerant of QUI (Jacobs and Labows, 1979). Some rodents avoid DS (Davis et al., 1986; Langley et al., 1987), while others demonstrate a preference for it (Davis et al., 1987). Although the reasons for these differences remain obscure, it is plausible that they reflect differences among species in their evolutionary history or ecology (Freeland and Janzen, 1974; Lindroth, 1988).

Management Implications. Bitter tastants are generally regarded as unpalatable to wildlife, despite growing evidence to the contrary. In the present experiments, guinea pigs were indifferent to concentrations of denatonium benzoate and denatonium saccharide that were nearly three orders of magnitude higher than those that humans perceive as intensely bitter (Mason, personal observation). These denatonium compounds are used as the principle active ingredient in some commercial repellents. Although species differences exist among herbivores in avoidance of bitter tastants, the present results are inconsistent with the notion that bitter chemicals can serve as broadly effective repellents, particularly against herbivores.

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ALLELOPATHY OF OATS. I. ASSESSMENT OF ALLELOPATHIC POTENTIAL OF EXTRACT OF OAT SHOOTS AND IDENTIFICATION OF AN ALLELOCHEMICAL

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Abstract—The allelopathic potential of oat (*Avena sativa* L.) extracts was investigated under laboratory conditions. The ethyl ether-, acetone-, and water-soluble fractions obtained from the extract of oat shoots inhibited the germination and growth of roots and hypocotyls of lettuce (*Lactuca sativa* L.). The inhibitory activity of the water-soluble fraction was maximum, followed by that of ethyl ether-soluble and acetone-soluble fraction. An active principle of the water-soluble fraction was isolated and its structure was determined by spectral data as L-tryptophan. L-Tryptophan inhibited the growth of hypocotyls and roots of lettuce seedlings at concentrations greater than 0.03 and 0.1 mM, respectively. These results suggested that L-tryptophan may be an allelochemical which affects the growth or germination of different plant species.

Key Words—Allelochemical, phytotoxicity, *Avena sativa*, lettuce, *Lactuca sativa*, oat, L-tryptophan.

INTRODUCTION

A number of crop and weed species have been reported to cause an inhibitory effect on the growth of other plant species, which is called allelopathy (Rice, 1974). Chemicals with allelopathic potential are present in many plants and in

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many organs, including leaves, flowers, fruits, and buds (Guenzi et al., 1967; May and Ash, 1990; Putnam and Tang, 1986; Rice 1984). Under certain conditions, these compounds may be released into the environment in sufficient quantities and with enough persistence to affect a neighboring or successional plant. In field situations, it has been shown that the release of allelochemicals occurred by exudation from living roots and decomposition of plant residues (Bhowmik and Doll, 1982; Inderjit and Dakshini, 1992; Putnam and Tang, 1986).

Evidence for allelopathy has accumulated in the literature over many years (Duke, 1986; Putnam, 1988; Rice, 1984). However, very little information is available about the allelopathic potential of very young seedlings of crops. The objective of this research was to obtain an understanding of the chemical basis of one such allelopathic system. In this paper we describe the assessment of the possible allelopathic potential of extracts obtained from young shoots of oat seedlings (*Avena sativa* L.) under laboratory conditions and identification of an allelochemical, which was present in the water extract of shoots, by its mass, IR, ^{1}H and ^{13}C NMR spectra.

METHODS AND MATERIALS

Plant Materials. Seeds of oat (*Avena sativa* L. cv. Victory) were sown on two sheets of moist filter paper (No. 2; Toyo, Ltd., Tokyo) in trays and grown at 25°C in daily cycles of 14 hr light–10 hr dark in a growth chamber. Light was provided from above with two white fluorescent lamps (3.2 W/m^2 at plant level; FL-20S-PG, 20 W; National, Tokyo). After seven days, shoots of the seedlings were harvested, rinsed with distilled water, immediately frozen on Dry Ice, and stored at −80°C until extraction of allelopathic substance(s).

Extraction. Oat shoots (1 kg fresh wt) were homogenized in 5 liters of 50% (v/v) aqueous cold acetone and the homogenate was filtered through filter paper (No. 1; Toyo, Ltd.). The residue was homogenized again, by using the same solvent as in the first extraction, and filtered. The two filtrates were combined and evaporated to dryness at 35°C in vacuo. The concentrate was divided into ethyl ether-soluble (2.1 g), acetone-soluble (4.8 g), and water-soluble (12.3 g) fractions.

Bioassay. Each of the ethyl ether-soluble, the acetone-soluble, and the water-soluble fractions was evaporated to dryness and the residues dissolved in a small volume of ethyl ether, acetone, or distilled water, respectively. Each of the solutions was transferred in toto to a sheet of filter paper (No. 2; Toyo, Ltd.) in a 9-cm Petri dish and dried. The filter paper in the Petri dish was moistened with 10 ml of distilled water, and 50 seeds of lettuce (*Lactuca sativa* L.) were sown on it. The concentration of the residues of each fraction in the

bioassay was 0, 0.03, 0.1, 0.3, 1 and 3% (w/v). Germination of the seeds and the length of roots and hypocotyls of the seedlings were determined after two and four days of incubation in the dark at 25°C, respectively.

Purification of Water-Soluble Fraction. The water-soluble fraction was chromatographed on a column (3 × 50 cm) of Amberlite XAD-2 (200 g; Organo Ltd., Tokyo) and eluted with water that contained increasing amounts of acetone (10% per step, v/v; 200 ml of eluate per step). The inhibitory activity of each fraction was determined using a bioassay of lettuce as described above. All the tested samples of the bioassay corresponded to 20 and 5 g fresh weight equivalent of the extracted oat shoots. Fractions obtained by elution with 40–50% acetone in water gave a brown oil (980 mg) having plant growth-inhibitory activity. The residue (240 mg) obtained after passing the active material through a C_{18} Sep-Pak cartridge (Waters, Tokyo) was subjected to reverse-phase HPLC (19 mm × 15 cm; μBondasphere 5μ C_{18} − 100 Å; Waters; eluted at flow rate of 4 ml/min with 10% aqueous methanol, v/v; detected at 220 nm). The biological activity was detected in the eluate of retention times of 61–63 min. After evaporation, the active residue (36 mg) was finally purified by a second HPLC step (10 mm × 50 cm; ODS AQ-325 S-5 120 Å; YMC Co. Ltd., Kyoto, Japan; eluted at a flow rate of 1.5 ml/min with 40% aqueous methanol, v/v, detected at 220 nm), yielding an active component (12 mg) that eluted with retention times of 31.5–32.8 min.

Structure of this active principle was analyzed by EI-MS, ^1H and ^{13}C NMR, and specific rotation. EI-MS (70 eV) *m/z* (relative intensity): 204 (M^+, 9), 130 (100), 102 (6) and 76 (4). ^1H NMR (400 MHz) δ (CD_3OD): 7.70 (1H, d, J = 7.9 Hz), 7.36 (1H, d, J = 8.0 Hz), 7.19 (1H, s), 7.12 (1H, dd, J = 7.1, 8.0 Hz), 7.04 (1H, dd, J = 7.1, 7.9 Hz), 3.85 (1H, dd, J = 4.0, 9.4 Hz), 3.52 (1 H, dd, J = 4.0, 15.3 Hz), and 3.14 (1H, dd, J = 9.4, 15.3 Hz). ^{13}C NMR (100 MHz) δ (CD_3OD): 175.17 (s), 139.20 (s), 129.29 (s), 125.91 (d), 123.55 (d), 120.91 (d), 120.14 (d), 113.22 (d), 110.40 (s), 57.55 (d), 29.25 (t). The specific rotation: $[\alpha]_D^{20}$ −30.5° (D_2O; c 0.1).

RESULTS AND DISCUSSION

Allelopathic Potential of Shoot Extract. The allelopathic potential of three kinds of fractions obtained from extract of shoots of oat seedlings was evaluated (Tables 1 and 2). These three fractions suppressed the germination and the growth of roots and hypocotyls of lettuce. However, more noticeable inhibition was observed in the bioassay of the water-soluble fraction. Three independent runs of similar extractions and bioassays gave virtually the same results.

Significant reductions in the germination and the growth of the roots and hypocotyls were observed as the extract concentration increased. Results are in

TABLE 1. ACTIVITY OF FRACTIONS OBTAINED FROM SHOOT EXTRACT OF OAT SEEDLINGS ON GERMINATION OF LETTUCE SEEDS[a]

Concentration (%, w/v)	Germination (% of control)		
	Ethyl ether	Acetone	Water
0	100	100	100
0.03	97.7 ± 5.1	95.5 ± 4.3	93.2 ± 6.3
0.1	88.6 ± 6.8	90.1 ± 5.2	70.5 ± 4.8
0.3	75.0 ± 5.4	86.4 ± 6.4	47.7 ± 5.2
1	65.9 ± 3.4	81.8 ± 5.4	27.3 ± 4.7
3	45.5 ± 4.7	68.2 ± 5.1	11.4 ± 6.4

[a]Germination of the seeds was determined after two days of incubation in the dark at 25°C. Means ± SE of results from three replicates of 50 seeds each are shown.

TABLE 2. ACTIVITY OF FRACTIONS OBTAINED FROM SHOOT EXTRACT OF OAT SEEDLINGS ON GROWTH OF ROOTS AND HYPOCOTYLS OF LETTUCE SEEDLINGS[a]

Concentration (%, w/v)	Root (% of control)			Hypocotyl (% of control)		
	Ethyl ether	Acetone	Water	Ethyl ether	Acetone	Water
0	100	100	100	100	100	100
0.03	87.3 ± 5.7	94.7 ± 4.5	74.3 ± 6.3	95.3 ± 5.3	98.2 ± 5.6	83.5 ± 5.5
0.1	74.1 ± 6.5	86.1 ± 6.0	52.1 ± 6.7	88.1 ± 5.7	93.5 ± 6.8	71.8 ± 6.2
0.3	61.9 ± 5.4	78.8 ± 7.6	33.5 ± 7.5	82.6 ± 6.9	91.7 ± 5.9	65.9 ± 7.2
1	48.5 ± 6.8	71.9 ± 6.9	19.3 ± 5.4	76.0 ± 7.0	87.6 ± 6.4	57.1 ± 7.5
3	35.5 ± 7.1	63.6 ± 5.4	11.2 ± 6.1	78.4 ± 6.8	82.1 ± 5.7	48.6 ± 5.8

[a]The length of the roots and hypocotyls was measured after four days of incubation in the dark at 25°C. Means ± SE of results from three replicates of 50 plants each are shown. Elongation of control seedlings was 19.9 ± 1.1 and 11.4 ± 0.7 mm for the roots and the hypocotyls, respectively.

agreement with previous investigations in that the activity of either water extracts or weed residue was directly related to the concentration of the residue rates (Caussanel, 1979). Such rate-dependent responses of the test plant to the fractions suggested that each fraction separated from shoot extract contained allelochemical(s), but that allelopathic potential of the water-soluble fraction was maximum, followed by the ethyl ether-soluble fraction and the acetone-soluble fraction (Tables 1 and 2).

The roots of lettuce apparently are more sensitive to the fractions than the hypocotyl (Table 2). This observation agrees with the findings of Stachon and

Zimdahl (1980), who found the ethanol extracts of Canada thistle more inhibitory to cucumber radicles than to hypocotyls.

Identification of Allelochemical in Water-soluble Fraction. The allelopathically active substance (12 mg) was isolated as a colorless powder from the water-soluble fraction (12.3 g) obtained from the extract of oat shoots (1 kg fresh wt). This compound was identified as L-tryptophan by comparison of its spectra of EI-MS, ^{1}H and ^{13}C NMR and its specific rotation with those of an authentic sample of L-tryptophan (Nakarai Inc., Kyoto, Japan).

Oats have been shown to exude scopoletin, coumaric acid, *p*-hydroxybenzoic acid and vanillic acid, which are reported to have allelopathic activity, from its roots after radicle protrusion (Fay and Duke, 1977; Pèrez and Ormeño-Nuñez, 1991; Schumacher et al., 1983). Five phenolic acids (ferulic, coumaric, syringic, vanillic, and *p*-hydroxybenzoic) were identified in the residues of mature oat plants, and the allelopathic potential of their compounds has been shown (Guenzi and McCalla, 1966; Guenzi et al., 1967). Moreover, hydroxamic acid was isolated from cereals including oats, and their allelopathic activity has been shown (Pèrez, 1990). However, it has not been reported so far that L-tryptophan is a allelopathic substance.

Bioassay of L-Tryptophan. The allelopathic activity of L-tryptophan isolated from the water-soluble fraction and also authentic L-tryptophan were tested with the lettuce bioassay in the same manner as described by Kato-Noguchi et al. (1994). Figure 1 shows that the activities were identical and L-tryptophan inhibited the growth of the roots and hypocotyls at concentrations greater than 0.03

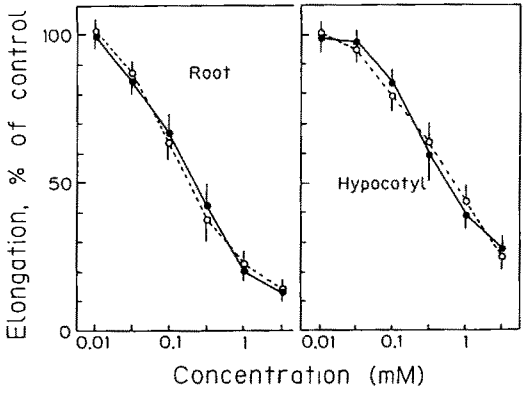

FIG. 1. Effects of isolated (○) and authentic (●) L-tryptophan on the growth of roots and hypocotyls of lettuce seedlings. The length of the roots and hypocotyls was measured after four days of incubation in the dark at 25°C. Means ± SE of results from three replicates of 50 plants each are shown. Elongation of control seedlings was 21.2 ± 1.4 and 12.1 ± 0.5 mm for the roots and the hypocotyls, respectively.

and 0.1 mM, respectively. These results suggested that L-tryptophan may act as an allelochemical to various plant species. In the following paper, we describe that L-tryptophan inhibited the growth of hypocotyls and roots of several plant species (Kato-Noguchi et al., 1994).

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ALLELOPATHY OF OATS. II. ALLELOCHEMICAL EFFECT OF L-TRYPTOPHAN AND ITS CONCENTRATION IN OAT ROOT EXUDATES

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Abstract—L-Tryptophan caused growth inhibition of roots and hypocotyls (or coleoptiles) of cockscomb (*Amaranthus caudatus* L.), lettuce (*Lactuca sativa* L.), cress (*Lepidium sativum* L.), timothy (*Phleum pratense* L.), rice (*Oryza sativa* L.), wheat (*Triticum aestivum* L.), and oat (*Avena sativa* L.); increasing the dose of L-tryptophan increased the inhibition. The concentrations for 50% inhibition of the root growth were 0.14, 0.15, 0.21, 0.79, 0.95, 1.7, and 2.4 mM for cockscomb, cress, lettuce, timothy, rice, wheat, and oat, respectively; the concentrations for 40% inhibition of the hypocotyl (or coleoptile) growth were 0.28, 0.33, 0.43, 2.7, 4.5, 7.2, and 15 mM for cockscomb, cress, lettuce, timothy, rice, wheat and oat, respectively. The levels of L-tryptophan in oat seedlings and in its root exudates were 29.3 mg/kg fresh wt and 0.25 mM under light conditions, and 21.1 mg/kg fresh wt and 0.18 mM under dark conditions, respectively. The presence of L-tryptophan in the root exudates coupled with its effect on growth suggested that L-tryptophan may play an important role in the growth inhibition of other plants in nature.

Key Words—Allelopathy, *Avena sativa*, oat, L-tryptophan, root exudates.

INTRODUCTION

Numerous workers have reported the presence of growth and germination inhibitors in various plants (Rice, 1984). In some cases the active principles have

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been isolated and characterized (Putnam, 1988). It has been further found that some of these compounds have a role in the interaction of species (Rice, 1984). Furthermore, the allelopathic potential of some crop species has been found to have promise in biological weed control (Leather, 1983; Owens, 1973; Patterson, 1981; Putnam et al., 1983; Putnam and Tang, 1986).

In the preceding paper (Kato-Noguchi et al., 1994), we reported that one allelochemical, with activity assessed by lettuce bioassay, was isolated from the water-soluble fraction obtained from the aqueous extract of shoots of oat seedlings, and it was identified as L-tryptophan by spectrometric analyses. The purpose of the present experiment was to evaluate the allelopathic potential of L-tryptophan to several plant species and to determine its concentration in oat seedlings and root exudates.

METHODS AND MATERIALS

Bioassay. Seeds of cockscomb (*Amaranthus caudatus* L.), cress (*Lepidium sativum* L.), lettuce (*Lactuca sativa* L.), timothy (*Phleum pratense* L.), rice (*Oryza sativa* L.), wheat (*Triticum aestivum* L.), and oat (*Avena sativa* L.) were used for bioassay.

L-Tryptophan (Nacalai Inc., Kyoto, Japan) was dissolved at various concentrations with distilled water, and the solution (10 ml) was added on a sheet of filter paper (No. 2; Toyo, Ltd.) in a 9-cm Petri dish. Fifty seeds (cockscomb, cress, lettuce, and timothy) or 30 seeds (rice, wheat, and oat) of each test plant were separately sown on the paper, and they were allowed to grow in the dark at 25°C for four days (cockscomb, cress, lettuce, timothy, and oat) and for five days (rice and wheat). Then the length of the roots and the hypocotyls (or coleoptiles) of the seedlings was measured, and the percentage elongation of growth was calculated by reference to the elongation of control seedlings.

All bioassays were repeated three times.

Plant Materials for Quantification of L-*Tryptophan.* Seeds of oat (*Avena sativa* L. Victory) were sterilized in a 2% (w/v) solution of sodium hypochlorite for 5 min, rinsed in distilled water for 30 min, and germinated on a filter paper in the dark at 25°C for two days. Germinated seedlings were selected and transferred, in groups of 100, to 24-cm Petri dishes each containing two sheets of filter paper moistened with 50 ml sterilized water. After being kept in the dark at 25°C for three days, some of the seedlings were grown in the dark (dark-grown seedlings) and the rest under continuous white light (3.2 W/m^2 at plant level; light-grown seedlings) at 25°C for one additional day. The seedlings and culture solution were separated and both were stored at −80°C for quantification of L-tryptophan. All instruments used in this part of the experiment were sterilized.

Quantification of L-*Tryptophan.* Plant material kept at −80°C was homogenized, and water-soluble fractions were extracted as described previously (Kato-Noguchi et al., 1994). After evaporation of the extract, the residue was dissolved in 10 ml distilled water and passed through a reverse-phase Sep-Pak cartridge (C_{18}; Waters, Tokyo). The sample of L-tryptophan purified in this way was injected onto a column for HPLC (10 mm × 50 cm; ODS AQ-325 S-5 120 Å; YMC Co. Ltd., Kyoto, Japan), eluted at flow rate of 1.5 ml/min with 40% aqueous methanol (v/v), and detected at 220 nm. The retention time of L-tryptophan was 32.1 min under these conditions. Quantification was performed by measuring the average areas of peaks from at least three injections of sample and calibrating. The overall recovery of L-tryptophan through the entire quantification process was 84 ± 2%, as calculated from five repetitive test runs with authentic L-tryptophan.

Culture solutions of oat seedlings were evaporated to dryness and treated in the same manner as the water-soluble fraction.

RESULTS AND DISCUSSION

Effects of L-*Tryptophan.* Figure 1 shows effects of L-tryptophan on the root growth of the test plants. L-tryptophan inhibited growth at concentrations greater than 0.03 mM. The dose–response curves of the plants for L-tryptophan were linear between 20 and 70% inhibition when the percentage elongation was plot-

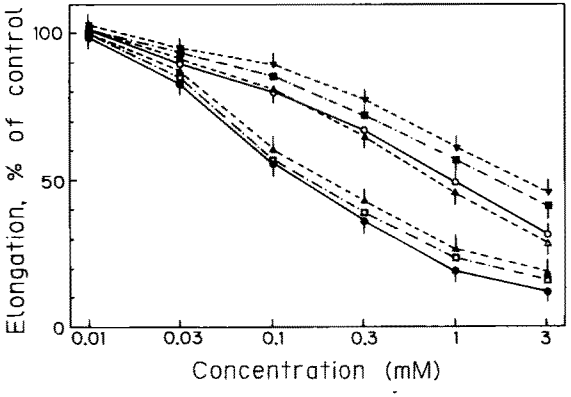

FIG. 1. Effects of L-tryptophan on the growth of roots of cockscomb (●), cress (□) lettuce (▲), timothy (△), rice (○), wheat (■), and oat (▼). Means ± SE of results from three replicates of 30–50 plants each are shown. Elongation of control seedlings was 19.7 ± 1.9, 29.1 ± 2.1, 21.5 ± 2.1, 20.8 ± 2.4, 30.7 ± 2.1, 32.7 ± 2.9, and 38.4 ± 2.5 mm for cockscomb, cress, lettuce, timothy, rice, wheat, and oat, respectively.

ted against the logarithm of the dose. The doses required for 50% inhibition, as interpolated from the dose–response curves, were 0.14, 0.15, 0.21, 0.79, 0.95, 1.7, and 2.4 mM for cockscomb, cress, lettuce, timothy, rice, wheat, and oat, respectively.

Figure 2 shows effects of L-tryptophan on the growth of hypocotyls (cockscomb, cress, and lettuce) and on the growth of coleoptiles (timothy, rice, wheat, and oat). The effectiveness on timothy, rice, wheat, and oat of L-tryptophan was very weak, and complete dose–response curves were obtained only with cockscomb, cress, and lettuce. The dose–response curves of cockscomb, cress, and lettuce were linear against the logarithms of doses between 20 and 60% inhibition. The doses required for 40% inhibition were 0.28, 0.33, 0.43, 2.7, 4.5*, 7.2*, and 15* mM for cockscomb, cress, lettuce, timothy, rice, wheat and oat, respectively. Asterisks indicate values were estimated by extrapolating from the dose–response curves.

L-Tryptophan inhibited the growth of roots and hypocotyls (or coleoptiles) of the test plants, and increasing the dose increased the inhibition (Figure 1 and 2). However, L-tryptophan was more effective on the roots of the test plants than on the hypocotyls (or coleoptiles) of the same plants. Moreover, the hypocotyls of Dicotyledoneae (cockscomb, cress, and lettuce) apparently were more sensitive to L-tryptophan than the coleoptiles of Monocotyledoneae (timothy, rice, wheat, and oat).

Quantification of L-Tryptophan. Table 1 shows the levels of L-tryptophan

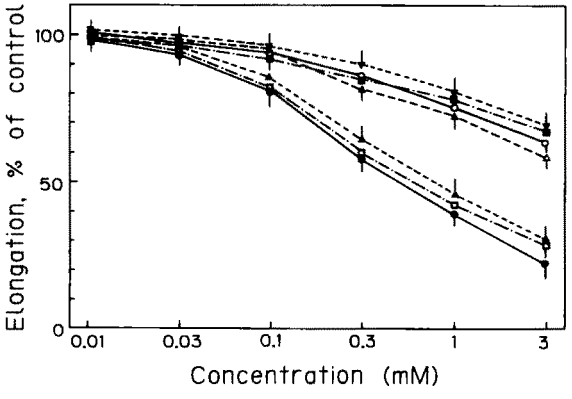

FIG. 2. Effects of L-tryptophan on the growth of hypocotyls of cockscomb (●), cress (□), and lettuce (▲) and on the growth of coleoptiles of timothy (△), rice (○), wheat (■), and oat (▼). Means ± SE of results from three replicates of 30–50 plants each are shown. Elongation of control seedlings was 7.5 ± 0.7, 14.1 ± 1.5, 12.1 ± 0.9, 13.1 ± 1.0, 17.9 ± 1.2, 35.7 ± 3.1, and 32.1 ± 2.9 mm for cockscomb, cress, lettuce, timothy, rice, wheat, and oat, respectively.

TABLE 1. LEVELS OF L-TRYPTOPHAN IN OAT SEEDLINGS AND ITS ROOT EXUDATES[a]

Treatment	Seedling (mg/kg fresh wt)$^{-1}$	Exudate (mM)
Dark-grown	21.1	0.18
Light-grown	29.3	0.25

[a]This experiment was repeated three times and the results were similar on each occasion.

in oat seedlings and in oat root exudates under dark and light growth conditions. Such levels in light-grown seedlings were about 1.4 times those in the dark-grown ones. It has been reported that organic compounds are exuded from living plant roots (Rovira, 1969), and stress and other factors such as light, moisture, and plant age and plant nutrition can increase root exudation (Nye and Tinker, 1977). The levels of L-tryptophan exuded by the roots are proportional to its content in the seedlings (Table 1). The result suggested that root exudation of L-tryptophan may depend on a specific regulating factor that is related to the concentration.

The detection of L-tryptophan in the root exudates of oat (Table 1) and the effect of L-tryptophan on the growth (Figures 1 and 2) suggested that L-tryptophan may play an important role in the growth inhibition of other plants in nature. The mechanism of action of L-tryptophan is under investigation.

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TESTS AND REFINEMENTS OF A GENERAL STRUCTURE-ACTIVITY MODEL FOR AVIAN REPELLENTS

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Abstract—We tested the robustness of a structure-activity model for avian trigeminal chemoirritants. Fourteen benzoates and acetophenones were tested using European starlings *Sturnus vulgaris* as a bioassay. In general, the previously proposed model was a reasonable predictor of repellency (i.e., irritant potency). We found that the presence of a phenyl ring was critical to repellency. Basicity of the molecule is the next most critical feature influencing repellency. The presence of an acidic function within the electron-withdrawing functionality seriously detracts from repellency. The presence or absence of an electron-withdrawing or -donating group may potentiate repellent effects, but its presence is not critical, so long as the phenyl ring is electron rich. Our data suggest that there is an *o*-aminoacetophenone/methyl anthranilate trigeminal chemoreceptor in birds analogous to the mammalian capsaicin receptor. Both receptors contain a benzene site. However, birds seem to lack the associated thiol/hydrogen-bonding site present in mammals which is needed to activate the benzene site. Rather, birds may possess an associated exposed charged site that in turn may interact with the stimulus to activate the benzene site. These differences may explain the differential sensitivity of birds and mammals to aromatic irritants.

Key Words—Acetophenones, benzoates, bird repellents, irritant, receptor model, structure-activity relationships, *Sturnus vulgaris*, trigeminal.

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INTRODUCTION

Many birds avoid edibles based upon chemosensory cues (Schuler, 1983; Jakubas et al., 1992; Mason et al., 1989). When avoidance is nonlearned and resistant to habituation, the bird is most likely responding to a chemical irritant (Clark and Mason, 1993). Mediation of irritation is via the chemically sensitive fibers (A-delta and C fibers) of the trigeminal nerve (Finger et al., 1990). These fibers are typically found in the highest densities around mucous membranes; for birds, this corresponds to the eyes and buccal and nasal cavities.

There is evidence that irritants are perceived similarly within a vertebrate class, but there are dramatic differences in perception between classes. Birds do not avoid familiar mammalian irritants such as ammonia, gingerol, zingerone, hydroquinones, and naphthalene (Dolbeer et al., 1989; Mason and Otis, 1990). Other mammalian irritants, such as piperine, allyl isothiocyanate and mercaptobenzoic acid, have some repellent effects, but only at high concentrations ($>10{,}000$ ppm) under specific presentation schemes (Mason and Otis, 1990). The most striking example of the difference between birds and mammals is for capsaicin. Mammals uniformly avoid capsaicin (the hotness in red chilies) at about 100–1000 ppm. Birds will readily consume up to 20,000 ppm (Solzcsanyi et al., 1986; Mason et al., 1991). Yet birds avoid methyl anthranilate (grape flavoring) at 1000 ppm (Mason et al., 1989), while mammals are indifferent or prefer the compound at concentrations up to 10,000 ppm (Furia and Bellanca, 1975).

Very little is known about comparative quantitative structure–activity relationships of trigeminal irritants among the taxa. The apparent differences between birds and mammals in response to irritants suggests that avian and mammalian trigeminal receptors may not share common structures (Solzcsanyi et al., 1986). Perception of chemical pain is hypothesized to be adaptive because it allows an animal to avoid potentially dangerous compounds. The existence of differences in perception between birds and mammals raises the question about the possible selective pressures extant at the beginning of each taxonomic line.

In an effort to better understand the potentiating factors responsible for differences in chemethesis between birds and mammals, we set out to expand upon and quantify the chemical structure–behavioral activity relationships of candidate trigeminal irritants for each class. This paper focuses on refinements and tests of a previously elucidated avian model (viz., Clark and Shah, 1991; Clark et al., 1991; Shah et al., 1991). The compounds tested herein were selected to test the robustness of our model.

METHODS AND MATERIALS

Study Subjects. Adult European starlings (*Sturnus vulgaris*) were captured at the Philadelphia Zoo using funnel traps and transported to the Monell Center via car. Starlings were individually caged (61 × 36 × 41 cm) under a 12:12

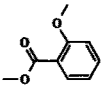
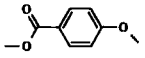
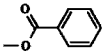
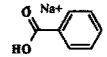
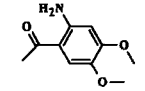
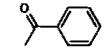
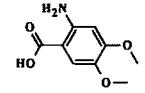
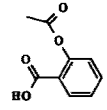
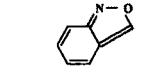
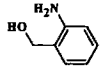
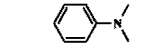
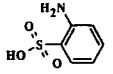
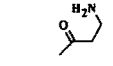
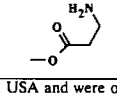
hr light–dark cycle for a two-week adaptation period and given free access to Purina Flight Bird Conditioner (Purina Mills, St. Louis, Missouri), water and oyster shell grit (United Volunteer Aviaries, Nashville, Tennessee). Starlings were chosen as test animals because previous experiments showed them to be good models of avian sensitivity (Clark and Smeraski, 1990; Clark and Shah, 1991).

Stimuli. We selected 14 chemicals on the basis of conformational, physical, and electronic attributes that could be used to test the repellency model previously generated (Figure 1). We attempted to achieve a maximum test concentration of 5000 mg/liter (0.5%); however, differences in water solubility precluded achieving this goal in many cases. Solubility data were obtained from the published literature or empirically. In all cases, concentrations were validated using UV spectrographic or HPLC methodology. Lower concentrations tested were obtained via serial dilution of the maximum concentration used. The rationale for stimulus selection is as follows.

Effects of Substituent Position. We previously found substituent position to be an important factor influencing repellency (Clark and Shah, 1991). Position of substituents affects the distribution of surface charges of the molecule, which in turn may be important for stimulus access to receptors. Groups such as $-NH_2$ and OH (and their derivatives, e.g., $-OCH_3$) act as strong activators toward electrophilic aromatic substitution, releasing their electrons via resonance rather than induction. The carbonium ion formed during electrophilic attack ortho or para to the substituent is stabilized by electron-donating groups (EDG). Furthermore when in the ortho position, the free NH_2/OH groups have a possibility of hydrogen bond interactions as in the previously tested methyl anthranilate and *o*-aminoacetophenone (Clark and Shah, 1991; Clark et al., 1991). We selected methyl 2- and methyl 4-methoxybenzoate to test the importance of substituent position in the absence of hydrogen bonding.

Effects of Substituent Position and Electron Richness of Phenyl Ring. If electron richness of the phenyl ring was the only critical feature for repellency (viz., Clark et al., 1991), then loading the ring with EDGs might be expected to enhance repellency. Previous experiments demonstrated that *o*-aminoacetophenone was a strong repellent (Clark and Shah, 1991). We selected 2-amino-4,5-dimethoxyacetophenone because it contained three EDGs. Anthranilic acid also was shown to be repellent, but less so because of its free carboxylic acid group (Clark et al., 1991). We selected 2-amino-4,5-dimethoxybenzoic acid to determine if repellency could be improved upon by loading the phenyl ring with EDGs.

Effects of Removing EDG and Altering EWG. Donation of lone pairs of electrons to the phenyl ring is associated with repellency (Clark and Shah, 1991). We selected methyl benzoate because we anticipated that the absence of an EDG on this molecule would result in a diminished repellency relative to the previ-

Code	Name	CAS	Structure
M2MOB (99%)	methyl 2-methoxybenzoate	606-45-1	
M4MOB	methyl 4-methoxybenzoate	121-98-2	
MB	methyl benzoate	93-58-3	
SB	sodium benzoate	532-32-1	
2A45DMAP	2-amino-4,5-dimethoxyacetophenone	4101-30-8	
AP	acetophenone	98-86-2	
2A45DMBA	2-amino-4,5-dimethoxybenzoic acid	5653-40-7	
ASA	acetylsalicylic acid	50-78-2	
ANTH	anthranil	271-58-9	
2ABAL	2-aminobenzyl alcohol	5344-90-1	
NNDMAN	*N,N*-dimethylanaline	121-69-7	
SAAM	2-aminobenzene sulfonic acid	88-21-1	
BALN	beta-alanine	107-95-9	
MEBALN	methyl ester of beta-alanine	2491-20-5	

All compounds were obtained from Aldrich, Milwaukee, Wisconson, USA and were of 99% + purity.

FIG. 1. Chemical structures, Chemical Abstract Service registry number (CAS), compound source, purity, and codes for stimuli.

ously tested methyl anthranilate. Acetophenone was chosen for similar reasons for comparison with the previously tested *o*-aminoacetophenone.

Sodium benzoate was selected because it did not have an EDG or the acidic proton of CO_2H, but it retained the carboxylate in anionic form, and, here, the negative charge is on the oxygen, not on the phenyl ring. Anthranil was selected because it possessed an electron-withdrawing group (EWG) but did not contain any substituent contributing to acidity due to the EWG.

We tested acetylsalicylic acid (aspirin) because it lacked an EDG, but in addition, substituents in the ortho position offered steric hinderance.

Effects of Removing EWG. 2-Aminobenzyl alcohol was selected because it represented a molecule with a strong basic EDG, but lacking an EWG. Thus, we anticipated that it would be a potent repellent as was found for veratryl alcohol (Shah et al., 1991). *N,N*-Dimethylaniline was selected for similar reasons, and for direct comparison to the previously tested dimethyl anthranilate (DMA) (Clark et al., 1991). If *N,N*-dimethylaniline proved to be a better repellent than dimethyl anthranilate, this would support the notion that an EWG is a detractor to repellency and that resonance per se is not critical to repellency. 2-Amino sulfonic acid was selected because of its stronger EWG, the effects of hetero atom(s), and also because it possesses acidity without a CO_2H group (relative to anthranilic acid).

Effects of Phenyl Ring. We selected β-alanine because its functionalities were similar to the previously tested anthranilic acid, but devoid of the phenyl ring. The methyl ester of β-alanine was selected because of similarity of functionalities to methyl anthranilate. If these functionalities are important per se, rather than the presence of a phenyl ring, then repellency should not be affected relative to the previously tested aminobenzoic acids.

Behavioral Assays. After adaptation, tap-water consumption was measured for 6 hr on each of three pretreatment days. Starlings were ranked according to mean water consumption and assigned to one of the six groups. The bird with the highest water consumption was assigned to the first treatment group, the bird with the second highest consumption was assigned to the second treatment group, and so forth to the final group, followed by a series of assignments from the final group back to group 1. Equality for water consumption among groups was validated using a one-way analysis of variance and was a prerequisite for further testing. A total of 36 birds was used for each experiment, with six birds per treatment group. Groups were randomly assigned to receive a specific concentration of a chemical during the treatment period.

After assignment to a concentration group, a series of no choice (one-bottle) tests was initiated. On the first day (pretreatment period), beginning at 0930 hr, consumption of tap water was recorded every 2 hr for the next 6 hr. On the second day, beginning at 0930 hr, birds were given their preassigned concentration of the chemical being tested. Consumption was recorded every 2 hr for

6 hr. After the test, birds were given free access to tap water. On the third day, beginning at 0930 hr, posttreatment water consumption of tap water was recorded every 2 hr for a total of 6 hr. In all cases, water was presented in 120-ml graduated Richter tubes. Starlings were visually isolated from one another as well as from the contents of the drinking tubes via opaque acrylic partitions.

So that comparisons of dose–response curves could be made from one experiment to another, consumption on the treatment and posttreatment days was standardized relative to pretreatment water intake. Experience indicated that absolute consumption varied seasonally, even after a two-week adaptation period. The ratio of treatment to pretreatment consumption was a measure of avoidance, while the ratio of posttreatment to pretreatment consumption was an index of carryover effects resulting from consumption of chemicals.

Characterization of Fluid Intake for Individual Compounds. A two-factor repeated measures analysis of variance was used to test whether an individual compound was repellent. The between-subjects factor was concentration group with six levels. The within-subjects (repeated) factor was period with two levels (treatment day and posttreatment day). The dependent variable was the fluid intake relative to the pretreatment fluid intake. Thus, if a chemical was repellent, its score would be less than 1.0. Duncan's multiple-range tests were used to test for post-hoc differences among means, with significance set at $P < 0.05$.

Avoidance may be attributable to sensory irritation of the trigeminal nerve and/or a postingestional conditioned aversion (Clark and Mason, 1993). Learned aversions are characterized by initial normal consumption, followed by reduced intake during subsequent observations periods. In contrast, trigeminally mediated repellency does not require learning. Trigeminal repellents cause irritation (pain) and are avoided upon initial contact. Thus, intake tends to be similar across sampling periods. Each chemical was evaluated for its mode of action using a two-way repeated measures anova. The dependent variable was absolute amount of treated water consumed. The between-subjects effect was concentration, while the within-subjects effect was hours with three levels. Only the interaction term and the main within-subjects effect were of interest. Duncan's multiple-range tests were used to determine post-hoc differences among means.

Comparison among Compounds: Operational Definitions of Sensitivity to Repellents. In most cases the dose–response relationship was best described by a nonlinear four parameter logistic equation of the form, R (response) $= (a - d)/[1 + (x/c)^b] + d$, where a is the asymptotic maximum relative consumption, b is the slope, d is the asymptotic minimum relative consumption, c is the inflection, and x is the concentration in milligrams per liter (Table 1). The minimum asymptotic consumption was constrained by a value of: mean − one standard error, in cases where the highest concentration tested was at the solubility limit of the compound. Otherwise, the minimum asymptotic value was constrained to be greater than zero, since negative intake was not a permissible

TABLE 1. PARAMETER VALUES FOR DOSE–RESPONSE RELATIONSHIPS OF CANDIDATE BIRD REPELLENTS

Code[a]	*a* maximum	*b* slope	*i* inflection	*d* minimum	intercept
M2MOB	.887	1.926	2013	0.01	
M4MOB	1.088	14.704	107	0.817	
2A45DMAP	1.036	1.966	170	0.262	
2A45DMBA		−0.035			1.135
MB	0.970	6.279	554	0.613	
AP	1.005	1.030	536	0.289	
SB		−0.035			0.911
ANTH	1.001	1.571	636	0.015	
ASA	1.067	4.967	1429	0.525	
2ABAL	1.200	1.068	435	0.180	
NNDMAN	0.880	1.257	827	0.300	
2ASAD		−0.154			1.345
BALN		0.010			0.959
MEBALN	1.011	8.093	3416	0.611	
OAP[a]	0.87	11.991	204	0.010	
MA[a]	1.03	1.745	980	0.060	

[a]Reference compounds used in the laboratory (Clark and Shah, 1991; Clark et al., 1991).

value. In the remaining cases, relative intake was independent of concentration, and a log linear equation was used to characterize the relationship. Selection of models was based upon minimization of the least squared error term.

There are several ways to estimate potency of a repellent. The slope is a measure of sensitivity to changes in irritant concentration. At the extremes, large values for slope indicate an "all-or-none" threshold response, i.e., birds may be insensitive to the repellent up to a threshold concentration, after which intake is maximally suppressed. Small values for slope indicate a graded threshold response, i.e., the suppression of fluid intake is more moderate per unit change of irritant concentration. Another measure of sensitivity is the displacement of the dose–response curve along the concentration axis as estimated by the inflection point. Low inflection values (leftward shift of the curve) indicate heightened responsiveness to irritant concentrations. High inflection values (rightward shift of the curve) indicate diminished responsiveness to the irritant. The final measure of sensitivity is suppression of fluid intake as measured by the minimum asymptotic consumption. Relying on a single measure, e.g. inflection, as used in toxicological studies, to categorize irritant potency may be misleading. For example, acetophenone and methyl benzoate have relatively similar inflection points (Table 1). Using this criterion, one would conclude that these two com-

pounds have similar potency. However, inspection of the minimum asymptotic intake shows that acetophenone yields a far greater suppression of fluid intake. Thus, the question becomes: which of the available dose–response indices is the most appropriate index of potency?

To better estimate which measures of sensitivity (or combinations thereof) should be used to characterize repellent potency, we used a principal-components analysis to define descriptive dimensions. z-transformed parameter values for the nonlinear logistic dose–response curves were used as variables. Similarities of compounds based upon a dissimilarity matrix (i.e., cosine) of principal axis scores were estimated using cluster analysis. A weighted average method was used to estimate linkage among groups. The variables weighted most heavily for each significant principal component axis were operationally defined as the relevant measures of sensitivity. These measure were then used to evaluate repellent potency relative to structural features of molecules.

RESULTS

Dose–Response Relationships of Individual Compounds. Maximum suppression of intake for methyl 2-methoxybenzoate was 15% of normal levels at 4578 mg/liter. The concentration-group profiles between the treatment and posttreatment periods for methyl 2-methoxybenzoate differed ($F = 3.21$, $df = 5$, $P = 0.02$). The post-hoc test for the day of treatment showed that intake for birds receiving 4578 mg/liter was less than for any other concentration group (Figure 2A, M2MOB). Intake for all groups during the posttreatment period returned to pretreatment levels. Absolute consumption differed across concentration groups and time ($F = 2.03$, $df = 2,60$, $P = 0.046$). Birds presented with concentrations of 915 mg/liter or more showed signs of enhanced avoidance during the fourth and sixth hours of exposure, suggesting that repellency was a postingestion conditioned response. At concentrations of 476 mg/liter or less consumption was constant across hours.

Intake of methyl 4-methoxybenzoate was 85% of normal at the highest concentration tested (Figure 2B, M4MOB), although the relative fluid intake profiles for the treatment and posttreatment days differed ($F = 2.80$, $df = 5,30$, $P = 0.034$). Post-hoc analysis across concentration-groups for the treatment day indicated that birds receiving 203 mg/liter differed from those receiving 20 mg/liter. There were no other differences among concentration groups. Fluid consumption returned to pretreatment levels for all groups during the posttreatment period. Absolute consumption for each of the concentration groups was similar as a function of time, indicating that the starlings showed no evidence of learned aversions ($P = 0.446$).

Intake of 2-amino-4,5-dimethoxyacetophenone was maximally reduced to

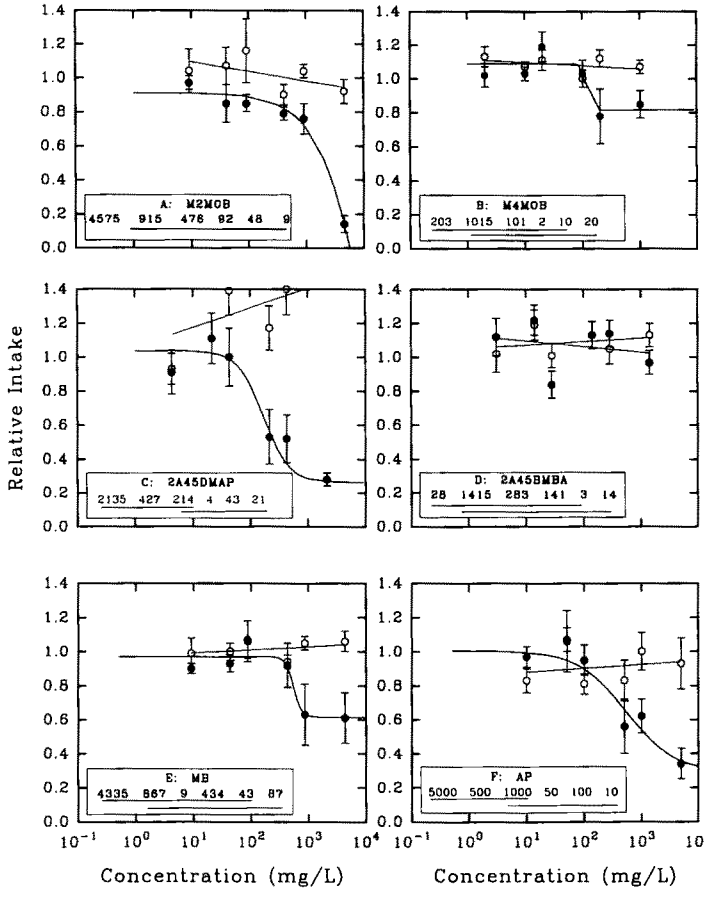


FIG. 2. Dose–response curves for (A) methyl-2-methoxybenzoate, (B) methyl-4-methoxybenzoate, (C) 2-amino-4,5-dimethoxyacetophenone, (D) 2-amino-4,5-dimethoxybenzoic acid, (E) methyl benzoate, and (F) acetophenone. Consumption is scaled relative to pretreatment water consumption. Solid dots depict fluid intake on the day the birds received chemical in their drinking water. Open circles depict relative water intake on the day following treatment. Vertical bars depict 1 SE. Inset: The Duncan's multiple-range post-hoc comparison for fluid intake for concentration groups on the day of treatment. Concentration groups are ranked based on relative fluid consumption, i.e., lowest to highest. Lines depict nonsignificant associations between concentration-group means.

28% of normal, with the overall pattern for relative fluid intake between the treatment and posttreatment periods differing ($F = 4.41$, $df = 5,30$, $P = 0.004$; Figure 2C, 2A45DMAP). The post-hoc test for fluid intake during the treatment period showed that consumption for the 2135 mg/liter and 427 mg/liter groups differed from that of groups receiving 43 mg/liter and below. Fluid intake was constant across time for each of the concentration-groups, indicating that repellency was sensory and not postingestionally conditioned ($P = 0.727$).

Intake of 2-amino-4,5-dimethoxybenzoic acid was not reduced relative to normal consumption. There was no significant interaction between concentration group and treatment period ($P = 0.408$), nor were there significant main effects for concentration or period ($P = 0.074$ and 0.665, respectively), i.e., fluid intake during the treatment and posttreatment periods was the same as for the pretreatment period for all test groups (Figure 2D, 2A45DMBA).

Fluid intake for methyl benzoate was reduced to only 63% of normal levels (Figure 2E, MB). Relative fluid intake differed for the treatment and posttreatment periods as a function of concentration ($F = 2.70$, $df = 5,30$, $P = 0.04$). The post-hoc test indicated that only the highest concentration tested (4335 mg/liter) differed from any of the lower concentrations tested. Posttreatment consumption was uniformly similar to pretreatment levels, suggesting that intake of methyl benzoate did not have any carryover effects. Neither was there evidence of a learned avoidance (bihourly) response during treatment administration ($P = 0.719$). Thus, the weak repellent effect was construed to be sensory in nature.

Maximal reduction of fluid intake for acetophenone was 34% of normal consumption (Figure 2F, AP). Relative intake for the treatment and posttreatment periods differed as a function of concentration ($F = 5.0$, $df = 5,30$, $P = 0.002$). The post-hoc comparison of relative fluid consumption during the treatment period indicated that intake for the 5000, 1000, and 500 mg/liter groups were similar, but differed from intake recorded for the 100, 50,10 mg/liter concentration groups. There was no evidence of a postingestional conditioned avoidance (i.e., absolute intake across time for all concentration groups was similar, $P = 0.465$).

Fluid intake for sodium benzoate was equal to normal (Figure 3A, SB). Relative intake profiles for the treatment and posttreatment periods were similar ($P = 0.119$). Although the dose–response curve for the treatment period indicated that there was no repellent effect, the significant period effect ($F = 29.93$, $df = 2,60$, $P \leq 0.001$) indicated that SB exerted a carryover effect. The increased loading experienced by birds in the higher concentration groups resulted in increased water consumption (higher than normal) during the posttreatment period. While there was no evidence of a sensorially mediated repellent effect, there was some evidence of a postingestional effect. The average concentration profiles across time were all similar ($P = 0.065$). However, there was a tendency

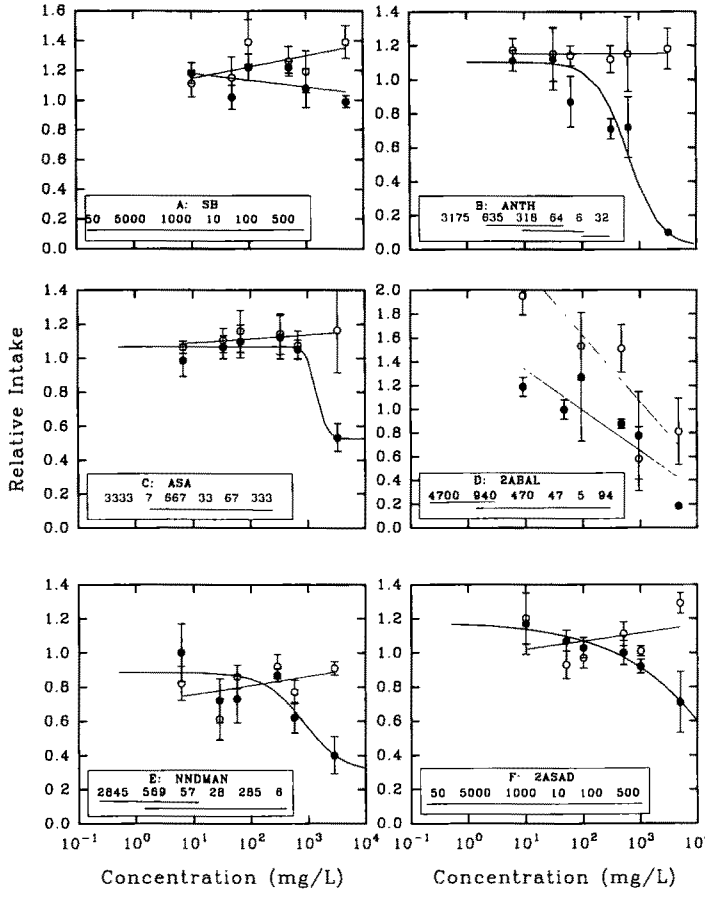


FIG. 3. Dose–response curves for (A) sodium benzoate, (B) anthranil, (C) acetylsalicylic acid, (D) 2-aminobenzyl alcohol, (E) *N,N*-dimethylanaline, and (F) 2-aminobenzene sulfonic acid. Refer to Figure 2 for description of figure particulars. Note the change of scale for intake for parts A and D.

for birds to drink less treated fluid as a function of time ($F = 28.93$, $df = 2,60$; $P < 0.001$). Thus, prolonged exposure (i.e., >4–6 hr) to SB might yield repellency in the context of a classical conditioned-avoidance paradigm.

Anthranil maximally suppressed fluid intake to 10% of normal consumption (Figure 3B, ATHN). The relative intake profiles for the treatment and posttreatment period were different ($F = 11.96$, $df = 5,30$, $P < 0.001$). The post-hoc test showed that intake for birds presented with 3175 mg/liter was different from

all other concentrations, while birds presented with 635 mg/liter differed from birds presented with 0.32 and 0.6 mg/liter. Absolute consumption across time for each of the concentration-groups was similar ($P = 0.90$), indicating the absence of a learned aversion.

At the highest concentration tested, acetylsalicylic acid reduced fluid intake to 55% of normal intake. The profiles for relative fluid intake differed across treatment periods ($F = 3.56$, $df = 5,30$, $P = 0.012$). Fluid intake by birds presented with 3333 mg/liter differed from all other concentrations (Figure 3C, ASA). There were no other concentration effects during the day of treatment. Water intake returned to pretreatment levels during the posttreatment period. Absolute consumption differed across concentration and time ($F = 2.34$, $df = 2,60$, $P = 0.021$). In general, for concentrations less than 667 mg/liter there was no indication of decreased intake as a function of time. At 3333 mg/liter, starlings tended to decrease consumption by the fourth and sixth hours, suggesting some postingestional influence on avoidance.

2-Aminobenzyl alcohol was not a sensory repellent; rather, it was a toxicant. Consumption was reduced at higher concentrations during the treatment day (Figure 3D, 2ABAL). However, the avoidance was likely due to malaise. This is evident from posttreatment consumption of water, where intake was inversely related to dose of the compound. Birds ingesting higher amounts of 2-aminobenzyl alcohol were more apt to experience malaise, while those ingesting lower doses increased their water consumption relative to pretreatment intake. Birds ingesting higher loads of 2-aminobenzyl alcohol were more apt to die: one bird from the 4700 mg/liter group died three days after ingestion of the compound, two birds from the 940 mg/liter group died one to two days after ingestion, and one bird from the 470 mg/liter group died two days after consuming treated water.

Relative fluid intake of *N,N*-dimethylaniline was reduced to 40% of normal intake. The relative profiles differed between the treatment and the posttreatment days ($F = 3.56$, $df = 5$, $P = 0.012$). The post-hoc test for fluid intake on the day of treatment showed that only the highest concentration (2845 mg/liter) differed from other concentration groups (Figure 3E, NNDMAN). Intake during the posttreatment day returned to pretreatment levels. There was no indication of a postingestion conditioned avoidance. Intake for all concentrations were similar across time ($P = 0.981$).

The maximum suppression of fluid intake for 2-aminobenzene sulfonic acid was 70% of normal. Nonetheless, relative fluid intake differed between the two treatment periods ($F = 5.68$, $df = 5,30$, $P \leq 0.001$). The post-hoc test indicated that the only difference among concentration groups was between the highest dose and the two lowest doses (Figure 3F, 2ASAD). Because the concentration group bihourly profiles were similar and there was no overall time effect, we

concluded that avoidance was attributable to sensory rather than postingestional effects.

β-Alanine was not repellent, with intake equal to normal across concentrations. The relative intake profiles across concentrations was similar between the two treatment periods ($P = 0.748$), and there was no concentration group effect ($P = 0.49$). However, there was a difference in overall intake between the treatment day and posttreatment day (Figure 4A, BALN; $F = 8.30$, $df = 1,30$, $P = 0.007$), with birds during the posttreatment period consuming relatively more water. Consumption was not related to any repellent feature of the treatment, however, because fluid intake during the treatment period was similar to pretreatment levels.

Fluid intake for the methylester of β-Alanine was suppressed to 65% of normal levels for the highest concentration tested, 4320 mg/liter. Relative intake across concentration groups differed between the treatment and posttreatment periods (Figure 4B; $F = 3.07$, $df = 5,30$, $P = 0.024$). Post-hoc tests revealed intake for the group receiving the 4320 mg/liter solution differed from groups receiving 86 mg/liter or less. Absolute consumption across concentration groups and time were similar ($P = 0.927$), indicating that there was no learned aversion.

Comparison among Compounds Using Multiple Estimates of Sensitivity. The importance of the dose-response curve parameters (Table 1) were evaluated using principal components analysis. Only the first two principal axes had eigenvalues greater than one: PC1 = 1.61 and PC2 = 1.13. These axes explained 40.3 and 28.3% of the variance among the dose-response parameters, respectively. The first principal component segregated the compounds along a suppression axis, while the second principal component segregated compounds along an inflection axis (Figure 5). Hereafter, the shift of the dose-response curve as influenced by the inflection value of the dose-response curve will be referred

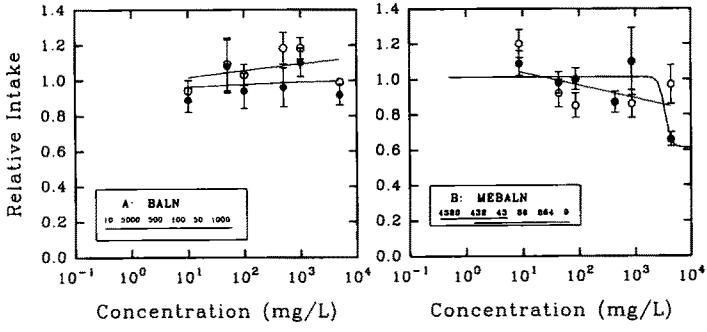


FIG. 4. Dose-response curves for (A) β-alanine and (B) the methyl ester of beta-alanine. Refer to Figure 2 for description of figure particulars.

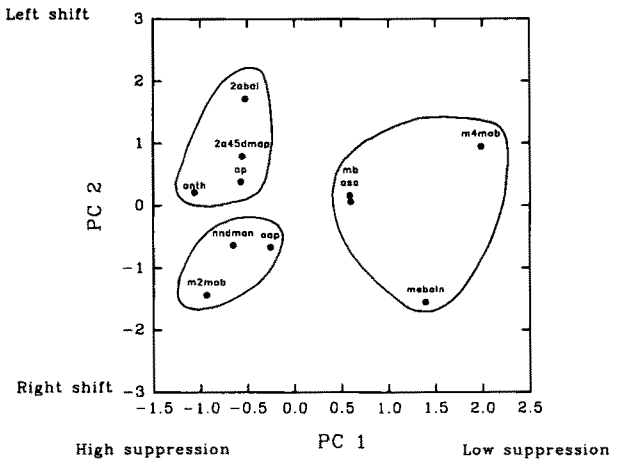


FIG. 5. A plot of the first two axes of a principal components analysis of the dose–response parameters. The first principal axis can be thought of as a suppression axis, while the second axis can be thought of as a inflection shift axis. Codes for compounds are defined in Table 1. Only those compounds described by the nonlinear logistic model were included in this analysis. OAP and MA were included as reference points. The circles enclosing compound codes are groups of similar dose–response principal components scores as determined by cluster analysis.

to as sensitivity to the compound. Compounds positioned in the (+,+) or (+,−) quadrants tended to weakly suppress fluid intake. Relative to two previously well-described bird repellents, methyl anthranilate and *o*-aminoacetophenone, these compounds did not strongly suppress fluid intake, nor did birds exhibit strong sensitivity to the compounds (Figure 6A). Thus, acetylsalicylic acid, methyl benzoate, the methyl ester of β-alanine, β-alanine, methyl 4-methoxybenzoate, sodium benzoate, 2-aminobenzene sulfonic acid amine, and 2-amino-4,5-dimethoxybenzoic acid all can be considered ineffective bird repellents. Compounds positioned in the (−,+) and the (−,−) quadrants tended to significantly suppress fluid intake (Figure 5). Of these repellent compounds, birds tended to be more sensitive to those in the (−,+) quadrant.

Effects of Altering Compound Structure Relative to Reference Repellent Molecules. Relative to MA, substitution of the amino with a methoxy group (e.g., methyl-2-methoxybenzoate) did not affect suppression, but there was a slight decrease in sensitivity (Table 1, Figure 6B). Alternatively, moving the methoxy substitution to the para position (e.g., methyl 4-methoxybenzoate) resulted in a substantial loss of suppressive activity (Figure 6A).

Loading the phenyl ring with EDGs (e.g., 2-amino-4,5-dimethoxyaceto-

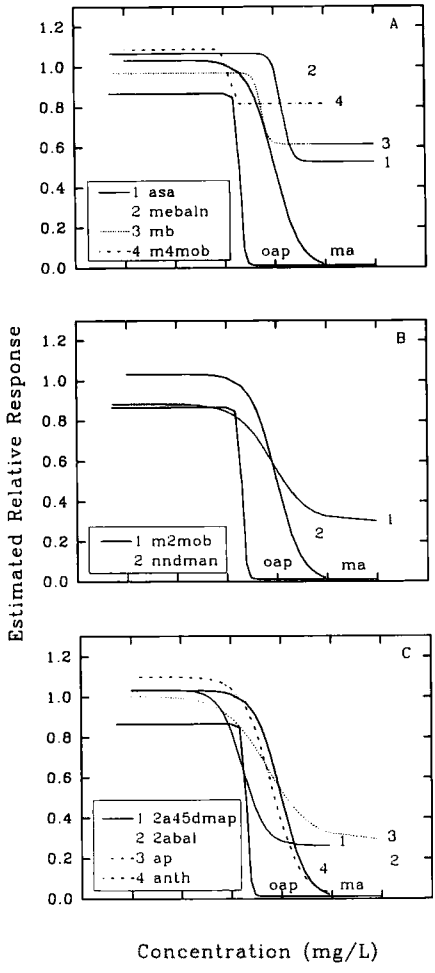


FIG. 6. A summary of the dose-response relationships for the 14 compounds tested. Curves are based upon least regression analyses of linear and nonlinear models (see text). Dose-response curves are groups based upon cluster relationships of principal component scores (Figure 5). (A) Compounds that are ineffective as repellents. These compounds have low suppressive activity and birds show low sensitivity, i.e., the response curve is shifted to the right of the reference compounds. (B) These compounds are repellent to varying degrees, all yielding significant suppressive activity. Birds show intermediate sensitivity to these compounds, i.e., the response curves are shifted to the right of the reference repellents, but left of the compounds defined as ineffective. (C) These compounds also exhibit suppressive activity, but birds are more sensitive to this group, i.e., the response curves are approximately the same as one of the reference repellents (MA).

phenone) did not substantially change the birds' sensitivity relative to the OAP reference, but it did result in a decrease in suppressive activity (Figure 6C).

Removing the EDG from MA (e.g., methyl benzoate) resulted in a decrease in suppression, but no substantial shift in sensitivity (Figure 6A). In contrast, there was a loss of sensitivity and suppressive activity for acetophenone (relative to OAP, Figure 6C). The EWG does not by itself detract from repellency. Anthranil possessed approximately the same level of sensitivity and suppression as MA (Figure 6C), indicating the importance of the acidic function of EWGs in detracting from repellency. The importance of acidity as a repellent detractor is further seen in the poor suppressive activity of 2-aminobenzene sulfonic acid.

Removing the EWG (e.g., *N*,*N*-dimethylaniline) decreased the suppressive activity but enhanced the functional response to concentration relative to MA. However, because *N*,*N*-dimethylanaline still clustered with other repellents, the data suggest that the absence of an EWG is not critical to repellency.

DISCUSSION

In general, our qualitative predictions about repellent activity were borne out by the data. However, the current study revealed several modifications to the general model. The presence or absence of EWGs or EDGs are important contributory factors for repellency, but the presence of both is not necessary for repellency. For example, despite the absence of an EDG and the consequent effect of electron depletion due to the EWG, acetophenone was repellent. However, the degree of suppression and sensitivity were diminished relative to responses observed for OAP (Table 1, Figure 6C). In the case of methyl benzoate, the diminution of suppressive activity relative to MA was sufficiently large so as to render methyl benzoate ineffective as a repellent (Figure 6A). Thus, while not critical for repellency, the EDG does contribute to enhancing repellency. Similarly the absence of an EWG does not eliminate repellency, but it does enhance repellent action. Birds were less sensitive to *N*,*N*-dimethylanaline and the compound's repellent action was less than either of the two reference molecules (Figure 6B).

It appears that the basicity of the EWG is an important feature regulating repellency of a compound. The presence of a carboxyl or other acidic function on the aromatic ring uniformly results in poor repellent action, irrespective of other attributes of the molecule. The detracting effects are amplified if the acidic function is within the EWG.

The presence of a negative charge on the phenyl ring is an additionally important feature for repellency. The salt of benzoic acid (sodium benzoate) tends to increase the delocalization of lone pairs of electrons between the two oxygens and carbon, resulting in a moderately more basic aqueous solution

(relative to the nonrepellent benzoic acid). Nonetheless, sodium benzoate is not repellent. Because the carboxylic carbon is less electropositive, repellency may be diminished. Benzaldehyde, which possesses a carbonyl group (i.e., is very reactive to nucleophilic attack) but lacks EDG substituents, is a potent repellent (Clark, in preparation). In this case, the alpha carbon is very electropositive and is open to nucleophilic attack. Furthermore, the molecule is more basic relative to sodium benzoate and benzoic acid. So long as substitution at the alpha carbon site is with relatively electron-donating groups, such as CH_3 (e.g., acetophenone), the molecule will exhibit some repellent action. The fact that acetophenone is only moderately repellent and benzaldehyde is strongly repellent, suggests that electrophilicity of the alpha carbon is important.

Simply loading the phenyl ring with EDGs may not yield a better repellent. The addition of OCH_3s in the 4- and 5-carbon positions of 2-aminobenzoic acid and 2-aminoacetophenone might mitigate the effects of electron donation by shifting the pi orbitals out of the phenyl ring's plane, thus causing steric inhibition. Another possibility is that the OCH_3s alter the charge distribution around the phenyl ring relative to the unsubstituted moieties.

Finally, the phenyl ring itself is critical to repellency. Neither β-alanine nor its methyl ester were repellent relative to *o*-aminoacetophenone and methyl anthranilate (the only difference between the pairs of molecules being the presence or absence of the aromatic ring).

In summary, the most critical features of an avian repellent (in order of importance) appear to be: (1) the presence of a phenyl ring; (2) the basicity of the molecule in general, and specifically, when an electron withdrawing group is present, the absence of an acidic function within the EWG functionality; and (3) the electronegativity of the phenyl ring. Steric effects and extreme delocalization of lone pairs of electrons (as occurs with meta isomers and aromatics multiply substituted with EDGs) tends to interfere with repellency. These features are typified by two well-described avian repellents, methyl anthranilate and *o*-aminoacetophenone (MA-OAP).

Considerable effort has been directed towards the identification of mammalian chemoreceptor types, but no work has focused on birds. For mammals, numerous models have been put forward to explain perception of chemically induced pain (Nielsen, 1991). These models generally belong to one of three categories. First, stimuli, e.g., acids, may cause physical damage to cells, resulting in release of endogenous substances such as bradykinins, serotonin, and histamine, which bind to specific receptor sites on chemoreceptive fibers, resulting in neuronal depolarization. Second, external stimuli may specifically bind to receptors directly. Third, external stimuli may adsorb near the chemoreceptive fiber surface and the interaction of the physicochemical effects may nonspecifically cause neuronal depolarization. It is important to bear in mind that the mechanism for sensory irritation need not be mutually exclusive. There is ample

evidence to suggest that all three types of mechanisms exist within trigeminal/somatosensory chemoreceptive fibers.

The form of a mammalian capsaicin receptor may consist primarily of a benzene site associated with a thiol/hydrogen bond donating site that reacts with the C—C double bond and hydrogen bond acceptor site (Solzcsanyi and Jancso-Gabor, 1975; Nielsen, 1991). Interaction with the thiol site is assumed to be critical for the activation of the benzene site. Thus, the long-chain alkyl and the aromatic OCH_3 and OH are all critical features of the mammalian capsaicin receptor. Capsaicin and its analogs do not influence avian irritation. However, vanillyl derivatives lacking the long alkyl chain do act as avian irritants (Shah et al., 1991). This suggests that the thiol site is absent in birds and in its place may be a charged protein. This protein would interact with the EDG, either as a hydrogen donor or via electrostatic attraction. Thus, the difference between the mammalian capsaicin receptor and the proposed avian methyl anthranilate/*o*-aminoacetophenone receptor may simply reflect the loss of expression of a thiol site. While this model clearly requires careful experimental consideration, it does suggest that a broader comparative approach toward vertebrate irritants is warranted.

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USE OF WATER AND EDTA EXTRACTIONS TO ESTIMATE AVAILABLE (FREE AND REVERSIBLY BOUND) PHENOLIC ACIDS IN CECIL SOILS[1]

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Abstract—Sterile and microbe reinfested Cecil A_p and B_t soil materials amended with 0 to 5 μmol/g of ferulic acid, *p*-coumaric acid, *p*-hydroxybenzoic acid, or vanillic acid were extracted after varying time intervals with water, EDTA, or NaOH to characterize sorption of cinnamic and benzoic acid derivatives and to determine the effectiveness of water and EDTA extractions in estimating concentrations of free and reversibly bound phenolic acids in soils. Basic EDTA (0.5 M, pH 8) extractions and water extractions provided good estimates of both free and reversibly bound cinnamic acid derivatives, but not of benzoic acid derivatives. Neutral EDTA (0.25 M, pH 7) and water extractions, however, were effective for both cinnamic and benzoic acid derivatives Rapid initial sorption of both cinnamic and benzoic acid derivatives was followed by slow long-term sorption of the cinnamic acid derivatives. Slow long-term sorption was not observed for the benzoic acid derivatives. The amount of sorption of phenolic acids in soil materials was directly related to the concentration of phenolic acids added to soil materials. The addition of a second phenolic acid to the soil materials did not substantially affect the sorption of each individual phenolic acid. Sodium hydroxide extractions, which were made only after phenolic acids in phenolic acid-amended and non-amended soil material were depleted by microbes, confirmed that neutral EDTA and water extractions of soils can be used to make accurate estimates of baseline (residual) levels of free and reversibly bound phenolic acids available to soil microbes and, thus, potentially to seeds and roots.

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[1]The use of trade names in this publication does not imply endorsement by the North Carolina Agricultural Research Service of products named, nor criticism of similar ones not mentioned.

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Key Words—EDTA extraction, water extraction, NaOH extractions, Cecil soil, ferulic acid, *p*-coumaric acid, *p*-hydroxybenzoic acid, vanillic acid, sorption, free phenolic acids, reversibly bound phenolic acids, allelopathy, soil microbial ecology.

INTRODUCTION

Allelopathic interactions involving phenolic acids have been frequently described (see reviews by Rice, 1984; Kuiters, 1990; Siqueira et al., 1991). However, most of these studies lacked satisfactory information about the available (i.e., free and reversibly bound) concentrations of phenolic acids in soils. Determining available concentrations of phenolic acids in soils is an initial step in establishing the importance of allelopathic interactions in natural and managed ecosystems. Estimates of phenolic acids in soils have been obtained by using various extractants and extraction procedures (Guenzi and McCalla, 1966; Turner and Rice, 1975; Chou and Patrick, 1976; Kaminsky and Muller, 1977, 1978; Whitehead et al., 1981, 1982, 1983; Dalton et al., 1983, 1987, 1989; Blum et al., 1991). The extractants most frequently used have been water, ethylenediamine tetraacetic acid (EDTA), and sodium hydroxide (NaOH). Water extractions recover primarily phenolic acids in soil solution, but most of the reversibly bound phenolic acids are not released from soil particles during water extraction. Alkaline NaOH extractions, in addition to extracting free and bound phenolic acids, can partially solubilize and hydrolyze the organic materials present in soils, thereby introducing phenolic acids that normally would not be in the soil solution (Kaminsky and Muller, 1978; Kaminsky, 1980). The amount of organic matter solubilized and hydrolyzed by NaOH extraction is directly related to the NaOH concentration and the extractant pH (Stevenson, 1982). Chelating agents, such as EDTA, recover phenolic acids in soil solution and a portion of the phenolic acids that are reversibly bound (Kaminsky and Muller, 1977; Kaminsky, 1980; Dalton et al., 1987; Blum et al., 1992). Although determinations have been attempted, it is still unclear how much of the reversibly bound fraction recovered by EDTA is actually available to soil organisms and how much of the reversibly bound phenolic acids available to soil organisms is not recovered by EDTA.

Kaminsky (1980) used phenolic acid dose–response curves for lettuce seed germination in aqueous solutions and soils to determine "estimates of phenolic acid availability in soils" (EPAS). The EPAS and phenolic acid concentrations in soils determined by neutral EDTA and NaOH extractions were then compared. Based on these comparisons, Kaminsky concluded that neutral EDTA extracted only available (i.e., free and reversibly bound) phenolic acids. Based on water and EDTA extractions of ferulic acid-amended sterile and microbe reinfested Cecil soil materials, Blum et al. (1992) concluded that basic EDTA

extractions provide good estimates of ferulic acid availability to soil microbes. In the research reported here, we provide additional information on the effectiveness of water and EDTA extractants in estimating concentrations of free and reversibly bound cinnamic (ferulic and *p*-coumaric) and benzoic (*p*-hydroxybenzoic and vanillic) acid derivatives available to soil microbes and, thus, potentially to seeds and roots.

METHODS AND MATERIALS

Samples from A_p and B_t horizons (from the same pedon) of a Cecil (Typic Kanhapludults, clayey, kaolinitic, thermic) soil from the Piedmont of North Carolina were air-dried, sieved (0.25 mm), and characterized. The Cecil soil material contained 2.5 and 36 g/kg of gibbsite, 5 and 84 g/kg Fe_2O_3, 37.5 and 450 g/kg kaolinite, and 37 and 2 g/kg of organic matter, for the A_p and B_t horizon, respectively (Dalton et al., 1987). Air-dried soil materials were stored at room temperature in the laboratory. One gram of soil was placed into a 15-ml Corex tube (Corning Inc., Rochester, New York), capped (Bacti-Capall, Sherwood Medical Industries), and autoclaved three times (15 min at 1.2 kg/cm^2 and 121°C) at three-day intervals. A 1.5 ml-aliquot of filter-sterilized (0.2-μm Supor-200 membrane filter; Gelman Sciences, Ann Arbor, Michigan) ferulic acid, *p*-coumaric acid, *p*-hydroxybenzoic acid, and/or vanillic acid (Sigma, St. Louis, Missouri), adjusted to pH 5 with NaOH, was added aseptically to tubes. Concentrations of phenolic acids added ranged from 1.25 to 5 μmol/g soil. Two-acid mixtures (2.5 μmol/g each) of some of these phenolic acids (Table 1) also were added. The racks of tubes containing the soil materials and phenolic acids were stored at 24°C in a dark incubator with a pan of water (experiment 1) or in closed plastic bags (all other experiments).

Soil Reinfestation. Soil extracts (2:1 water–soil mixture, v/w) for reinfestation were obtained from soil–sand mixtures (1:2 soil–sand mixture totaling 150 g) that had been treated with 5 ml of a phenolic acid mixture (0.5 mM of ferulic acid, *p*-coumaric acid, and vanillic acid), 15 ml water, and 10 ml of double-strength Hoagland's solution (Hoagland and Arnon, 1950) and incubated in the dark at 30°C for three days (Blum and Shafer, 1988). Phenolic acids were added to enhance phenolic acid-utilizing microbial populations. No phenolic acids were detected by HPLC analysis in the soil extracts used to reinfest soil materials.

After a specific number of days (Table 1), half the tubes received 0.5 ml of filtered (Whatman No. 42) soil extract obtained from the appropriate Cecil soil material and 0.5 ml double-strength Hoagland's solution (pH 5). This procedure reinfested the soil with microorganisms. The other half, i.e., controls, received 0.5 ml filter-sterilized soil extract and 0.5 ml filter-sterilized double-

TABLE 1. EXPERIMENTAL PROTOCOLS

Experiment	Phenolic acids	Concentrations (μmol/g)[a]	Time of microbial reinfestations (days)[b]	Extractants	Time of extractions (days)[b]	
					Sterile	Reinfested
1A	ferulic *p*-coumaric vanillic	1.25, 2.5, 3.75 and 5	21	0.5M EDTA, pH 8 water	42	42
1B	two-acid mixtures	2.5 plus 2.5	21	0.5 M EDTA, pH 8 water	42	42
2	ferulic *p*-coumaric	2.5	24	0.25 M EDTA, pH 7 water	14 24 34 48 62 124	28 32 36 49 62
				0.5 M EDTA, pH 8	14 24	28
3	vanillic *p*-hydroxybenzoic	2.5[c]	50	0.25 M EDTA, pH 7 water 1 M NaOH[d]	14 25 36 48	74
4	ferulic *p*-coumaric	2.5[c]	50	0.5 M EDTA, pH 7 1 M NaOH	74	74

[a] Molecular weights of ferulic acid, *p*-coumaric acid, *p*-hydroxybenzoic acid, and vanillic acid are 194.2, 164.2, 138.1, and 168.2, respectively.
[b] Number of days after the addition of phenolic acids.
[c] Soil materials to which no phenolic acids were added were also extracted 24 days after reinfestation.
[d] Used only for day 74 extractions.

strength Hoagland's solution. Tubes and their contents were vortexed at approximately weekly intervals after reinfestation.

Soil Extractions. Soil samples in the tubes were extracted with water, EDTA, or 1 M NaOH at varying time intervals before or after reinfestation (Table 1). Extractant (2.5 ml) was added to each tube so that the final solution volume was approximately 5 ml. Actual water content in the tubes for each extraction period was determined gravimetrically from a subset of test tubes (105°C).

EDTA extractants were prepared as follows: Disodium ethylenediamine tetraacetic acid (374.24 g) was added to 500 ml water in a beaker. Sodium hydroxide pellets were added very slowly as the EDTA–water suspension was stirred rapidly until the EDTA was dissolved. The resulting solution had a pH of approximately 8.5. The pH of this solution was adjusted to 8 or 7 with concentrated hydrochloric acid. The final volume of the solution was brought to 1 or 2 liters, depending on the concentration required in the tubes [e.g., to 1 liter (0.5 M) for 0.25 M desired in tube].

For the water extractions, tubes were vortexed and then centrifuged immediately after the addition of water. For the EDTA or NaOH extractions, tubes were vortexed immediately after the addition of the extractant and 5 hr later just before centrifugation. Tubes were stored at room temperature in the dark during the 5-hr extraction period. Tubes were centrifuged for 10 min at 12,100*g*. Humic acids were precipitated by adjusting NaOH extracts to a pH of approximately 2.5. The resulting supernatants were filter-sterilized (0.2-μm membrane filters) and the extracted phenolic acids were quantified by HPLC.

Phenolic Acid Isolation and Quantification. Phenolic acids were isolated and quantified with a Waters (Milford, Massachusetts) fully automated HPLC equipped with a model 484 absorbance detector set at 254 nm. A 4-μm particle size Nova-pak C_{18} Radial Pak cartridge in a RCM-100 cartridge holder was eluted for 20 min with 22% methanol, 0.5% ethyl acetate, 0.9% acetic acid, and 76.6% water for the cinnamic acid derivatives, and 17% methanol, 0.4% ethyl acetate, 0.8% acetic acid, and 81.8% water for the benzoic acid derivatives to separate and quantify the phenolic acids. Background phenolic acid content of soils was determined by eluting extracts with a methanol–ethyl acetate–acetic acid–water gradient (Blum et al., 1991). Identification and quantification were confirmed by comparing retention times and areas with those of standard amounts of the appropriate phenolic acids. Standard phenolic acids were purchased from Sigma Chemical Company (St. Louis, Missouri).

Stability of Phenolic Acids in Solution. To test the stability of cinnamic acid and benzoic acid derivatives in extractant solutions, 0.5 mM concentrations of ferulic acid, *p*-coumaric acid, vanillic acid, or *p*-hydroxybenzoic acid in water (pH 4, 5.5, 7.5 or 8.5), 0.5 or 0.25 M EDTA (pH 7, 7.5 or 8.0) solution, or 1 M NaOH (pH 12.5) solution were analyzed by HPLC after 5 min, 5 hr,

and/or 24 hr storage in the dark at room temperature. The pH 4 water solutions were used as controls.

Data Analyses. Data ($N = 2$) from the various experiments described in Table 1 were analyzed with SAS (1988) PROC ANOVA and PROC GLM. Since coefficients of variation for the data were very small, most $<1\%$, two replicate samples were adequate.

RESULTS

Experiment 1A. Recovery of ferulic acid, *p*-coumaric acid, and vanillic acid from soil materials was significantly affected by initial concentration added, extractant (water, 0.5 M, pH 8 EDTA), soil materials (Cecil A_t, B_p), presence or absence of microbes, and all interactions of these factors.

Water and 0.5 M pH 8 EDTA were good extractants for the estimation of free and reversibly bound cinnamic acid derivatives. This was not the case for benzoic acid derivatives, which were not stable in the 0.5 M, pH 8 EDTA extractant. With the exception of vanillic acid, EDTA extracted more phenolic acids from either soil materials than did water (Table 2). Approximately 8% more vanillic acid was extracted from sterile soil material by water than by EDTA. We subsequently determined that the cinnamic acid derivatives, ferulic acid, and *p*-coumaric acid, were not detectably modified over a 5-hr period in 0.5 M, pH 8 EDTA solution. The benzoic acid derivatives, vanillic acid and *p*-hydroxybenzoic acid, were very unstable in the presence of 0.5 M pH 7.5 and pH 8 EDTA. Within 5 min after these benzoic acid derivatives were added to 0.5 M, pH 8 EDTA solution, 63 and 73% of the initial vanillic acid and *p*-hydroxybenzoic acid, respectively, were no longer detectable by HPLC analysis. Concentrations of the benzoic acid derivatives were not modified over 5 hr in water solutions ranging from pH 4 to pH 8.5. Subsequently, tests with various concentrations of EDTA over a range of pH values suggested that both *p*-hydroxybenzoic and vanillic acids are fairly stable in 0.25 M, pH 7 EDTA for up to 24 hr. A comparison of the effectiveness of 0.25 M, pH 7 EDTA and 0.5 M, pH 8 EDTA in the recovery of ferulic acid and *p*-coumaric acid suggested that 0.25 M, pH 7 EDTA was as good or better than 0.5 M, pH 8 EDTA (unpublished data) in extracting these cinnamic acid derivatives from Cecil soil materials.

The amount of phenolic acids recovered by water or 0.5 M, pH 8 EDTA in the absence of microbes was related directly to the initial concentration (Table 2). In the presence of microbes, linear relationships between initial concentrations and recovery 21 days after reinfestation were not always evident due to microbial metabolism of these phenolic acids.

The percentage of the phenolic acids sorbed to the soil materials (not

TABLE 2. PARTIAL REGRESSION COEFFICIENTS, P, AND R^2 VALUES FOR RECOVERY OF DIFFERENT CONCENTRATIONS OF FERULIC ACID, p-COUMARIC ACID, AND VANILLIC ACID FROM CECIL A_p AND B_t SOIL MATERIALS[a]

Compound	Soil Microbes	Extractant	Intercept	Linear	P	r^2
A_p						
Ferulic acid	Absent	EDTA	0.008	0.512	0.0001	0.99
	Absent	H_2O	−0.047	0.210	0.0001	0.99
	Present	EDTA	0.008	0.004	0.0006	0.88
	Present	H_2O			NS[b]	
p-Coumaric acid	Absent	EDTA	0.009	0.810	0.0001	0.99
	Absent	H_2O	−0.020	0.300	0.0001	0.99
	Present	EDTA	0.003	0.004	0.0035	0.78
	Present	H_2O			NS	
Vanillic acid	Absent	EDTA	0.046	0.315	0.0001	0.95
	Absent	H_2O	0.098	0.371	0.0001	0.99
	Present	EDTA			NS	
	Present	H_2O	0.002	0.002	0.0475	0.51
B_t						
Ferulic acid	Absent	EDTA	0.001	0.596	0.0001	0.99
	Absent	H_2O	0.056	0.097	0.0001	0.99
	Present	EDTA	0.012	0.015	0.0001	0.94
	Present	H_2O			NS	
p-Coumaric acid	Absent	EDTA	−0.048	0.799	0.0001	0.99
	Absent	H_2O	0.054	0.156	0.0001	0.99
	Present	EDTA			NS	
	Present	H_2O			NS	
Vanillic acid	Absent	EDTA	0.010	0.242	0.0001	0.99
	Absent	H_2O	0.038	0.265	0.0001	0.99
	Present	EDTA			NS	
	Present	H_2O			NS	

[a] Dependent variable: micromoles phenolic acid recovered per gram of soil; independent variable: micromoles phenolic acid added per gram of soil.
[b] Not significant at 0.05.

recovered by water) ranged from approximately 57% for vanillic acid in Cecil A_p soil material to 89% for ferulic acid in Cecil B_t soil material (Figures 1–3). Sorption is defined as any chemical process that causes loss of phenolic acids from solution when interacting with the soil phase, including surface-mediated oxidation or polymerization into organic matter. Sorption of all phenolic acids was approximately 10% greater in B_t than in A_p soil materials. EDTA recovered 40% of the sorbed ferulic acid from the Cecil A_p soil material and 54% from

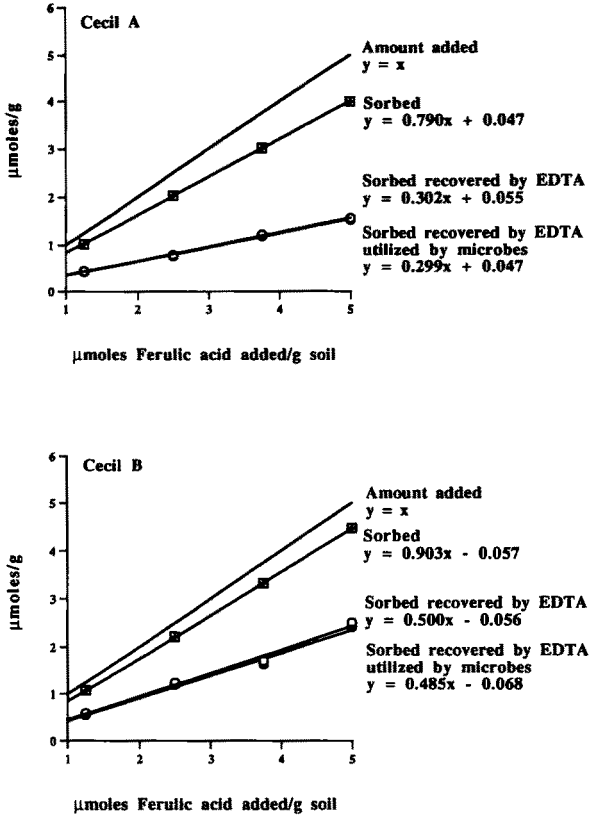

FIG. 1. Sorption of 1.25–5 µmol ferulic acid/g soil material (42 days after addition), sorbed ferulic acid recovered by 0.5 M, pH 8 EDTA, and sorbed ferulic acid recovered by EDTA that was utilized by soil microbes in Cecil A_p and B_t soil materials in the presence (21 days after reinfestation) or absence of microbes. Points are mean values (N = 2). The absence of standard error bars indicates that the standard error bars are smaller than the symbol representing the mean.

the Cecil B_t soil material. EDTA recovered approximately 74% of the sorbed *p*-coumaric acid from the A_p and B_t soil materials. In addition to the free phenolic acids, more than 94% of the sorbed phenolic acids that were recovered by the EDTA extractant could be metabolized by soil microbes.

Experiment 1B. Recovery and sorption of ferulic acid, *p*-coumaric acid, and/or vanillic acid in experiment 1B were similar to those of experiment 1A. The recoveries of individual phenolic acids from soil materials that had been treated with two phenolic acids, when significant, were only slightly modified

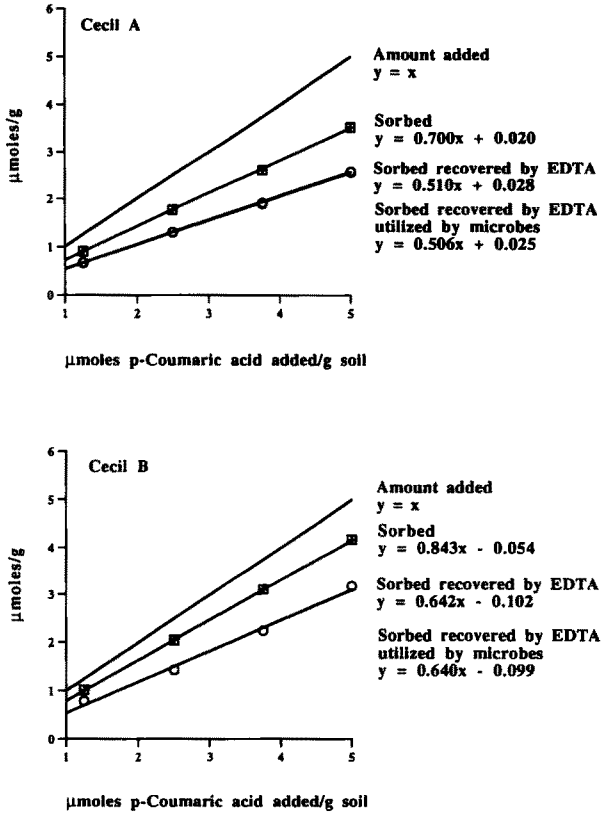

FIG. 2. Sorption of 1.25–5 μmol *p*-coumaric acid/g soil material (42 days after addition), sorbed *p*-coumaric acid recovered by 0.5 M, pH 8 EDTA, and sorbed *p*-coumaric acid recovered by EDTA that was utilized by soil microbes in Cecil A_p and B_t soil materials in the presence (21 days after reinfestation) or absence of microbes. Points are mean values ($N = 2$). The absence of standard error bars indicates that the standard error bars are smaller than the symbol representing the mean.

($\pm$ <5%) by the presence of the second phenolic acid (data not presented). Such small changes suggested that sorption of individual phenolic acids in two-acid mixtures were independent of the other phenolic acid present in these hydrated soil materials.

Experiment 2. A comparison of sorption (i.e., amount not recovered by water extraction from sterile soil materials) of ferulic acid and *p*-coumaric acid in experiment 1 (see text) and experiment 2 (Table 3) indicated a 20% lower sorption in experiment 2. Metabolism of reversibly sorbed phenolic acids by

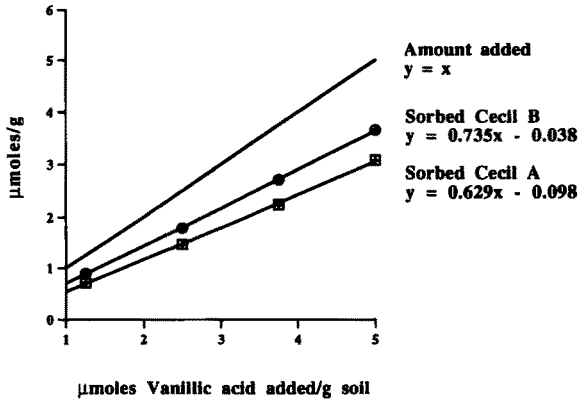

FIG. 3. Sorption of 1.25–5 µmol vanillic acid/g soil material (42 days after addition) in Cecil A_p and B_t soil materials in the absence of microbes. Points are mean values (N = 2). The absence of standard error bars indicates that the standard error bars are smaller than the symbol representing the mean.

TABLE 3. AMOUNTS OF FERULIC ACID AND *p*-COUMARIC ACID ADDED TO CECIL SOIL MATERIALS SORBED AFTER 62 DAYS, SORBED RECOVERED BY 0.25 M EDTA (pH 7), AND SORBED RECOVERED BY EDTA UTILIZED BY SOIL MICROBES

Compound	Soil	Sorbed[a]	Sorbed recovered by EDTA[b]	Sorbed recovered by EDTA utilized by microbes[c]
Ferulic	A_p	1.40 ± 0.005 (56)	0.42 ± 0.009 (30)	0.42 ± 0.009 (100)
	B_t	1.75 ± 0.013 (70)	0.96 ± 0.029 (55)	0.96 ± 0.029 (100)
p-Coumaric acid	A_p	0.99 ± 0.009 (40)	0.42 ± 0.023 (42)	0.41 ± 0.023 (98)
	B_t	1.42 ± 0.003 (59)	1.06 ± 0.012 (71)	1.03 ± 0.012 (98)

[a] Sorbed = amount not recovered by water extraction from sterile soil materials ± standard error. Values in parentheses are percentage based on 2.5 µmol/g initially added to soil materials.

[b] For sterile soils, the difference in recovery between water and EDTA (0.25 M, pH 7) extractions ± standard error. Values in parentheses are percentages of sorbed phenolic acids that were recovered by EDTA.

[c] Differences between sorbed phenolic acid recovered by EDTA extraction (EDTA − water) from sterile soil materials and soil materials reinfested with microbes ± standard error. Values in parentheses are percentages of the recovered phenolic acids that were utilized by microbes.

microbes, however, was similar in the two experiments. The only obvious difference between these two experiments was that test tube racks holding the soil samples were stored in closed plastic bags in experiment 2. The bags were used to reduce evaporation of water from the tubes in the incubator. Tubes from experiment 2 contained 10–15% more water at soil extraction time than did tubes from experiment 1.

Approximately 18% of the cinnamic acid derivatives added to soil materials could not be recovered by EDTA extractions made after the first 14 days (Table 4; Figures 4 and 5). Most of this sorption probably occurred immediately after the addition of the phenolic acids to the soil (Dalton et al., 1989). After 124 days, this fraction constituted 58 and 42% of ferulic acid and 19 and 21% of *p*-coumaric acid added to Cecil A_p and B_t soil materials, respectively.

Microbial metabolism of phenolic acids was much more rapid than the slower sorption (day 14 to 124) in sterile soil materials and was greater in the A_p horizon than the B_t horizon soil materials over the first 14 days after reinfestation (Figures 4 and 5). The differences in rates between A_p and B_t horizon materials were most likely due to differences in the initial microbial populations (Blum and Shafer, 1988). For both soils, microbial metabolism slowed considerably one week after reinfestation; this was probably due either to nutrient limitations or to the rates of release of the reversibly bound forms.

Experiment 3. All sorption of the benzoic acid derivatives occurred prior to day 14 (Figures 6 and 7), most likely immediately upon addition of benzoic acids to the soil materials (Dalton et al., 1989). Sorption (amount not recovered by water extraction) for both *p*-hydroxybenzoic acid and vanillic acid was 10%

TABLE 4. PARTIAL REGRESSION COEFFICIENTS, P, AND R^2 VALUES FOR RECOVERY OF FERULIC ACID AND *p*-COUMARIC ACID FROM STERILE CECIL A_p AND B_t SOIL MATERIALS DURING DAY 14 TO DAY 124 AFTER ADDITION OF PHENOLIC ACID[a]

Compound	Soil	Extract	Intercept	Linear	Quadratic	P	r^2
Ferulic acid	A_p	EDTA	2.219	−0.014	3.78×10^{-5}	0.0001	0.99
		H_2O	1.917	−0.017	5.38×10^{-5}	0.0001	0.99
	B_t	EDTA	2.167	−0.009	2.20×10^{-5}	0.0001	0.98
		H_2O	0.733	0.003	-3.14×10^{-5}	0.0001	0.89
p-Coumaric acid	A_p	EDTA	2.136	−0.002		0.0136	0.47
		H_2O	1.868	−0.008	2.93×10^{-5}	0.0001	0.99
	B_t	EDTA	2.090	0.0006	-1.16×10^{-5}	0.0001	0.89
		H_2O	NS[b]				

[a] Dependent variable; μmoles phenolic acid recovered/g soil; independent variable: days after addition of phenolic acids.
[b] Not significant at 0.05.

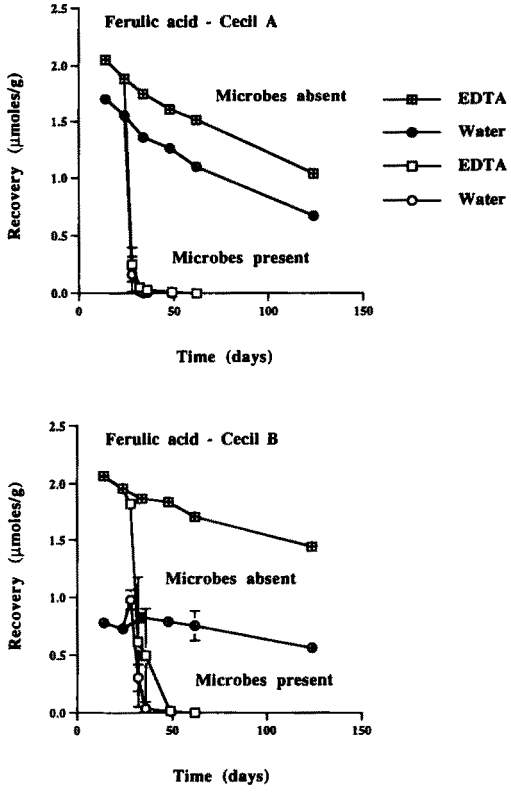

FIG. 4. Recovery of 2.5 µmol ferulic acid/g soil material from Cecil A_p and B_t soil materials by 0.25 M, pH 7 EDTA and water in the absence of microbes (days 14–124) or in the presence of microbes (day 24 to 62). Soil materials were reinfested on day 24. Points are mean values ($N = 2$). The absence of standard error bars indicates that the standard error bars are smaller than the symbol representing the mean.

for Cecil A_p and 43% for Cecil B_t. The amount of benzoic acid derivatives not recovered by EDTA extractions was 0.6% for Cecil A_p and 5% for Cecil B_t.

Experiment 4. In the presence of microbes, recovery by EDTA eventually ceases due either to the depletion of the reversibly bound forms or the inability of the EDTA extraction procedure to recover the more tightly, but still reversibly, bound forms. A more rigorous extraction procedure that would not create artifacts (e.g., oxidation products) could help in identifying the actual cause of the decline in recovery by EDTA. Unfortunately, an ideal extraction procedure does not exist. We used a 5 hr–1 M sodium hydroxide extraction procedure.

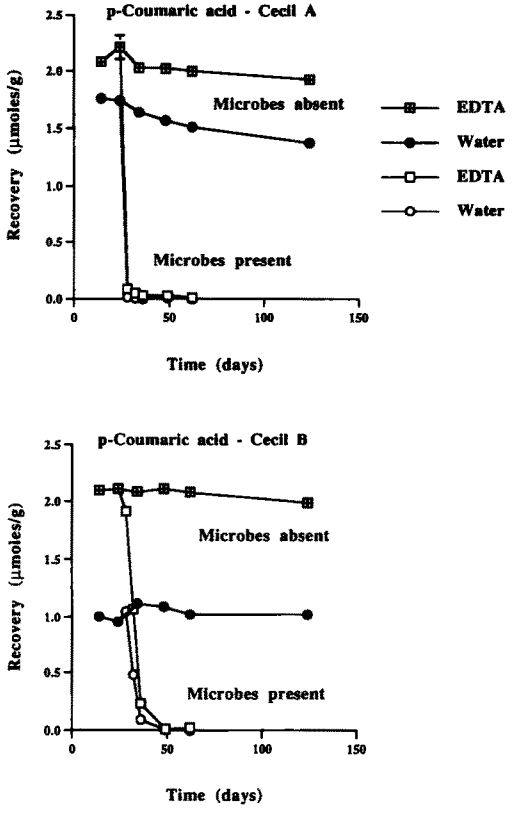

FIG. 5. Recovery of 2.5 μmol *p*-coumaric acid/g soil material from Cecil A_p and B_t soil materials by 0.25 M, pH 7 EDTA and water in the absence of microbes (days 14–124) or in the presence of microbes (days 24–62). Soil materials were reinfested on day 24. Points are mean values (N = 2). The absence of standard error bars indicates that the standard error bars are smaller than the symbol representing the mean.

Concentrations of phenolic acids were not modified over 5 hr in 1 M, pH 12.5 NaOH solution.

Sodium hydroxide extractions were made only after EDTA extractions of microbe reinfested soil materials recovered <0.01 μmol/g soil phenolic acids. Recoveries of phenolic acids by 1 M, pH 12.5 NaOH from phenolic acid-amended soils were equal to or greater than those from soils that had not been amended with phenolic acids (Figure 8). The differences in the amount recovered from amended and nonamended phenolic acids soils represented sorbed phenolic acids that were not recoverable by EDTA and, thus, not available to microbes.

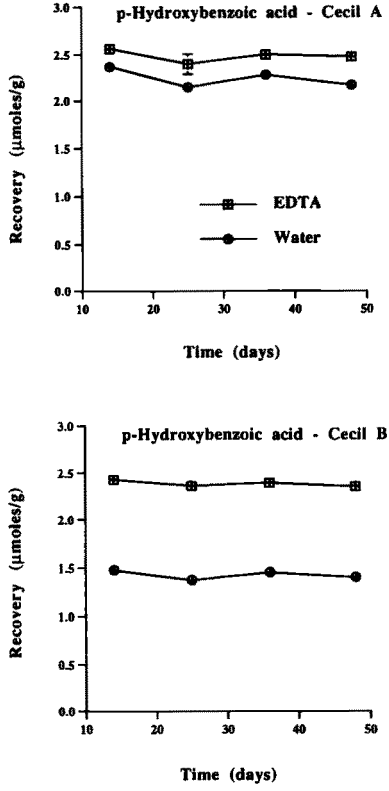

FIG. 6. Recovery of 2.5 μmol *p*-hydroxybenzoic acid/g soil material from Cecil A_p and B_t soil materials by 0.25 M, pH 7 EDTA and water in the absence of microbes (days 14–48). Points are mean values ($N = 2$). The absence of standard error bars indicates that the standard error bars are smaller than the symbol representing the mean.

These results suggest that 0.25 M EDTA (pH 7) extractions closely estimated free and reversibly bound cinnamic and benzoic acid derivatives in Cecil soils. The difference in phenolic acids extracted from soil by EDTA and water would, thus, constitute the reversibly bound phenolic acids that can be released from soil particles by the action of microbes.

DISCUSSION

The bulk solution pH values at the start and just prior to the addition of extractants ranged between 4.5 and 5.3 in the absence of microbes. In the presence of microbes, the pH values ranged from 5 to 6.5. The pK_a values for

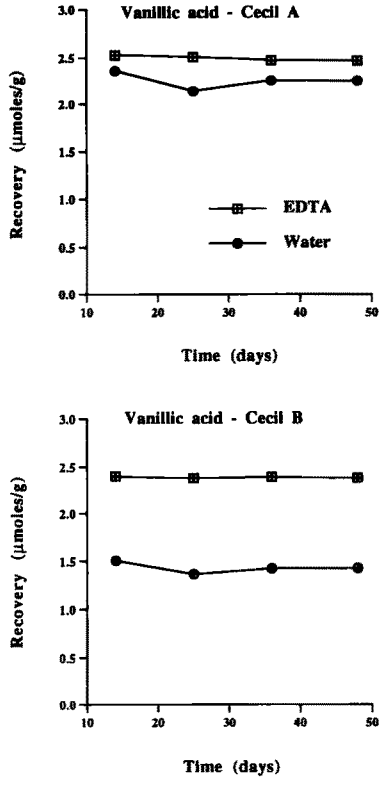

FIG. 7. Recovery of 2.5 μmol vanillic acid/g soil material from Cecil A_p and B_t soil materials by 0.25 M, pH 7 EDTA and water in the absence of microbes (days 14–48). Points are mean values (N = 2). The absence of standard error bars indicates that the standard error bars are smaller than the symbol representing the mean.

simple phenolic acids (e.g., ferulic acid, vanillic acid, etc.) are approximately 4.5; thus, both anionic and protonated (nonionic) forms were present in these soil systems. The anionic forms, for example, could bind to positively charged sites (e.g., Al and Fe-oxyhydroxides) on soil surfaces (Watson et al., 1973; Parfitt et al., 1977) and indirectly to the negative surface sites (e.g., clays) by way of multivalent cation bridges (Greenland, 1965, 1971). The protonated nonionic forms could be sorbed by the soil organic matter (Chiou, 1989; Hasset and Banwart, 1989) and/or polymerized into humic substances in the soil (Martin et al., 1972; Martin and Haider, 1976; Haider et al., 1977; Wang et al., 1978, 1986). The binding strengths of the resulting complexes vary. Some complexes (e.g., those resulting from anion exchange or cation bridging) are easily dis-

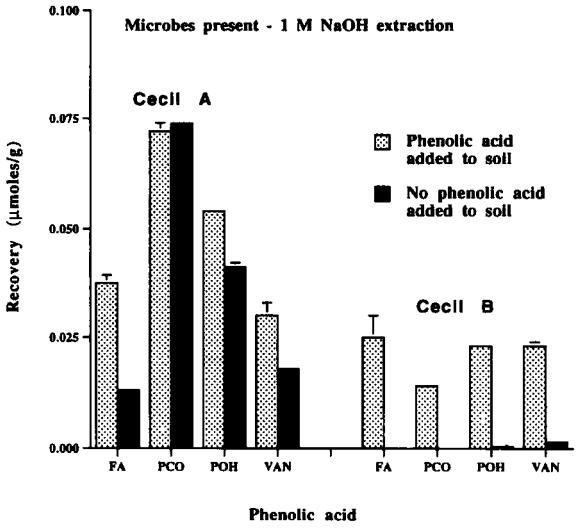

FIG. 8. Recovery of ferulic (FA), *p*-coumaric (pCO), *p*-hydroxybenzoic (POH), and vanillic (VAN) acids by 1 M NaOH 24 days after reinfestation of Cecil A_p and B_t soil materials amended with (74 days after addition of 2.5 μmol phenolic acid/g soil material) or without phenolic acids. Means ± standard errors ($N = 2$).

rupted; others (e.g., those resulting from ligand exchange, oxidation reactions with mineral surfaces, incorporation into humic substances) are fixed into recalcitrant organic matter and are thus unavailable to microbes in the short term. Chelates released by microorganisms (Duff et al., 1963) in the bulk soil or in the rhizosphere could easily disrupt complexes resulting from anion exchange or cation binding. How rapidly phenolic acids released by microbes into the soil solution are resorbed by soil particles, metabolized by microbes, taken up by seeds or roots, or leached will, of course, depend upon soil and species characteristics.

In work reported here, we have characterized the direct linear relationship between sorption and the phenolic acid concentration added to soil, the noncompetitive sorption of phenolic acids in mixtures, the slow longer-term sorption (day 14 to day 124) of ferulic acid and *p*-coumaric acid, and the lack of longer-term sorption for *p*-hydroxybenzoic acid and vanillic acid in sterile Cecil A_p and B_t soil materials. Similar behavior for other organic compounds, particularly pesticides, in a variety of soil materials have been summarized by Giles et al. (1960), Chiou (1989), and Weber and Miller (1989). In addition, we have demonstrated that neutral EDTA (0.25 M, pH 7) and water extractions of soil samples can be used effectively in estimating levels of free and reversibly bound

(i.e., available to microbes) phenolic acids in Cecil soils. Kaminsky (1980), using correlations between estimates of phenolic acid availability based on lettuce seed germination rates and EDTA and NaOH extractions of sandy clay loam soils, also concluded that neutral EDTA solution is an effective extractant for estimating available (free and reversibly bound) phenolic acids to seeds in soils. Since EDTA extractions appear to be ineffective in extracting phenolic acids bound in plant debris (Blum et al., 1992), potential sources of phenolic acids that may be released by microbial decomposition in and on the soil are not included in these EDTA and water extracts.

To establish the presence of allelopathic interactions in natural or managed ecosystems will require the characterization of sources (inputs) of phenolic acids in soils (i.e., the amounts, the timing, and the rates of release of phenolic acids) and the "competitive nature" of the various sinks (e.g., clays, organic matter, microbes, roots, seeds) in soils for phenolic acids. Water and EDTA extractions of field soils will provide data on background (residual) levels of free and reversibly bound phenolic acid, but no data on inputs or the partitioning of phenolic acids to the various sinks in the soil. The relative "competitive nature" of soil sinks for phenolic acids, however, can be determined with water and EDTA extractions in the laboratory environment.

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MECHANISM OF AGGREGATION BEHAVIOR IN *Maladera matrida* ARGAMAN (COLEOPTERA: SCARABAEIDAE)

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Abstract—Adult *Maladera matrida* Argaman (Coleoptera: Scarabaeidae: Melolonthinae) males emerge from soil for an active period at dusk, a few minutes before the females. Adults are found during most of the active hours on the foliage in aggregations composed of an equal sex ratio. The mechanism of aggregation behavior of *M. matrida* beetles was studied in a Y-shaped olfactometer. No evidence was found for the existence of an aggregation pheromone released either by males or by females, but behavior tests indicate that adult *M. matrida* beetles, males as well as females, are attracted to volatiles of an injured host plant. The following scenario is suggested: Males emerge daily from soil at dusk, a few minutes before the females, and immediately start feeding. Additional males are attracted to the injured host's volatiles and form aggregations. When females emerge from soil, the attractant volatiles are concentrated in spots, and the females join the aggregations, forming an equal sex ratio.

Key Words—Coleoptera, Scarabaeidae, *Maladera matrida*, aggregation, plant volatiles, olfactometer, attractant, behavior

INTRODUCTION

Maladera matrida Argaman (Coleoptera: Scarabaeidae, Melolonthinae), a polyphagous pest, was first detected in Israel in 1983 (Klein and Chen, 1983), was described as a new species by Argaman (1986, 1990) and has lately been reported

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also from Saudi Arabia (Anonymous, 1991). Adult beetles are found feeding on foliage in irrigated fields, orchards, ornamental home gardens, and parks; the grubs develop in soil around root systems, causing damage mainly to root crops such as potatoes, sweet potatoes, and peanuts. Adult *M. matrida* beetles emerge from soil at dusk to feed and mate (Gol'berg et al., 1989).

Our preliminary field observations have indicated that emergence of males from soil for the active period at dusk always precedes that of females by a few minutes. Among scarabaeids, seasonal protandry was reported in various June beetles (Kard and Haim, 1990), whereas daily preemergence of males was seen in two species of *Cyclocephala* (Potter, 1980).

Preliminary observations also indicate that *M. matrida* adults aggregate on foliage in an equal sex ratio. In other scarabaeids, aggregations have been described in the Japanese beetle, *Popillia japonica* Newman (Ladd, 1970), and in two species of *Cyclocephala* (Potter, 1980), in which one female, or a mating couple, were surrounded by several males. Aggregations of equal sex ratio were reported in the green June beetle, *Cotinis nitida* (L.) (Domek and Johnson, 1988, 1990) and in other Coleoptera, e.g., the nitidulids *Carpophilus hemipterus* (L.) (Bartelt et al., 1990a), *C. lugubris* Murray (Lin et al., 1992), and *C. freemani* Dobson (Bartelt et al., 1990b); the chrysomelid, *Phyllotreta cruciferae* (Goeze) (Peng and Weiss, 1992); the scolytid, *Dendroctonus pseudotsugae* Hopkins (Knope and Pitman, 1972), and others.

Protandry may evolve under natural selection by conferring an advantage upon males that emerge early and maximize the number of females located during the time of activity (Potter, 1980), whereas the advantages of aggregation may lie in providing protection and an opportunity to locate food or mates (Bailey, 1991). A combination of protandry and aggregation of equal sex ratio may provide several of these advantages.

Several possibilities have been described for pheromone-mediated aggregation behavior in insects: (1) aggregation pheromone produced and released by insects (Phillips and Burkholder, 1981); (2) synergism between host plant volatiles and aggregation pheromone produced by insects (Bartelt et al., 1990a, b; Dowd and Bartelt, 1991; Lin et al., 1992); (3) contact with host plant required for production of aggregation pheromone by insects (Peng and Weiss, 1992); (4) aggregation pheromone released by insects while feeding (Domek and Johnson, 1988); (5) pheromone produced by insects as a derivative of consumed host plant materials (Eisner and Meinwald, 1987); (6) attractant produced by an interaction between microorganisms and feeding insects (Domek and Johnson, 1990); and (7) attractant produced by microorganisms developing upon damaged host plant (Dolinski and Loschiavo, 1973).

The goal of the present study was to elucidate the mechanism of aggregation behavior in *Maladera matrida* beetles.

METHODS AND MATERIALS

Beetles. M. matrida was reared in a climatic room at 27°C, 50–70% relative humidity, and 16:8 hr light–dark. The beetles were sexed at the pupal stage, because it is difficult to distinguish adult males from females. Fifteen to 20 beetles of one sex were placed in plastic boxes, 10 × 10 × 25 cm, containing humid sand and *Duranta repens* L. leaves as a food substrate. Beetles used in this study, either as attractant sources or as subjects attracted to a volatile source, were 10–35 days old and were starved for 48 hr prior to the experiments.

Bioassay Setup. All experiments were held in an olfactometer (Figure 1), composed of a vacuum pump connected, through a flowmeter, to a Y-shaped glass tube (stem 2 × 14 cm, each arm 2 × 10 cm). Each of the two arms led to a spherical glass trap, followed by a glass bulb containing a volatile source. Another flowmeter was connected by Tygon hoses to the glass bulb and to a bottle of activated charcoal. The assay beetles were released individually at the starting point and would walk or fly against the air current toward the attractant source, falling into the spherical trap, from which they would be removed to avoid interruption of the system. The airflow along the stem tube and in both arms was 244 ml/min. A smoke test demonstrated a laminar airflow in the stem tube and in both arms.

Because the beetles are active from sunset on for 2.5 hr (Gol'berg et al., 1989), the experiments began when the lights were switched off and lasted for 2.5 hr under red light (located above the olfactometer), which in preliminary observations appeared to have no effect on the behavior of the beetles.

Because of the short daily activity period of the beetles, only one set of experiments was held in a given day, and the behavior of only one sex, either males or females, of *M. matrida* was tested toward a given attractant. Every set of experiments was repeated five times on different days, with 20–55 subjects tested in a given day. At the end of an experiment set, the olfactometer was rinsed with analytical grade acetone.

Treatments. The following sets of experiments were conducted in order to determine whether *M. matrida* beetles are attracted to one or both sexes of their own species, to an interaction between their own species and a host plant, or

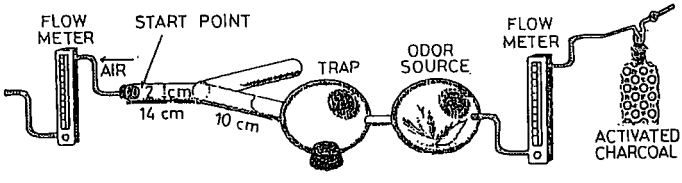

FIG. 1. The olfactometer.

to any insect–host interaction. In any given experiment, different sources of attractant were placed in the bulb of each arm of the olfactometer.

1. Seven *M. matrida* males vs. seven *M. matrida* females, no food; a total of 121 males and 158 females were individually tested in this experiment against these attractant sources.

2a. Blank (no volatiles) vs. seven *M. matrida* males feeding on five leaves of *D. repens*; 118 males and 136 females of *M. matrida* were tested.

2b. Blank (no volatiles) vs. seven *M. matrida* females feeding on five leaves of *D. repens*; 129 males and 113 females were tested.

3a. Seven nonfeeding *M. matrida* males vs. seven *M. matrida* males feeding on five leaves of *D. repens*; 130 males and 107 females were tested.

3b. Seven nonfeeding *M. matrida* females vs. seven *M. matrida* females feeding on five leaves of *D. repens*; 131 males and 122 females were tested.

4. Seven *M. matrida* females feeding on five leaves of *D. repens* vs. seven *M. matrida* males feeding on five leaves of *D. repens*; 140 males and 132 females were tested.

5. Five leaves of *D. repens* vs. seven *M. matrida* males feeding on five leaves of *D. repens*; 111 males and 126 females were tested.

6. Four fifth-instar nymphs of the desert locust, *Schistocerca gregaria* (Forskål) (Orthoptera: Acrididae), feeding on five leaves of *D. repens* vs. seven *M. matrida* males feeding on five leaves of *D. repens*; 148 males and 171 females were tested.

7. Four nonfeeding fifth-instar nymphs of *S. gregaria* vs. four *S. gregaria* nymphs feeding on five leaves of *D. repens*; 119 males and 137 females of *M. matrida* beetles were tested.

Only active beetles (found above soil) were used as an attractant source and as subjects in the behavior tests. Beetles that chose a direction and fell into the trap (Figure 1) within 5 min were recorded as a "response," while those that did not choose a certain arm within 5 min or moved in the opposite direction (i.e., with the air current) were scored as "no response." Every five runs, the olfactometer was turned 180° in order to change the direction of the volatile sources, to avoid bias from uncontrolled directional factors.

Statistical Analysis. The results were analyzed as two-choice data. Choices of any arm were pooled as a response category, versus the no-response category. The nonresponsive individuals (less than 10% in any experiment) were omitted from further calculations, and the choice of one arm was compared to the choice of the other. G statistics (Sokal and Rohlf, 1969) were used for replicated goodness-of-fit tests. The heterogeneity of the replicates for each experiment was tested first and was found to be nonsignificant ($P >> 0.1$), indicating that replicates of the same experiment did not differ significantly between different days. A second goodness-of-fit test was performed for the pooled data. Both

tests were used to compute the significance of deviation from expectation (1 : 1) for each experiment ($\alpha = 5\%$).

RESULTS AND DISCUSSION

In preliminary experiments, seven *M. matrida* males without food were placed in one arm of the olfactometer vs. no volatile source in the other. Male and female subjects ($N = 78$ and 54, respectively) did not differ significantly ($P < 0.05$) in their choice of arm. When seven *M. matrida* females without food were placed in one arm of the olfactometer vs. no volatiles in the other, females ($N = 66$) and males ($N = 76$) exhibited similar nonpreference toward either arm. These preliminary results indicated that *M. matrida* beetles, males as well as females, are not attracted to volatiles emitted by conspecific beetles per se. Nevertheless, the first set of experiments was carried out to test the possibility of differences in attraction to the different sexes.

1. Nonfeeding M. matrida *females vs. nonfeeding* M. matrida *males.* Male as well as female subjects did not differ in their response toward either males or females ($P >> 0.05$) (Figure 2A and B). Therefore, the possibility of an aggregation pheromone, produced and released by either one of the sexes in the absence of food (Phillips and Burkholder, 1981), could be excluded. Inasmuch as beetles serving as an attractant source were fed until 48 hr prior to testing, the possibility of a pheromone produced by the insects as a derivative of consumed host plant materials (Eisner and Meinwald, 1987) was excluded as well.

2a. Blank vs. M. matrida *males feeding on* D. repens *leaves.* Male and female subjects did not differ significantly in their choice of arm. Both males and females were significantly more attracted to the feeding males (73.3 ± 2.1% males and 69.7 ± 1.9% females of response category beetles, $P << 0.05$) than to the blank (Figure 3A).

2b. Blank vs. M. matrida *females feeding on* D. repens *leaves.* There was

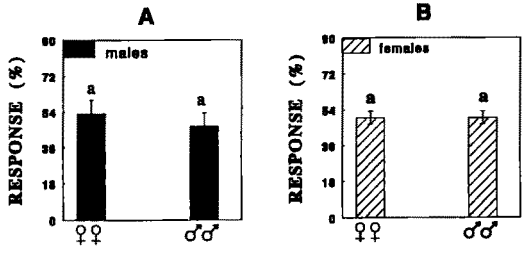

FIG. 2. Nonfeeding *Maladera matrida* females vs. nonfeeding *M. matrida* males. (A) Testing the behavior of 121 *M. matrida* males ($P >> 0.05$). (B) Testing the behavior of 158 *M. matrida* females ($P >> 0.05$).

no significant difference between the choice of arm by either male or female subjects. Most of the responding beetles were attracted to the feeding females (70.6 ± 5.0% and 68.2 ± 4.2%, respectively) (Figure 3B).

3a. Nonfeeding M. matrida *males vs.* M. matrida *males feeding on* D. repens *leaves.* Male as well as female subjects were significantly more attracted to the feeding males (67.0 ± 3.1% and 69.4 ± 6.5%, respectively) (Figure 4A).

3b. Nonfeeding M. matrida *females vs.* M. matrida *females feeding on* D. repens *leaves.* Male as well as female subjects were significantly more attracted to the feeding females (66.6 ± 5.1% and 60.3 ± 3.2%, respectively) (Figure 4B).

These results suggested that *M. matrida* beetles are attracted to feeding beetles of both sexes, rather than to beetles alone. Therefore, the following set of experiments was conducted in an attempt to distinguish between the attraction of feeding males and feeding females.

FIG. 3. (A) Blank vs. males of *M. matrida* feeding on *Duranta repens* leaves. Testing the behavior of 118 males and 136 females of *M. matrida* ($P \ll 0.05$). (B) Blank vs. females of *M. matrida* feeding on *D. repens* leaves. Testing the behavior of 129 males and 113 females of *M. matrida*. ($P \ll 0.05$).

FIG. 4. (A) Non-feeding *M. matrida* males vs. *M. matrida* males feeding on *D. repens* leaves. Testing the behavior of 130 males and 107 females of *M. matrida* ($P \ll 0.05$). (B) Non-feeding *M. matrida* females vs. *M. matrida* females feeding on *D. repens* leaves. Testing the behavior of 131 males and 122 females of *M. matrida* ($P \ll 0.05$).

4. M. matrida females feeding on D. repens *leaves vs.* M. matrida *males feeding on* D. repens *leaves.* Female subjects did not exhibit significant preference to either feeding females or feeding males ($P >> 0.05$); however, male subjects were significantly more attracted to feeding females (63.3 ± 2.4%) (Figure 5). The latter finding led us to the assumption that a sex pheromone released by the females might be involved in male attraction. However, the fact that males did not exhibit significant attraction toward nonfeeding females may suggest that a sex pheromone is released by the females while feeding on the host plant. This assumption is supported by field observations (G. Yarden, personal communication). Since we were looking for an aggregation factor, which should be attractive to both males and females (Alcock, 1982), females were excluded as an attractant source from subsequent tests, to avoid influence of a female sex pheromone on male behavior.

The next test was performed in order to distinguish the influence of volatiles emitted by the host plant from those of the feeding males.

5. D. repens *leaves alone vs.* M. matrida *males feeding on* D. repens *leaves.* Male as well as female subjects were significantly more attracted to the feeding males (69.2 ± 1.6% and 64.9 ± 5.0%, respectively) (Figure 6).

The results obtained so far could be explained by the presence of an attractive factor resulting from the feeding action of adult *M. matrida* beetles of both sexes, a mechanism suggested for aggregation-inducing behavior by Domek and Johnson (1988). In order to test this hypothesis and to distinguish volatiles emitted by the feeding beetles from those emitted by a damaged host plant, the feeding action of the beetles was mimicked by placing fifth-instar nymphs of *Schistocerca gregaria* (Forskål) on the host plant.

6. S. gregaria *nymphs feeding on* D. repens *leaves vs.* M. matrida *males feeding on* D. repens *leaves.* Male as well as female subjects did not favor feeding beetles over feeding locusts (Figure 7). The possibility that an aggregation pheromone is produced upon contact between host plant and beetle (Peng

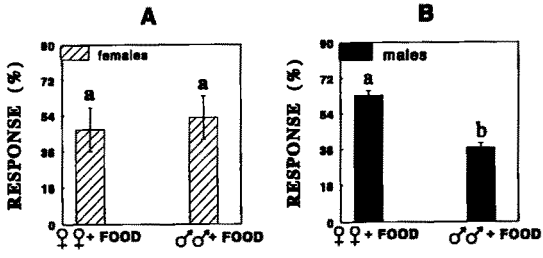

FIG. 5. *M. matrida* females feeding on *D. repens* leaves vs. *M. matrida* males feeding on *D. repens* leaves. (A) Testing the behavior of 132 females of *M. matrida* ($P >> 0.05$). (B) Testing the behavior of 140 males of *M. matrida* ($P << 0.05$).

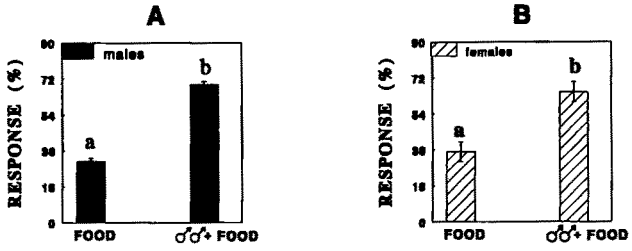

FIG. 6. Intact leaves of *D. repens* vs. *M. matrida* males feeding on *D. repens* leaves. (A) Testing the behavior of 111 *M. matrida* males ($P \ll 0.05$). (B) Testing the behavior of 126 *M. matrida* females ($P \ll 0.05$).

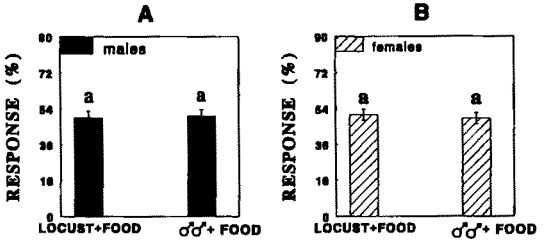

FIG. 7. *S. gregaria* nymphs feeding on *D. repens* leaves vs. *M. matrida* males feeding on *D. repens* leaves. (A) Testing the behavior of 148 *M. matrida* males ($P \gg 0.05$). (B) Testing the behavior of 171 *M. matrida* females ($P \gg 0.05$).

and Weiss, 1992) or that of synergism between food volatiles and an aggregation pheromone (Bartelt et al., 1990a,b; Lin et al., 1992) were therefore discounted.

The following experiment was carried out to ascertain that *M. matrida* was not attracted to the locusts themselves.

7. Nonfeeding S. gregaria *nymphs vs.* S. gregaria *nymphs feeding on* D. repens *leaves.* Males and females of *M. matrida* were significantly more attracted to feeding *S. gregaria* nymphs (65.4 ± 3.1% and 63.7 ± 5.9%, respectively) (Figure 8). In field observations, aggregations of *M. Matrida* adults were seen close to feeding larvae of *Spodoptera littoralis* (Buisduval) (Lepidoptera: Noctuidae). Inasmuch as the beetles exhibited a similar degree of attraction to feeding insects belonging to three different orders, it may be assumed that they are not attracted to volatiles emitted by the insects while feeding, but rather to the volatiles of the injured host plant itself. Attraction to injured (cracked) host plant was reported by Trematerra and Giragenti (1989) and by Walgenbach et al. (1987). The possibility that microorganisms in the food were involved in inducing aggregation behavior (Dolinski and Loschiavo, 1973) in *M. matrida* was not tested.

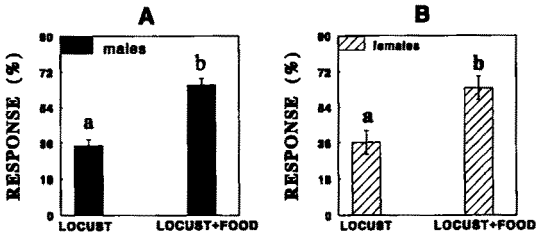

FIG. 8. Nonfeeding *S. gregaria* nymphs vs. *S. gregaia* nymphs feeding on *D. repens* leaves. (A) Testing the behavior of 119 *M. matrida* males ($P << 0.05$). (B) Testing the behavior of 137 *M. matrida* females ($P << 0.05$).

Our observations and experiments indicate the following scenario: Males emerge daily from soil at dusk, a few minutes before females, and immediately start feeding. Additional males are attracted to the injured host plant volatiles and form aggregations. When females emerge from the soil, the attractant volatiles are concentrated in spots, and the females join the aggregations, forming an equal sex ratio.

M. matrida is a polyphagous pest, and since the same aggregation pattern was seen on numerous host plants (Gol'berg et al., 1989; Harari et al., in preparation), it may be assumed that the volatiles emitted from the injured plant are general odor components such as leaf alcohols, aldehydes, and derivatives. Such green odor components are known to affect the behavior of many insects, e.g., the Colorado potato beetle, *Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae); the cabbage maggot, *Delia radicum* (L.) (Diptera: Anthomyiidae); the carrot rust fly, *Psila rosae* (Fabricius) (Diptera: Psilidae), and others (Visser, 1986).

The type of aggregation behavior described herein may either be an adaptive behavior, enabling the beetle to respond to volatiles emitted by an injured host plant to ensure food availability (Shorey, 1973) and mate location (Bailey, 1991) in a patchy environment, or may play a role in sexual selection. Preliminary field observations have indicated that there may be a sexual advantage to aggregation, as most of the copulations in *M. matrida* populations seem to occur within the aggregations, but these observations have to be corroborated.

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LEAF AGE AFFECTS COMPOSITION OF HERBIVORE-INDUCED SYNOMONES AND ATTRACTION OF PREDATORY MITES

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Abstract—We investigated the olfactory response of the predatory mite *Phytoseiulus persimilis* to cucumber leaves infested with prey, the herbivorous spider mite *Tetranychus urticae*. The predators responded to volatiles from young rather than old infested cucumber leaves. GC-MS analysis of the headspace of spider mite-infested, artificially damaged and undamaged cucumber plants showed that herbivore-induced plant volatiles were present among the volatiles of both old and young infested cucumber leaves. The major components of the herbivore-induced plant volatiles were (3*E*)-4,8-dimethyl-1,3,7-nonatriene and (*E*)-β-ocimene: these compounds are known to attract the predatory mites. In addition, we found three oximes (2-methylbutanal *O*-methyloxime, 3-methylbutanal *O*-methyloxime, and an unknown oxime) in the headspace of both old and young infested cucumber leaves. 3-Methylbutanal *O*-methyloxime and the unknown oxime were much more abundant in the headspace of infested old cucumber leaves. The potential adaptive value of differential attractiveness of cucumber plant leaves of different age is discussed.

Key Words—Cucumber plant, *Tetranychus urticae*, *Phytoseiulus persimilis*, Acari, herbivore-induced synomone, tritrophic interaction, oximes.

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INTRODUCTION

Undamaged plants produce volatiles that are sometimes considered "possible insect attractants" (e.g., del Rosario et al., 1984; Buttery and Ling, 1984; Lwande et al., 1989; Liu et al., 1989). When a leaf is under attack by herbivores, the blend of leaf volatiles is expected to change; mechanical damage inflicted by herbivores causes an increased release of six-carbon type green leaf volatiles (Hatanaka, 1981; Whitman and Eller, 1990). Moreover, decompartmentalization of plant components as well as the interaction of herbivore- and plant-derived chemicals could lead to new volatiles (Boland et al., 1992). Indeed, leaves infested by herbivores produce allelochemicals that attract the natural enemies of the herbivore (Dicke and Sabelis, 1988a; Dicke et al., 1990a,c; Takabayashi et al., 1991a,b; Turlings et al., 1990a; Vet and Dicke, 1992). For example, upon being infested by spider mites, plants start emitting volatiles that attract predatory mites (Dicke and Sabelis, 1988; Dicke et al., 1990a; Takabayashi et al., 1991b).

When allelochemicals are adaptive to both emitter and receiver, they are classified as synomones (Dicke and Sabelis, 1988b). Thus the plant volatiles produced in response to herbivore damage and that recruit carnivorous natural enemies are called herbivore-induced synomones (HIS) (Vet and Dicke, 1992).

Not all plants within a population are expected to produce HIS or to produce them in the same concentration (Sabelis and de Jong, 1988). Furthermore, it is known that variation in HIS production may arise from various sources: the herbivore species (Dicke, 1988; Sabelis and van de Baan, 1983; Takabayashi et al., 1991a; Turlings et al., 1990b), the plant species (Dicke and Sabelis, 1988a; Takabayashi and Dicke, 1993; Takabayashi et al., 1991a; Turlings et al., 1990b), and plant cultivar (Dicke et al., 1990c; Takabayashi et al., 1991a). In this paper, we report variation in HIS production within plant individuals. Changes in the composition of the volatile blend emitted by *T. urticae*-infested leaves and the behavioral response of the predatory mites are associated with the age of the leaf in a tritrophic system consisting of cucumber plants, two-spotted spider mites (*T. urticae*), and the predatory mites (*P. persimilis*).

METHODS AND MATERIALS

Plants. Lima bean plants (*Phaseolus lunatus* cv. Carolina or Sieva) and cucumber plants (*Cucumis sativus* cv. Santo F1) were grown in a greenhouse in the Department of Entomology, Wageningen Agricultural University (20–30°C, 50–70% relative humidity, photoperiod of at least 16 hr). Lima bean plants three to four weeks after germination and 50 to 80-cm long cucumber plants were used in the experiments.

Mites. The predatory mites (*Phytoseiulus persimilis* Athias-Henriot) were

obtained from Koppert BV. (Berkel and Rodenrijs, The Netherlands). They were reared in the laboratory for about two years on spider mites (*Tetranychus urticae*) on lima bean leaves at 20–30°C, 50–70% relative humidity, and 16:8 hr light–dark regime. Four weeks before the first experiments, predators from this population of *P. persimilis* were used to start a second predator population that was reared on *T. urticae* on cucumber (*Cucumis sativus*, cv. Santo F1) leaves. Both young and old infested cucumber leaves were used for this predator rearing. These two predator populations are referred to as *P. persimilis* (Li) and *P. persimilis* (Cu) respectively.

Satiated *P. persimilis* (Li) were individually kept in a plastic tube (10 mm diam. 40 mm long) for 15 hr at 20–30°C, 50–70% relative humidity, and 16:8 hr light–dark regime. These predatory mites are referred to as starved *P. persimilis* (Li).

Bioassay. A Y-tube olfactometer was used for experiments (Takabayashi and Dicke, 1992).

The response of *P. persimilis* is known to be dependent on rearing history (Dicke et al., 1990b): *Phytoseiulus persimilis* (Li) preferred spider mite-infested lima bean leaves over spider mite-infested cucumber leaves (a mixture of young and old leaves), but after female predators had been transferred to spider mite-infested cucumber leaves for only seven days they preferred the volatiles from infested cucumber leaves over the volatiles from infested lima bean leaves (Dicke et al., 1990b). Therefore, we tested the response of *P. persimilis* (Cu) (ca. > 4-week old colony) and *P. persimilis* (Li) (ca. 2-year old colony) to the volatiles from infested young and old cucumber leaves.

Since starvation may increase the degree of attraction of predatory mites (Sabelis and Dicke, 1985), the response of starved *P. persimilis* (Li) was also tested. Each predatory mite was observed individually for a maximum of 5 min. Only adult females were used.

Statistics. Bioassay data were subjected to a sign test to determine the significant difference in attraction between the results and the null hypothesis (50:50 distribution of predators over the two olfactometer arms).

Samples for Bioassay and Chemical Analysis. One to three weeks after the two-spotted spider mites were introduced, the cucumber plants were considered infested. Both infested and uninfested leaves were classified as young leaves (length ca. 5–7 cm) or old leaves (length ca. 10–17 cm).

To collect the volatiles of artificially damaged cucumber leaves, uninfested cucumber leaves were damaged with carborundum on a wet cotton pad to imitate spider mite damage. For the chemical analyses, uninfested leaves and artificially damaged leaves were not separated into young and old leaves as described above.

Collection and Identification of Leaf Volatiles. Clean air, filtered through silica gel (250 ml), a mixture of molecular sieves 5A and 13X (250 ml), activated

charcoal (250 ml), and Tenax-TA (90 mg), was drawn over detached cucumber leaves in a glass flask (5 liters) and subsequently through a Tenax-TA-containing glass tube (Chrompack: 90 mg) where the leaf volatiles were trapped. About 10–30 infested leaves were used as samples. Airflow rate was 150 ml/min. Leaf volatiles were desorbed and injected with a Thermodesorption Cold Trap injector (TCT: Chrompack) to a gas chromatograph mass spectrometer (GC-MS: VG MM7070F) equipped with a fused silica capillary column (Supelcowax 10, 60 m × 0.25 mm ID, 0.25-μm film thickness). The ionization voltage was 70 eV. Oven temperature was programmed from 40°C (hold time: 4 min) to 100°C at 2°C/min and then to 270°C at 6°C/min. The chemical structure of each compound was elucidated by comparison of the mass spectra with those of authentic chemical samples or by comparison with data in the mass spectrum library of the Department of Organic Chemistry, Wageningen Agricultural University. (*E*,*E*)-α-Farnesene was identified from the mass spectra and from a comparison of the retention time with that of synthetic (*E*,*E*)-α-farnesene in two different fused silica capillary columns (DB-WAX and DB-1, 30 m × 0.25 mm ID, 0.25 μm film thickness).

Synthetic Chemicals. Most synthetic chemicals were obtained from commercial sources. In addition, (3*E*)-4,8-dimethyl-1,3,7-nonatriene and (3*E*,7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene were synthesized as described previously (Dicket et al., 1990a), (*E*,*E*)-α-farnesene was obtained from J. Gut, TNO, Division of Technology for Society, Delft. The oximes (2-methylbutanal *O*-methyloxime and 3-methylbutanal *O*-methyloxime) were synthesized by D. de Bie, Department of Organic Chemistry, Wageningen Agricultural University.

RESULTS

Attraction of Predatory Mites: Effects of Leaf Age, Predator Rearing History and Predator Starvation. When infested young cucumber leaves versus uninfested young cucumber leaves were offered in the olfactometer, satiated *P. persimilis* (Li) preferred infested young leaf volatiles (Figure 1). In contrast, when infested old leaves versus uninfested old leaves were offered in the olfactometer to satiated *P. persimilis* (Li), there was no preference (Figure 1).

We also tested the response of *P. persimilis* (Cu) to the volatiles from infested young and old leaves. The results were the same as those for *P. persimilis* (Li): the predators preferred young infested cucumber leaves over uninfested young leaves but did not prefer infested old leaves over uninfested old leaves (Figure 1).

Starvation does not yield a response different from that of satiated *P. persimilis* (Li) (Figure 1).

When seven young infested leaves were compared in the olfactometer to

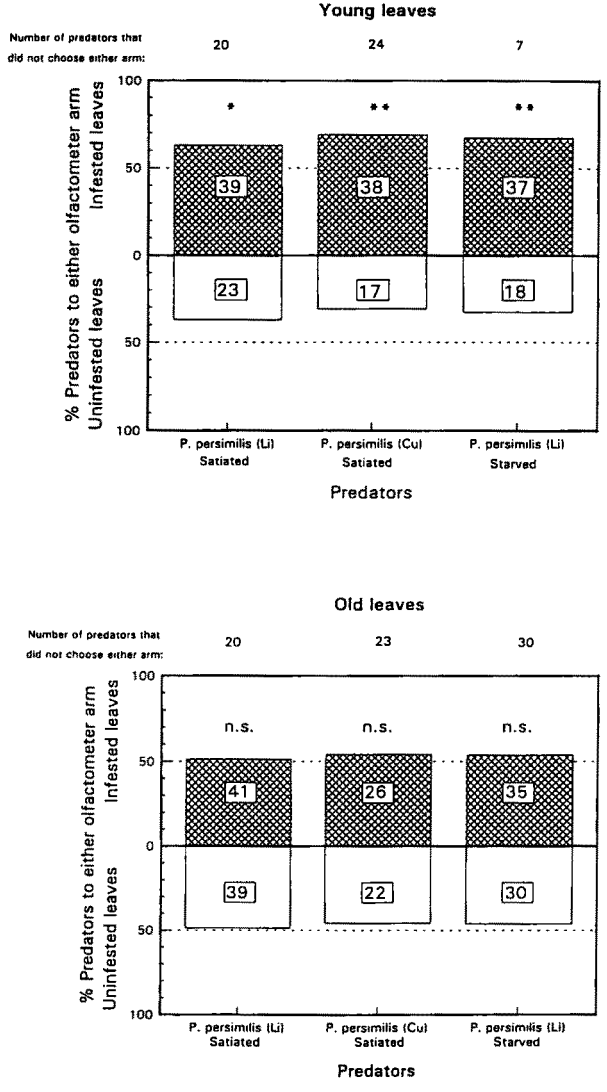

FIG. 1. Response in Y-tube olfactometer to *P. persimilis* females to the volatiles from cucumber leaves infested by *T. urticae* versus those from uninfested cucumber leaves. Numbers in bar segments indicate the numbers of predators that made a specific choice in the olfactometer. Sign test on numbers was used to evaluate differences from 50:50 distribution over two olfactometer arms. No-choice predators were not included. **0.001 $< P <$ 0.01, *0.01 $< P <$ 0.05, n.s.: not significant. Number of leaves used: 5–7. Number of spider mites per leaf: ca. 600–800.

clean air, *P. persimilis* (Li) preferred the volatiles of young infested leaves (left bar, Figure 2). However, when seven young infested leaves plus seven old infested leaves versus clean air were compared, *P. persimilis* (Li) did not show a preference for the infested leaf volatiles (right bar, Figure 2).

Chemical Analysis

Volatiles of Uninfested Versus Spider Mite Infested Cucumber Leaves (Table 1). The main volatiles from uninfested undamaged cucumber leaves were (*Z*)-3-hexenyl acetate (53.0%) and (*Z*)-3-hexenol (34.9%); 3-octanone (2.7%), 2-hexenal (1.7%), 1-hexanol (1.4%), and hexanal (1.2%) were minor components. In contrast, the main volatiles from spider mite-infested cucumber leaves were (*E*)-β-ocimene and (3*E*)-4,8-dimethyl-1,3,7-nonatriene. These compounds were not emitted by the uninfested undamaged leaves or the artificially damaged leaves. In addition, nitrogenous compounds (2-methylbutanal *O*-methyloxime, 3-methylbutanal *O*-methyloxime, an unknown oxime, 2-methylpropanenitrile, 2-methylbutanenitrile and 3-methylbutanenitrile), two terpenoids [(*E*,*E*)-α-far-

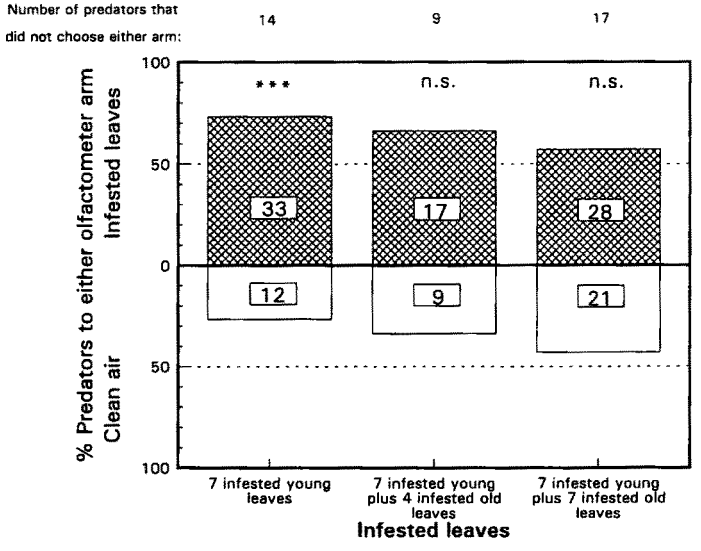

FIG. 2. Response in Y-tube olfactometer of *P. persimilis* (Li) females to the volatiles from cucumber leaves infested by *T. urticae* versus clean air. Numbers in bar segments indicate the numbers of predators that made a specific choice in the olfactometer. Sign test on numbers was used to evaluate differences from 50:50 distribution over two olfactometer arms. No-choice predators were not included. ***$P < 0.001$, n.s.: not significant. Number of spider mites per leaf: ca. 50–100.

TABLE 1. COMPOUNDS IDENTIFIED IN HEADSPACE VOLATILES OF CUCUMBER LEAVES WITH DIFFERENT TREATMENTS

	Relative amount (mean)			
	Undamaged leaves ($N = 2^a$)	Leaves damaged with carborundum ($N = 2$)	Leaves infested by *T. urticae*	
			Young ($N = 2$)	Old ($N = 2$)
Aldehyde				
Pentanal	0.2			
2-Hexenal	1.7	6.5		
Hexanal	1.2	1.6		
2-Pentenal	0.2	0.4		
Heptanal	0.1	0.1		
Decanal	0.3	0.2		
Nonanal	0.2	0.9		
Alcohol				
(*Z*)-3-Hexen-1-ol	34.9	36.0	0.4	0.6
(*E*)-3-Hexen-1-ol		0.1		
1-Hexanol	1.4	2.4		
2-Ethyl-1-hexanol	0.2	0.2		0.4
1-Butanol	0.1			
1-Penten-3-ol	0.1	tr[b]		
1-Pentanol	0.1			
Benzyl alcohol	0.6			
Ketone				
3-Octanone	2.7			
1-Penten-3-one	0.4	0.3		
6-Methyl-5-heptene-2-one	0.2	0.1		
2-Nonanone	0.9			
Ester				
n-Butyl acetate	0.7	0.1		
n-Hexyl acetate	0.3	1.1	1.1	
Iso-pentyl acetate				0.6
(*Z*)-3-Hexen-1-yl acetate	53.0	47.2	1.2	1.0
3-Hexenyl formate		tr		

TABLE 1. CONTINUED

	Relative amount (mean)			
	Undamaged leaves ($N = 2^a$)	Leaves damaged with carborundum ($N = 2$)	Leaves infested by *T. urticae*	
			Young ($N = 2$)	Old ($N = 2$)
Terpenoid				
Limonene	0.4	tr	tr	
(*Z*)-β-Ocimene			0.3	1.0
(*E*)-β-Ocimene			24.9	38.4
(3*Z*)-4,8-Dimethyl-1,3,7-nonatriene			2.5	0.1
(3*E*)-4,8-Dimethyl-1,3,7-nonatriene			53.8	19.8
unknown terpenoid			0.4	
Linalool	0.1			
(*E*,*E*)-α-Farnesene			4.0	1.9
(3*E*,7*E*)-4,8,12-Trimethyl-1,3,7,11-tridecatetraene			3.4	0.6
Nitrile				
2-Methylpropanenitrile			0.1	
2-Methylbutanenitrile			0.8	
3-Methylbutanenitrile			2.5	2.4
Oxime				
2-Methylbutanal *O*-methyloxime			0.8	0.5
3-Methylbutanal *O*-methyloxime			3.8	32.0
Unknown oxime			0.1	0.8
Others				
2-Ethylfurane	0.2	0.2		
Caproic acid	0.8	0.7		
Methyl salicylate			tr	
Unidentified minor peaks	1.2	2.4	0.4	

[a] Number of replications of GC-MS analysis.
[b] Compounds that were found at less than 0.1%.

nesene and (3*E*,7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene], and methyl salicylate (trace amount) were only found among the volatiles of infested cucumber leaves but not among those of uninfested undamaged leaves or artificially damaged leaves.

Volatiles from Spider Mite-Infested Young and Old Cucumber Leaves. Examples of ion chromatograms of infested young cucumber leaf volatiles and infested old cucumber leaf volatiles are given in Figure 3. Certain peaks were found in the headspace of infested leaves but not in the headspace of uninfested leaves or in the headspace of artificially damaged leaves, and thus represent herbivore-induced volatiles. No volatiles have been recorded in headspace analysis of spider mites (M. Dicke and M.A. Posthumus, unpublished data). To compare the total amounts of volatiles emitted by infested young and infested

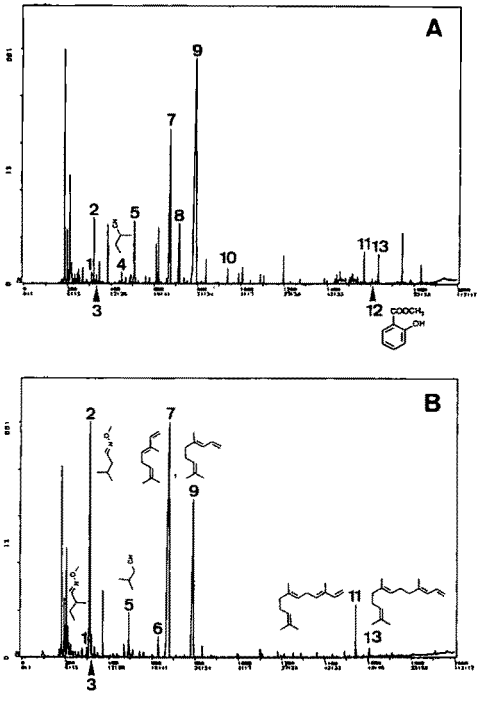

FIG. 3. Total ion chromatogram of infested young cucumber leaf volatiles (A) and infested old cucumber leaf volatiles (B). Peak 1: 2-methylbutanal *O*-methyloxime, 2: 3-methylbutanal *O*-methyloxime, 3: unknown oxime, 4: 2-methylbutanenitrile, 5: 3-methylbutanenitrile, 6: (*Z*)-β-ocimene, 7: (*E*)-β-ocimene, 8: (3*Z*)-4,8-dimethyl-1,3,7-nonatriene, 9: (3*E*)-4,8-dimethyl-1,3,7-nonatriene, 10: unknown terpenoid, 11: α-farnesene, 12: methyl salicylate, 13: (3*E*,7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene.

old leaves, we compared the total peak area of the herbivore-induced volatiles (arbitrary units) and adjusted this for the total leaf surface area sampled (in square centimeters), the number of spider mites and the sampling time (in minutes). The amounts of herbivore-induced volatiles appeared to be similar for old and young infested leaves: 8.4×10^{-5} for young leaves (total peak area 27,870; total leaf area 1500 cm^2; number of spider mites 13,000; total sampling time 17 min; $N = 2$) and 9.4×10^{-5} for old leaves (total peak area 21,923; total leaf area 2500 cm^2; number of spider mites 5500; total sampling time 17 min; $N = 2$). Thus, infested old and young leaves do not differ much with regard to quantitative aspects of spider mite-induced volatiles.

Qualitatively, however, several differences have been recorded. One of the characteristic differences between the blends of volatiles from infested young and infested old cucumber leaves was the relative amount of the two oximes, 3-methylbutanal *O*-methyloxime and the unknown oxime (Table 1). Infested young leaves produced less oximes (3-methylbutanal *O*-methyloxime 3.8%; unknown oxime 0.1%) than infested old leaves (3-methylbutanal *O*-methyloxime 32.0%; unknown oxime 0.8%). The reverse was the case for the nitriles: infested young leaves produced slightly more nitriles (2-methylpropanenitrile 0.1%, 2-methylbutanenitrile 0.8%, and 3-methylbutanenitrile 2.5%) than infested old leaves did (2-methylpropanenitrile not detected, 2-methylbutanenitrile not detected, and 3-methylbutanenitrile 2.4%). The relative amounts of two of the main herbivore induced plant chemicals, (*E*)-β-ocimene and (3*E*)-4,8-dimethyl-1,3,7-nonatriene, differ according to the age of the cucumber leaf. (*E*)-β-Ocimene was produced less in infested young leaves (24.9% and 38.4% among the volatiles of infested young and old leaves, respectively). On the other hand, (3*E*)-4,8-dimethyl-1,3,7-nonatriene was produced relatively more in infested young leaves (53.8% and 19.8% among the volatiles of infested young and old leaves respectively).

DISCUSSION

Our study shows that the response of the predatory mite *P. persimilis* towards infochemicals from spider mite-infested cucumber leaves is affected by the age of the infested leaves: infested young leaves attracted the predators while infested old leaves did not. This response was compared for predators reared under different conditions because the response of the predators is known to be dependent on rearing condition (host plant on which the spider mites are reared; Dicke et al., 1990b) and starvation (Sabelis and Dicke, 1985). Thus, the response of *P. persimilis* to the infested young leaves and to the infested old leaves is not affected by its previous predation experience. In the experiments of Dicke et al. (1990b) where *P. persimilis* (Li) and *P. persimilis* (Cu) differ in their

response to infested cucumber leaves, a mixture of old and young leaves were used. At that time the differential attraction of old and young leaves was unknown. Our current data show that *P. persimilis* (Li) and *P. persimilis* (Cu) do not differ in the response to only infested young or only infested old leaves. However, *P. persimilis* (Li) were not attracted by a mixture of infested old and infested young leaves (Figure 2) whereas *P. persimilis* (Cu) are attracted to a mixture (Dicke et al., 1990b). This suggests that *P. persimilis* (Cu) is less affected by the masking of the attractive volatiles from the infested young leaves by the volatiles from the infested old leaves (see below).

As no volatiles have been recorded in headspace analysis of *T. urticae* spider mites (M. Dicke and M.A. Posthumus, unpublished data), the compounds that characterized the infested cucumber leaf volatiles are herbivore-induced plant volatiles (see also Dicke et al., 1990a,b and Takabayashi et al., 1991b for evidence of plants producing synomones in response to *T. urticae* infestation). Our data show that infested old and young cucumber leaves emit similar amounts of spider-mite induced volatiles, when corrected for leaf surface area, number of spider mites and sampling time. The spider mite-induced volatiles were terpenoids ((*E*)-β-ocimene, (3*E*)-4,8-dimethyl-1,3,7-nonatriene, (3*E*,7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene, and (*E*,*E*)-α-farnesene), oximes (2-methylbutanal *O*-methyloxime, 3-methylbutanal *O*-methyloxime and unknown oxime), and nitriles (2-methylpropanenitrile, 2-methylbutanenitrile and 3-methybutanenitrile). The difference in attraction between infested young and old cucumber leaves is likely to be due to differences in blend composition with respect to these herbivore-induced volatiles.

Some of these herbivore-induced volatiles are known to be attractive to *P. persimilis*. (*E*)-β-Ocimene and (3*E*)-4,8-dimethyl-1,3,7-nonatriene, which are also emitted by *T. urticae*-infested lima bean leaves (Dicke et al., 1990a), attract *P. persimilis* (Li) and *P. persimilis* (Cu) (Dicke et al., 1990a; J. Takabayashi and M. Dicke, unpublished data). The other two terpenoids, (*E*,*E*)-α-farnesene and (3*E*,7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene, did not attract *P. persimilis* (Li), while (*E*,*E*)-α-farnesene did not attract *P. persimilis* (Cu) when offered singly (Dicke et al., 1990a; J. Takabayashi and M. Dicke, unpublished data). However, it remains to be investigated how mixtures of these chemicals affect the behavior of the predators.

The most uncommon herbivore-induced plant volatiles found in this study are the oximes and nitriles. To our knowledge, these chemicals have only been found as plant leaf volatiles in the headspace of apple leaves infested by *T. urticae* (Takabayashi et al., 1991a). 2-Methylbutanal *O*-methyloxime, 3-methylbutanal *O*-methyloxime, and the unidentified oxime were not emitted from uninfested undamaged cucumber leaves or mechanically damaged cucumber leaves. 3-Methylbutanal *O*-methyloxime is also found in trace amounts (less than 0.3%) in the headspace of apple leaves (cv. Summer red) infested by *T.*

urticae (Takabayashi et al., 1991a). Because no volatiles have been recorded in headspace analysis of *T. urticae* individuals (M. Dicke and M.A. Posthumus, unpublished data), and these three oxime compounds have not been recorded in the headspace of *T. urticae*-infested lima bean leaves (Dicke et al., 1990), *T. urticae*-infested *Solanum luteum* leaves, *T. urticae*-infested tomato leaves (Takabayashi and Dicke, 1993), *T. urticae*-infested apple leaves (cv. Cox orange) and *Panonychus ulmi*-infested apple leaves (cv. Summer red) (Takabayashi et al., 1991a), their emission is probably not related to two-spotted spider mites themselves, but to the interaction of the spider mites and the cucumber leaf. In addition, one of the most quantitative differences between infested young cucumber leaf volatiles and infested old cucumber leaf volatiles was the relative amount of 3-methylbutanal *O*-methyloxime and unknown oxime: they were emitted in higher amounts from spider mite-infested old cucumber leaves than from spider-mite infested young cucumber leaves. Infested old leaves (not attractive for the predator) emitted higher amounts of 3-methylbutanal *O*-methyloxime (32.0%) than infested young leaves (attractive for the predator) (3.8%). This suggested that the oximes decrease the attraction of the predatory mites by *masking* the attractive compounds. This hypothesis was supported by the results shown in Figure 2: the attractiveness of infested young cucumber leaves (left bar, Figure 2) was neutralized by adding an equal number of infested old leaves (right bar, Figure 2). However, these data are not conclusive on the role of the oximes in the lack of attractiveness of infested old leaves to the predators.

Not all plants are expected to produce synomones or to produce them in the same concentrations. The model of Sabelis and de Jong (1988) predicted that there is a rather wide range of conditions for synomone-producing plants to coexist with conspecific plants that spend their energy in other ways. In this study, we found that the above predicted polymorphism was also present within individual cucumber plants. We postulate the reason of this polymorphism as follows.

In searching for prey, wingless *P. persimilis* must climb up a cucumber plant or move from leaf to leaf in the canopy. In this searching process, the predators are likely to pass older leaves before reaching younger leaves. Normally the predator would stay in the first prey colony that it finds. If the infested old cucumber leaves attract and arrest the predators, the predatory mites are likely to stay on old infested leaves, thereby leaving the young leaves exposed to herbivory. Obviously, the young parts of the plant contribute more to plant fitness than old leaves. Young leaves may be more vulnerable to the spider mite attack for several reasons. Rapidly growing plant species or tissues often invest less in defense (Coley et al., 1985). A plant competes for light with its neighbors and growth may be more important than defense: slow-growing plants may be inhibited because of shading (Edwards et al., 1992). However, once a plant is infested by spider mites, it is more important to defend the growing parts, and

thus invading predatory mites should be directed to the growing tips. This may explain why young leaves are more attractive than old leaves.

Some unanswered questions remain: (1) Are predators intercepted in plants by prey colonies on old leaves when they are on their way to or exposed to cues from prey-infested young leaves? (2) Do oximes, when added to the headspace of infested young leaves, mask their attraction? (3) If so, why should the plant mask attractive volatiles, if it can also suppress the production of these? (4) Is the preference of predators for young infested cucumber leaves advantageous for the predators themselves? (5) Is the phenomenon observed here specific to cucumber plants or is it a general phenomenon? Answering this last question will provide insight into the possible general problem with which plants are confronted regarding defending old and young leaves. To answer these questions, there is a need for further behavioral studies on the function of herbivore-induced volatiles of cucumber plants, especially the oximes, which were found in larger amounts from infested old cucumber leaf volatiles.

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DETOXIFICATION OF CEREAL PLANT ALLELOCHEMICALS BY APHIDS: ACTIVITY AND MOLECULAR WEIGHTS OF GLUTATHIONE *S*-TRANSFERASE IN THREE SPECIES OF CEREAL APHIDS

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Abstract—The activity and molecular weights of glutathione *S*-transferase were studied in three species of cereal aphid: *Metopolophium dirhodum*, *Sitobion avenae*, and *Rhopalosiphum padi*. The highest level of glutathione *S*-transferase activity was recorded in extracts from *M. dirhodum* and the lowest from *S. avenae*, and extracts of larvae were more active than those from adults. The activity of this enzyme was higher in extracts of *S. avenae* and *R. padi* previously fed on a moderately resistant wheat variety than on a susceptible variety. Gel filtration followed by SDS-PAGE revealed three protein bands in the active fractions. The first had a molecular weight of 28,500 and the second of 27,500 and were present in all three species of aphid. The third protein differed in the three species, having a molecular weight of 26,000 in *S. avenae*, 25,500 in *M. dirhodum*, and 24,000 in *R. padi*.

Key Words—Grain aphid, *Sitobion avenae*, *Metopolophium dirhodum*, *Rhopalosiphum padi*, Homoptera, Aphididae, detoxifying enzymes, glutathione *S*-transferase, resistance, cereal allelochemicals.

INTRODUCTION

Resistance to aphids in cereals is strongly associated with the level of secondary plant metabolites such as phenolic compounds, hydroxamic acids, and indole

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alkaloids (Beck et al., 1983; Zuniga et al., 1985; Leszczynski et al., 1989). Although these substances do not exist in high concentrations in phloem sap, on which the aphids feed, there is evidence that aphids are exposed to them (Campbell and Binder, 1984; Leszczynski and Dixon, 1990; Wink and White, 1991; Dorschner and Kenny, 1992). However, herbivorous insects are well adapted to living on plants (Dreyer and Campbell, 1987; Pimentel, 1988). Aphids, in common with other insects, can accumulate toxic secondary plant substances, excrete them in their honeydew, or detoxify them by means of enzymes. The activity of the primary detoxifying enzymes, or so-called poly-substrate monooxygenases, is dependent on cytochrome P-450. They convert the allelochemicals into more polar, reactive substances, which are further metabolized by the secondary enzymes (Ahmed et al., 1986). In the second phase of detoxification, these reactive substances are made less toxic by conjugation with glutathione, glucose, phosphate, or sulfate and then stored or excreted (Smith, 1968; Leszczynski et al., 1992).

An important mechanism in the second phase of detoxification is the conjugation of reduced glutathione with one of a range of lipophilic xenobiotics, which is catalyzed by glutathione *S*-transferase (E.C. 2.5.1.18) (Habig et al., 1974). It has also been suggested that this enzyme may also participate in the primary detoxification of secondary plant substances (Clark and Shamaan, 1984; Wadleigh and Yu, 1987; Lee, 1991). Initially, these enzymes were classified on the basis of the substrate conjugated, and glutathione *S*- transferases that promote reactions with aryl, alkyl, arylalkyl or epoxide substrates were identified (Smith, 1968; Boyland and Chasseaud, 1969). Subsequently, several subunits of glutathione *S*-transferases were identified in mammalian and insect tissues. In insects, subunits of these enzymes have been reported in house flies (Motoyama and Dauterman, 1978; Clark et al., 1984), fruit flies (Yawetz and Koren, 1984), cockroaches (Usui et al., 1977), grass grubs (Clark et al., 1985), and the larvae of several species of Lepidoptera (Yu, 1989).

Leszczynski and Dixon (1992) reported the presence and activity of these enzymes in grain aphids. In this paper the level of activity, structure, and molecular weights of the glutathione *S*-transferase (GST) in the three most abundant species of cereal aphid are reported.

METHODS AND MATERIALS

Two cultivars of winter wheat were used, Grana, which is moderately resistant to the aphid *S. avenae*, and Emika, which is susceptible (Leszczynski, 1987). The three species of cereal aphid studied: grain aphid (*Sitobion avenae* (F.)), bird cherry oat aphid (*Rhopalosiphum padi* (L.)), and rose grain aphid (*Metopolophium dirhodum* (W.)), came from stock cultures kept at the University of East Anglia.

The effect of the wheat allelochemicals on the activity of GST was determined by transferring the aphids from barley, which lacks hydroxamic acids, to 4-day-old seedlings of wheat, on which they fed for 48 hr.

The activity of the glutathione *S*-transferase was measured using a slightly modified version of Lindroth's (1989) method. Protein content was determined by Bradford's (1976) method.

Gel filtration of the aphids' cytosolic fraction was done using a FPLC apparatus (Pharmacia) equipped with a Superose TM 12 column, which had previously been equilibrated with 0.1 M Na phosphate buffer of pH 7.5 in 0.15 M NaCl. The cytosolic extract from 2000 aphids fed on seedlings of Emika was lyophilized and suspended in a small volume of 0.1 M Na phosphate buffer pH 7.5. After centrifugation at 14,000*g* for 30 min, 200 μl of the supernatant was loaded onto the FPLC column. During separation, the column was eluted with 20 ml of 0.1 M Na phosphate buffer of pH 7.5 in 0.15 M NaCl (flow rate 0.5 ml/min). Fractions of 0.25 ml were collected and their glutathione *S*-transferase activity determined.

The active fractions were separated by means of SDS-PAGE on 12.5% slab gels by Laemmli's (1970) method and then stained with Coomassie brilliant blue R-250. The staining was calibrated using Sigma SDS-6 Dalton Mark VI as described by Weber and Osborn (1969).

The results given in the table and figures are averages of three replicates. The activity of the glutathione *S*-transferase in the aphids' tissues were subjected to an analysis of variance followed by Duncan's test.

RESULTS

Glutathione *S*-transferase was found in the cytosolic extracts of all three species of cereal aphid. Gel filtration revealed that there was a single peak in enzymic activity in each of the cereal aphids, which was not associated with any of the peaks in absorbance at 280 nm (Figure 1A). SDS-PAGE revealed three protein bands in the glutathione *S*-transferase active fractions (Figure 1B). The first had a molecular weight of 28,500 and was present in similar quantities in all three species. The second had a molecular weight of 27,500 and of the three aphids, *M. dirhodum* had the most and *S. avenae* least. The third fraction differed between species, having a molecular weight of 26,000 in *S. avenae*, 25,500 in *M. dirhodum*, and 24,000 in *R. padi* (Figure 2).

Enzymic activity was greatest in the extracts from *M. dirhodum* and lowest in those from *S. avenae* (Table 1). It was greater in extracts from larvae than in those from adult alatae and apterae in all three species. With the exception of *M. dirhodum*, the activity of glutathione *S*-transferase was significantly higher in the aphids fed on the moderately resistant wheat variety, Grana, than in those fed on the susceptible variety, Emika (Table 1).

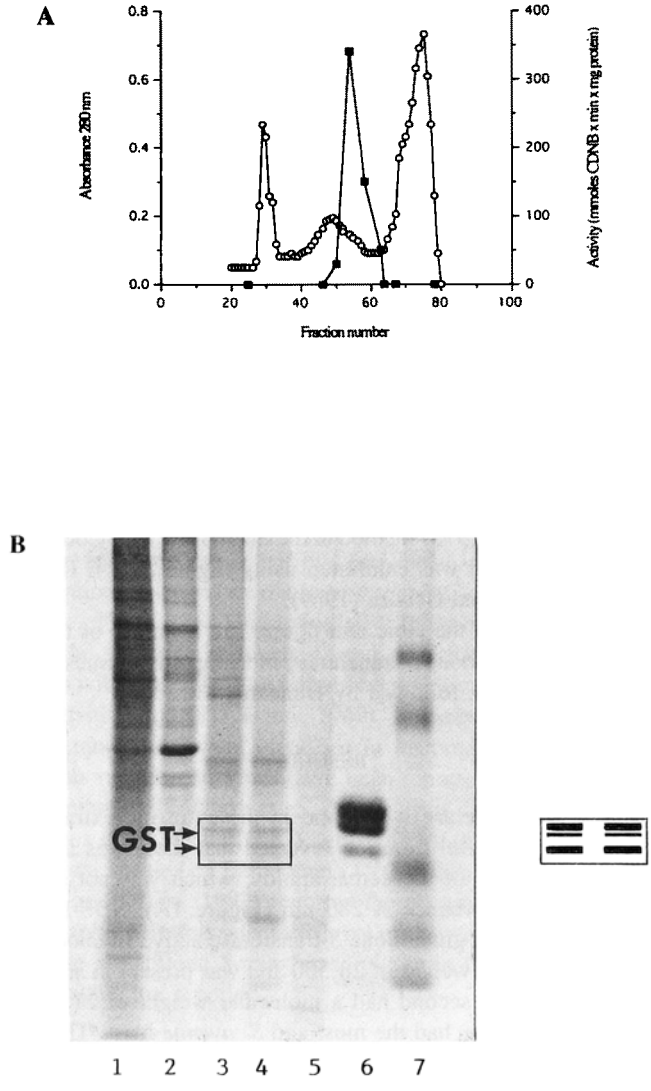

FIG. 1. (A) Profile of the absorbance at 280 nm and the activity of glutathione *S*-transferase in the cytosolic fraction of an extract of the grain aphid *S. avenae* (-○- absorbance 280 nm; -■- GST activity). (B) SDS-PAGE separation of the cytosolic extract and FPLC active fractions of the glutathione *S*-transferase in extracts from grain aphids: lane 1, *S. avenae* total cytosolic fraction; 2, fractions 47–48 (after gel filtration); 3, fractions 53–54; 4, fractions 55–57; 5, fractions 58–60; 6, GST bovine standard; 7, Dalton Mark VI proteins.

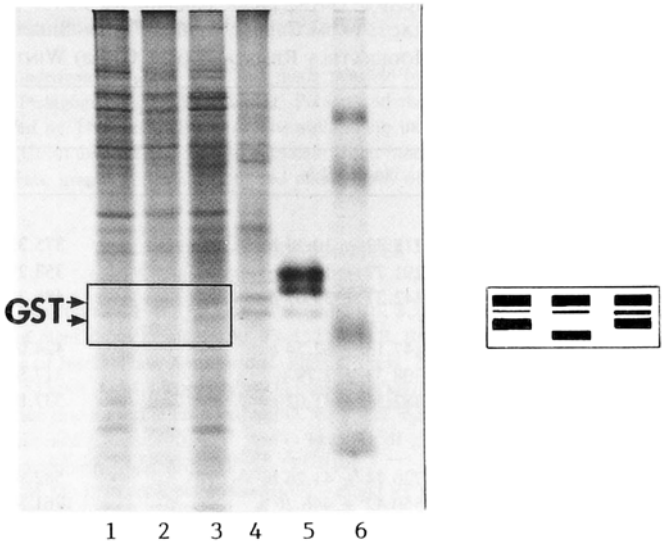

FIG. 2. SDS-PAGE separation of the glutathione *S*-transferase of three species of cereal aphid: lane 1, *S. avenae* total cytosolic fraction; 2, *R. padi* total cytosolic fraction; 3, *M. dirhodum* total cytosolic fraction; 4, fractions 53–54 (collected after gel filtration) showed highest activity of GST); 5, GST bovine standard; 6, Dalton Mark VI proteins.

DISCUSSION

These results clearly demonstrate that there was a single peak of activity of glutathione *S*-transferases present in the cytosolic fraction of the tissue extracts from all three species of cereal aphid. A similar result has been reported for the Mediterranean fruit fly (Yawetz and Koren, 1984). SDS-PAGE of the active fractions revealed three protein bands, which suggested that the cereal aphids' glutathione *S*-transferase might consist of three subunits. The molecular weights of the subunits ranged from 24,000 to 28,500 and are comparable to those of other insects (Motoyama and Dauterman, 1978; Cochrane et al., 1987; Grant and Matsumura, 1989). As GST is made up of several subunits, it may be able to detoxify several cereal allelochemicals.

As reported previously for cereal aphids, the activity of glutathione *S*-transferase in the tissues of *S. avenae* was significantly correlated with the concentration of allelochemicals in the wheat they had fed on, e.g., phenols, hydroxamic acids, and indole alkaloids (Leszczynski et al., 1989; Leszczynski and Dixon, 1990). In the present study a similar trend was observed in the bird-cherry oat aphid, *R. padi*. It is known that different plant xenobiotics can induce

TABLE 1. ACTIVITY OF GLUTATHIONE S-TRANSFERASE (Nanomoles CDNB per Minute per Milligram Protein) IN EXTRACTS FROM CEREAL APHIDS FED ON SEEDLINGS OF SUSCEPTIBLE (var. Emika) AND MODERATELY RESISTANT (var. Grana) WINTER WHEAT

	Wheat[a]	
Aphid	susceptible	resistant
S. avenae		
Alatae	218.77 ± 10.28 h	375.33 ± 64.79 f
Apterae	291.77 ± 10.94 g	353.29 ± 20.69 g
Larvae	442.27 ± 39.07 d	485.33 ± 15.46 d
R. padi		
Alatae	447.13 ± 62.54 e	424.00 ± 89.35 e
Apterae	308.33 ± 2.79 f	317.86 ± 22.63 h
Larvae	487.54 ± 41.02 c	537.86 ± 11.23 c
M. dirhodum		
Alatae		
Apterae	726.74 ± 41.26 b	582.94 ± 8.61 b
Larvae	1550.42 ± 446.26 a	761.50 ± 80.77 a

[a] Values in a column not followed by the same letter are significantly different ($P < 0.01$; Duncan's test).

or inhibit glutathione *S*-transferase activity in insects (Wadleigh and Yu, 1987; Rose et al., 1989; Lee, 1991). This induction or inhibition depends on the nature and concentration of the plant allelochemicals (Dowd, 1990). Our results show a higher activity of the enzyme in the grain aphid and bird-cherry oat aphid, fed on resistant varieties of cereals, which contain high concentrations of allelochemicals. Thus we assume that glutathione *S*-transferase is important in detoxifying cereal secondary metabolites and might enable these two species of aphids to thrive on cereals.

Moreover the enzymes showed a much higher level of activity in the aphids *M. dirhodum* and *R. padi*, which host-alternate between broad-leaved plants and grasses, than in *S. avenae*, which is confined to grasses. Similarly there is a much lower level of activity of these enzymes in the specialist velvet bean caterpillar than in the generalist armyworm (Wadleigh and Yu, 1988). Thus, oligo- and polyphagous insects, which feed on a wide range of host plants with different xenobiotics, may have much better developed glutathione *S*-transferases than monophagous species. However, in the case of aphids there was no difference in the number of subunits of glutathione *S*-transferases associated with the increase in activity. The results presented support the idea that glutathione *S*-transferases are important in the biochemical interactions between aphids and cereals. However, for a fuller understanding of the glutathione-dependent detox-

ification mechanism in cereal aphids further work is needed on the specificity of the subunits of glutathione *S*-transferase.

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ROLE OF DOUGLAS FIR (*Pseudotsuga menziesii*) CARBOHYDRATES IN RESISTANCE TO BUDWORM (*Choristoneura occidentalis*)

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Abstract—The current year's growth of Douglas fir contains galactose, unusual in that this carbohydrate makes up 78.7% of the total carbohydrate fraction. An agar diet study was undertaken to determine the effects of galactose, other carbohydrates, and terpenes on western spruce budworm larval mortality, growth rate, and adult biomas production. All concentrations of the carbohydrates and terpenes tested, as well as other mineral elements not tested, were typical of the current year's foliage of Douglas fir. In experiment I, the diet containing 5.61% total carbohydrate did not significantly affect larval mortality when compared to the control diet. However, diets containing 9.45% and 15% total carbohydrate concentrations significantly increased larval mortality 64% and 96.1%, respectively, when compared to the control. Also in experiment I, terpenes alone (78.9% morality) and terpenes in combination with 9.45% and 15% total carbohydrates significantly increased larval mortality (97.2% and 100%, respectively) when compared to mortality on the control diet (44%). To determine which carbohydrate was causing the adverse effect, 6% glucose, 6% fructose, and 6% galactose were placed individually and in combination with terpenes in diets in experiment II. The 6% galactose diet significantly increased larval mortality and reduced growth rate when compared to the control, glucose, and fructose diets. Glucose resulted in 16% less larval mortality, significantly enhanced female larval growth rate and pupal weight, but did not affect male larval growth rate and pupal weight, when compared to the control. Fructose resulted in a significant decrease in larval mortality and a general trend of enhanced female and male larval growth rate and pupal weight. Larval mortality on terpenes alone was not significantly different from the control, but terpenes with 6% galactose increased larval mortality and decreased female and male growth rate and pupal weight sig-

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nificantly when compared to glucose–terpene and fructose–terpene diets. No significant interactions were found between carbohydrates and terpenes in either experiment.

Key Words—Douglas fir, *Pseudotsuga menziesii*, galactose, terpenes, sugars, carbohydrates, resistance, western spruce budworm, mortality, *Choristoneura occidentalis*, Lepidoptera, Tortricidae.

INTRODUCTION

The definition of foliage quality with respect to phytophagous insects has been well addressed but perhaps not well understood (Stamp, 1990; Redak and Cates, 1984; Mattson et al., 1983; Reese, 1983; White, 1969, 1974). Foliage quality is generally considered to reflect nutritional components as well as the adverse effects of allelochemicals. The favorable role of nitrogen in the relationships between insects and plants has received the most attention (Cates et al., 1987; Hedin, 1983; Scribner and Slansky, 1981; Rosenthal and Janzen, 1979; McNeil and Southwood, 1978), but low levels of nitrogen and other macronutrients and imbalances in primary nutrients have been suggested as possible mechanisms conferring plant resistance to insects (Clancy et al., 1988; Dixon, 1973). Studies involving the utilization of carbohydrates by herbivorous insects have suggested that these compounds favorably affect insect growth (Altman and Dittmer, 1968) and have been shown generally to be feeding stimulants and attractants to the eastern spruce budworm (*Choristoneura fumiferana*) (Heron, 1965). However, studies involving carbohydrates are of limited number compared to those done with nitrogen, and of these carbohydrate studies, few indicate that individual carbohydrates may adversely affect herbivore fitness.

Three carbohydrates, glucose, fructose, and sucrose, have been suggested as the major constituents of conifer foliage (Little, 1970; Chalupa and Fraser, 1968) and are thought to be readily utilized by budworm larvae. Harvey (1974) has shown that most sugars in balsam fir and white spruce increased growth rates of the eastern spruce budworm and promoted greater pupal and adult weight. Clancy (1991) found "resistant" trees to be higher in sugars as compared to "susceptible" trees, and suggested that an artificial diet with 6.4% sugar content was optimal for budworm obtained from a colony. Further studies indicated that at high levels, and in combination with 1.2% N in the diet, sucrose was associated with reduced numbers of larvae in the F_1, F_2, and F_3 generations (Clancy, 1992). We were unable to find information concerning the effects of other individual carbohydrates on the budworm and no studies on the interaction between soluble carbohydrates and secondary metabolites on phytophagous insects.

The objectives of this paper were to determine: (1) the effect of soluble carbohydrates, either individually or in combination, found in the current year's

foliage of Douglas fir on the western spruce budworm (*Choristoneura occidentalis* Freeman), and (2) to evaluate the interaction between soluble carbohydrates and terpenes found in the same foliage. These objectives were generated because results from a previous study indicated that significant seasonal changes occurred in both soluble carbohydrates and secondary metabolites in the current year's growth of Douglas fir (*Pseudotsuga menziesii*) (Zou and Cates, 1994). The total soluble carbohydrates characterizing this foliage were found to be galactose (78.7%), fructose (5.6%), glucose (5.2%), and sucrose (3.2%).

METHODS AND MATERIALS

Egg masses from a nondiapausing colony of budworm obtained from Robert McCron, Integrated Pest Management Institute, Sault Ste. Marie, Ontario, Canada, were used. Larvae were permitted to emerge in a controlled environmental chamber (16:8 hr photoperiod, 24°C, 45% relative humidity) in 10-cm-diameter Petri dishes containing standardized diet (Robertson, 1979) in which nitrogen and carbohydrates were modified to resemble average quantities found in the current year's growth of Douglas fir. Third-instar larvae were randomly assigned to an experimental diet and individually placed in a snaptop Petri dish (9 × 50 mm) to prevent cannibalism.

General Diet Preparation. The concentrations of terpenes and carbohydrates used in the experimental diets were determined from concentrations found in the current year's growth of 125 Douglas fir trees located at our field study sites (Cates and Zou, 1990). These were modified as follows for soluble carbohydrate and nitrogen. Sugar-free wheat germ was prepared following the methods of Harvey (1974). Wheat germ was ground in a Wiley mill (1-mm screen), and extracted with diethyl ether in a Soxhlet extractor for 6 hr. The diethyl ether extract was then extracted with 50% methanol (3 × 350 ml for each 70-g aliquot) to remove carbohydrates. The diethyl ether residue consisting of lipids was placed back into the diet. Ether was completely removed from the diet by rotor-evaporation. The osmotic activity of each of the experimental diets containing carbohydrates and terpenes, when compared to the control diets (noncarbohydrate), was measured using a Vapor Pressure Osmometer (model 5500). The difference in osmotic activity between the experimental and control diets was relatively small (90 mm/kg). Nitrogen concentrations of all diets were adjusted by varying vitamin-free caesin in the diet so that all diets were composed of 1.26% total nitrogen. The diets also contained 3.7 mg/g phosphorus, 3.3 mg/g potassium, 3 mg/g calcium, 16 mg/g zinc, and 76 mg/g iron, all of which were near the average for these nutrients in Douglas fir needles located at our study sites. Nitrogen content was determined by an Autoanalyzer II, and the elements by Atomic Absorption Spectrophotomer (Perkin Elmer 500). Ter-

pene and carbohydrate concentrations were determined by capillary gas chromatography (Hewlett Packard 5890).

Experimental Diets. For experiment I, a total of eight experimental diets were formulated (Table 1). For experiment II, 99% pure β-D-fructose, D-(+)-galactose, and β-D-glucose, purchased from J.T. Baker, Inc. and the U.S. Biochemical Corporation, were verified for purity and placed in the experimental diet, which had been extracted for carbohydrates. Concentrations of each of these individual sugars when added to the appropriate diet (diet 2, glucose; diet 3, galactose; diet 4, fructose) was 6% (Table 3 below). Diet 5 contained no carbohydrate or terpene, and diets 6–8 contained 0.07% total terpene in addition to 6% of an individual carbohydrate. The percent composition of terpenes used for this set of diets is given in Table 1.

Budworm parameters. Initial budworm weight of third-instar larvae was determined by averaging the weight of several cohorts, each consisting of 10 third-instar larvae. Pupae were weighed within 12 hr of pupation, adults were allowed to emerge, frozen immediately after emergence, and then dried for 48 hr at 60°C to obtain adult dry biomass. Larval growth rate was calculated using the formula g/t, where g = the weight gained during the larval feeding period, and t = the duration of the feeding period in days. Larval mortality also was recorded.

Statistical Analyses. The feeding trials were designed as factorial experiments using two factors, soluble carbohydrates and terpenes, with four and two

TABLE 1. DIET COMPOSITIONS FOR EXPERIMENT I USED TO REAR BUDWORM LARVAE AND THE LARVAL MORTALITY ON EACH DIET[a]

Diet	Terpenes (%)[b]	Carbohydrates (%)[c]	Mortality (%)
1	0	0	44.0[a]
2	0	5.61	43.4[a]
3	0	9.45	64.0[b]
4	0	15.0	96.1[d]
5	0.08	0	78.9[b]
6	0.08	5.61	82.5[bc]
7	0.08	9.45	97.2[cd]
8	0.08	15.0	100.0[d]

[a] Categorical analysis was used to compare larval mortality on each treatment diet as compared to the other diets (control = 0 terpenes, 0 carbohydrates). Mortality values followed by the same letter are not significantly different (chi-square test, $P < 0.05$).

[b] Terpenes included tricyclene 4%, α-pinene 16.32%, camphene 27.42%, β-piene 8%, myrcene 1.17%, limonene 6.13%, terpinolene 0.64%, bornyl acetate 35.68%, geranyl acetate 0.475%, citronellyl acetate 0.05%.

[c] Carbohydrates included in each of diets 6, 7, and 8 were mannose 1.2%, fructose 8.9%, galactose 77.1%, glucose 6.7%, sucrose 5.2%.

levels, respectively, for a total of eight combinations. Individual larvae were considered as the basic experimental unit, and each budworm was randomly assigned to each experimental cell resulting in 25 replications per cell. The larval mortality, larval growth rate, pupal weight, and adult dry weight biomass data were transformed using the square root of the arcsine to stabilize the independent variance. Larval mortality, growth rate, and pupal weight were subjected to two-way MANOVA. Both soluble carbohydrate and terpene effects were analyzed as well as the interaction between these factors. In addition, budworm mortality was subjected to two-way categorical analysis; data were expressed as the number died vs. the number surviving, and comparisons between each treatment were made using chi-square. Statistical analyses followed SAS programs (SAS Institute, Inc., 1985).

RESULTS

Results from experiment I indicated that at the 9.45% and 15.0% levels total carbohydrates significantly increased larval mortality when compared to the noncarbohydrate control and the 5.61% diets (Table 1). All diets containing terpenes and carbohydrates significantly increased mortality when compared to the control diet. In addition, diets containing terpenes often resulted in significantly greater larval mortality than diets that varied only in total carbohydrate levels except for the 15% carbohydrate and terpene–15% carbohydrate diets (mortality of 96.1% and 100%, respectively). A chi-square categorical analysis indicated that carbohydrates and terpenes separately had significant effects on mortality, but no significant interaction occurred between total carbohydrate and total terpene content (Table 2). The high level of larval mortality resulted in too few pupae and adults to carry out further statistical analyses on larval growth rate and adult biomass production.

Since total carbohydrates were shown in experiment I to adversely affect budworm mortality, a second experiment was done to determine the effect of

TABLE 2. CHI-SQUARE TEST COMPARING EFFECTS OF TOTAL CARBOHYDRATES AND TOTAL TERPENE CONCENTRATION FROM EXPERIMENT I ON BUDWORM MORTALITY

Compound class	Larval mortality	
	Σ^2	P
Carbohydrates	18.15	0.0004
Terpenes	11.12	0.001
Carbohydrates × terpenes	2.76	0.43

individual carbohydrates, as well as combinations of individual carbohydrates and total terpenes, on larval mortality, larval growth rate, and pupal weight. Larvae reared on galactose showed significantly greater mortality when compared to the control, glucose, and fructose diets. Fructose plus terpenes had the same larval mortality as the control (diet 1) and was not significantly different from the terpene–noncarbohydrate diet. Galactose plus terpene was significantly different from all diets except the galactose and glucose–terpene diets (Table 3). Mortality due to glucose plus terpene was significantly greater than the control, 6% glucose, 6% fructose, and the terpene–fructose diets. Chi-square analysis indicated that individual carbohydrate- and terpene-containing diets had a significant effect on larval mortality ($P = 0.0001$; $P = 0.003$, respectively), but the interaction between terpenes and carbohydrates was not significant (Table 4).

TABLE 3. DIET COMPSITIONS OF INDIVIDUAL CARBOHYDRATES USED TO REAR BUDWORM LARVAE FOR EXPERIMENT II AND LARVAL MORALITY ON EACH DIET[a]

Diet	Terpenes (%)	Carbohydrates (%)[b]	Mortality (%)
1	0	0	36^{bc}
2	0	6	20^{ab}
3	0	6	68^{e}
4	0	6	8^{a}
5	0.07	0	48^{bcd}
6	0.07	6	56^{de}
7	0.07	6	68^{e}
8	0.07	6	36^{b}

[a] Categorical analysis was used to compare laval mortality on each treatment diet to the other diets (control = 0 terpenes, 0 carbohydrates). Mortality values followed by the same letter are not significantly different (chi-square test, $P < 0.05$).
[b] Diets 2 and 6 contained glucose, diets 3 and 7 contained galactose, diets 4 and 8 contained fructose.

TABLE 4. CHI-SQUARE TEST COMPARING EFFECTS OF INDIVIDUAL CARBOHYDRATES AND TOTAL TERPENE CONCENTRATION FROM EXPERIMENT II ON BUDWORM MORTALITY

	Larval mortality	
Compound Class	Σ^2	P
Carbohydrates	20.88	0.0001
Terpenes	8.69	0.003
Carbohydrates × terpenes	5.25	0.15

Glucose and fructose resulted in greater larval growth rates and pupal weights for females as compared to the noncarbohydrate control diet (Tables 5 and 6). Average male growth rate and pupal weight on the glucose diet were not significantly different from the control, and male growth rate on the fructose diet was significantly different from the control but pupal weight was not. Galactose significantly reduced male larval pupal weight and showed a strong trend in reducing male larval growth rate, female pupal weight, and female larval growth rate when compared to the control. Male and female larvae reared on diets containing fructose and glucose were significantly higher in larval growth rate and pupal weight than those reared on the galactose diet. Therefore, the general pattern was that male and female larval growth rates and pupal weights were highest on diets containing fructose, followed by the glucose diet, then the control noncarbohydrate diet, and finally, were lowest on the galactose diet. When terpenes were present in the diets, few significant differences among diets with regard to male and female larval growth rates and pupal weights were observed (Tables 5 and 6). Exceptions to this were the galactose–terpene diet vs. the glucose–terpene diet and vs. the fructose–terpene diet where the galactose–terpene diet resulted in significantly lower female and male growth rates and pupal weights when compared to the other two diets. A two-way MANOVA showed that carbohydrates significantly affected female and male larval growth rate and pupal weight, that terpenes showed a trend toward significantly affecting

TABLE 5. FEMALE AND MALE LARVAL GROWTH-RATE AND PUPAL WEIGHT OF LARVAE REARED ON DIETS CONTAINING INDIVIDUAL CARBOHYDRATES AND TERPENES AND INDIVIDUAL CARBOHYDRATES[a]

	Female		Male	
Diet[b]	Growth rate (mg/day) $X \pm SD$	Pupal weight (mg) $X \pm SD$	Growth rate (mg/day) $X + SD$	Pupal weight (mg) $X + SD$
1 ncho	5.4 ± 3	100 ± 30	4.6 ± 2	81 ± 12
2 glu	8.8 ± 3	140 ± 30	5.6 ± 3	78 ± 25
3 gal	3.8 ± 3	80 ± 50	3.1 ± 2	58 ± 24
4 fru	11 ± 3	140 ± 30	7.9 ± 3	88 ± 28
5 nchot	4.9 ± 1	100 ± 10	3.9 ± 2	71 ± 15
6 glut	6.2 ± 3	120 ± 30	7.0 ± 2	90 ± 13
7 galt	3.5 ± 3	70 ± 50	2.7 ± 1	54 ± 13
8 frut	9.2 ± 3	140 ± 30	6.8 ± 3	86 ± 26

[a] Diet composition for experiment II for terpenes are in the footnotes of Table 1. Statistical comparisons are in Table 6.

[b] ncho = carbohydrate free diet, glu = 6% glucose, gal = 6% galactose, fru = 6% fructose; t = 0.07% terpene added to the above diets.

TABLE 6. STATISTICAL RESULTS COMPARING ALL POSSIBLE COMBINATIONS OF DIETS FROM EXPERIMENT II FOR EACH SEX FOR LARVAL GROWTH RATE AND PUPAL WEIGHT[a]

	Female		Male	
Diet comparison[b]	Growth rate	Pupal weight	Growth rate	Pupal weight
ncho vs. glu	0.021	0.027	0.306	0.799
ncho vs. gal	0.097	0.110	0.098	0.022
ncho vs. fru	0.004	0.039	0.005	0.431
glu vs. gal	0.0001	0.0002	0.005	0.026
glu vs. fru	0.174	0.825	0.041	0.255
gal vs. fru	0.0001	0.0003	0.0001	0.001
nchot vs. glut	0.447	0.409	0.023	0.161
nchot vs. galt	0.202	0.094	0.345	0.221
nchot vs. frut	0.005	0.085	0.024	0.282
glut vs. galt	0.05	0.018	0.005	0.020
glut vs. frut	0.045	0.410	0.835	0.669
galt vs. frut	0.0002	0.001	0.005	0.034
ncho vs. nchot	0.795	0.853	0.490	0.501
glu vs. glut	0.090	0.279	0.261	0.251
gal vs. galt	0.902	0.840	0.729	0.888
fru vs. frut	0.245	0.905	0.494	0.861

[a] Actual growth rates and pupal weights for each sex are found in Table 4. Comparisons were by ANOVA.

[b] ncho = carbohydrate free diet, glu = 6% glucose, gal = 6% galactose, fru = 6% fructose; t = 0.07% terpene was added to the diet.

female larval growth rate and pupal weight ($P = 0.062$), and that no significant interaction occurred (Table 7).

DISCUSSION

Carbohydrates found in the foliage of conifers vary from species to species, but the principal sugars are glucose, fructose, and sucrose (Little, 1970; Chalupa and Fraser, 1968). Douglas fir foliage is similar in that glucose, fructose, sucrose, and mannose were found in the current year's foliage, but significantly different in that 78.7% of the total soluble carbohydrate is galactose (Zou and Cates, 1994). Previous studies with balsam fir and white spruce indicated that the carbohydrate composition in host tissues promoted larval development and survival (Albert et al., 1982; Harvey, 1974; Heron, 1965), although adverse effects of sucrose at very high concentrations have been reported (Clancy, 1992). It is evident from the studies presented in this paper that 6% galactose adversely affected western spruce budworm larval mortality, growth rate, and pupal weight.

TABLE 7. TWO-WAY MANOVA COMPARING EFFECTS OF TOTAL TERPENE CONCENTRATION AND INDIVIDUAL CARBOHYDRATE CONCENTRATION FROM EXPERIMENT II ON BUDWORM LARVAL GROWTH RATE AND PUPAL WEIGHT

	Laval growth rate/pupal weight			
	Female		Male	
Compound class	*F*	*P*	*F*	*P*
Carbohydrates	11.69	0.001	7.18	0.001
Terpenes	2.93	0.062	0.23	0.78
Carbohydrates × terpenes	0.61	0.71	0.43	0.85

pupal weight. This result provides further support that both primary nutrients and secondary metabolites warrant investigation as to their potential adverse effect on the western spruce budworm and other insect herbivores (Clancy, 1991; Cates et al., 1987). Furthermore, these data suggest that investigations utilizing some total measurement of either a complex group of primary nutrients (e.g., total soluble carbohydrates) or secondary metabolites (e.g., total terpenes, total phenolics, total tannins) may not provide the refinement needed to determine the effects of individual compounds on herbivores (Cates and Zou, 1990; Cates et al., 1987).

The high concentrations of galactose found in the foliage of Douglas fir trees, as compared to average concentrations of 3.2% for sucrose, 5.2% for glucose, and 5.6% for fructose, deserves further investigation. The studies reported here were carried out using colony budworm. The effects of galactose and galactose plus individual terpenes and other secondary metabolites need to be determined using natural budworm populations. Although no interactions between carbohydrates and terpenes were significant, 6% glucose in the presence of the terpenes was different from the control and the 6% glucose diet. This suggests that different sugars may interact with terpenes in different ways. Furthermore, individual terpenes with individual carbohydrates also warrants investigation.

We are now investigating whether galactose is present as a free hexose in other conifers that the budworm prefers. Although the information is incomplete for all potential and utilized hosts, galactose appears to be a constituent of raffinose in conifers other than Douglas fir and can be found in low concentration during heavy insect feeding times (Harvey, 1974; Little, 1970). Douglas fir may possess a different metabolic pathway that leads to the production of galactose, and this hypothesis, as well as the mechanism of action against the budworm, are currently under investigation. In addition, we are testing the effects of ga-

ently to nitrogen and terpenes than do natural populations (Cates et al., 1987; Cates and Nay, unpublished data). Finally, data in this paper again verify that terpenes increase budworm larval mortality (Cates and Zou, 1990; Cates et al., 1987), despite the fact that the concentrations used in this study were average to slightly below average concentrations found in natural foliage. However, the reason that these concentrations increased mortality may be due to our using a nondiapausing, colony-reared source of budworm.

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INTERACTIONS BETWEEN *Kalmia* AND BLACK SPRUCE: ISOLATION AND IDENTIFICATION OF ALLELOPATHIC COMPOUNDS

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Abstract—Aqueous extracts of fresh leaves and organic soil of northern sheep laurel (*Kalmia angustifolia* var. *angustifolia*) were found to be inhibitory to the growth of black spruce (*Picea mariana*) germinants. Primary root growth of black spruce was more affected by the extracts than was shoot growth. The growth inhibition caused by the leaf extract was most pronounced under acidic conditions (pH 3–4). The aqueous extract of *Kalmia* leaves contained ferulic, vanillic, syringic, gentisic, *m*-coumaric, *p*-coumaric, *o*-hydroxyphenylacetic, and *p*-hydroxybenzoic acids as well as some other unknown compounds. These compounds were isolated from the aqueous extract of *Kalmia* leaves by ethyl acetate extraction and identified using thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC). Bioassay indicated that the overall toxicity of the phenolic compounds to black spruce appeared to increase in the order of *o*-hydroxyphenylacetic, *p*-hydroxybenzoic, vanillic, *p*-coumaric, gentisic, syringic, ferulic, and *m*-coumaric acids.

Key Words—Allelopathy, toxicity, growth inhibition, *Kalmia angustifolia*, sheep laurel, phenolic compounds, black spruce.

INTRODUCTION

Lack of growth and eventual death of planted conifer seedlings in forest sites dominated by *Kalmia angustifolia* (hereafter referred to as *Kalmia*) has been reported by several authors (Candy, 1951; Richardson, 1975; Wall, 1977). In

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central Newfoundland this has been a major silvicultural problem, particularly in the nutritionally poor site types where *Kalmia* proliferates rapidly following disturbance such as forest clear-cutting and burning. Natural regeneration of conifers also suffers from *Kalmia* interference (Thompson and Mallik, 1989). Extensive underground rhizome systems and stem base sprouting provide effective methods of vegetative regeneration in *Kalmia* (Mallik, 1993). This multi-stemmed ericaceous plant has a competitive advantage over conifers because of its very efficient regeneration strategies. In addition, *Kalmia*-induced site degradation has been attributed to the accumulation of large amounts of organic matter, increasing acidity, iron pan formation (Damman, 1971, 1975), and paludification and nutrient "lock up" (Rowe and Scotter, 1973).

It has been suggested that long-term occupancy of a site by *Kalmia* may preclude any tree regeneration and convert the productive forest site into a permanent ericaceous heath (Meades, 1986). Similar organic accumulation and soil acidity changes have been reported for other ericaceous plants of cool, moist, temperate climate, particularly *Calluna vulgaris* and *Erica cinerea* (Grubb et al., 1969; Gimingham, 1972). These and other heath-forming species in western Europe have been also reported to produce allelopathic substances in soil (Ballester et al., 1977; Carballeira, 1980; Jalal and Read, 1983a,b; Hobbs, 1984). Handley (1963) reported *Calluna*-induced "growth check" in sitka spruce (*Picea sitchenis*). Robinson (1971, 1972) reported that root exudate of *Calluna vulgaris* contained allelopathic compounds that were inhibitory to some mycorrhizal fungi of conifer seedlings. Jalal et al. (1982) and Jalal and Read (1983a,b) isolated and identified a number of phenolic compounds from *Calluna* plant and soil material. Some of these compounds were highly phytotoxic and were believed to contribute to the exclusion of other plants including trees from ericaceous heath (Read, 1984).

Peterson (1965) reported that dry leaf extracts of *Kalmia* in boiling water and ethanol inhibit primary root growth of black spruce (*Picea mariana*). Recently, Mallik (1987) and Thompson and Mallik (1989) demonstrated that water extracts of *Kalmia* plant components and soil significantly reduced the early growth of black spruce and balsam fir (*Abies balsamea*) seedlings. It is now evident that *Kalmia* can induce black spruce growth inhibition: (1) by direct competition due to its vigorous vegetative and reproductive growth following disturbance, (2) by producing allelopathic substances, and (3) by increasing paludification and acidification of the habitat.

A solution must be found to counteract the growth check of black spruce on *Kalmia* sites. The goal of our research was to find a way to enhance black spruce regeneration on sites dominated by *Kalmia*. This might be achieved either by controlling *Kalmia* growth or by reducing the *Kalmia*-induced growth inhibition in black spruce. Since treatments with herbicides, NPK fertilization, and cutting and burning have been unsuccessful in controlling *Kalmia* (Mallik 1990,

1991), we adopted an alternative approach. We hypothesized that allelopathy and soil acidity were greater problems for black spruce than competition from *Kalmia* and that inoculation of black spruce with appropriate mycorrhizial fungi capable of withstanding the *Kalmia* allelopathy would help improve black spruce growth. We have conducted various experiments to test these hypotheses. The objective of the present paper was to test whether *Kalmia* leaves contain water-soluble substance(s) that inhibit seedling growth of black spruce and if changes in pH of leaf extract have an effect on seedling root growth. Attempts were made to isolate and identify the allelopathic compounds from *Kalmia* leaves.

METHODS AND MATERIALS

Collection of Organic Soil and Leaf Samples. Samples of the humus (H) layer (Pritchett, 1979) were collected from a *Kalmia* site immediately adjacent to a black spruce stand in Terra Nova National Park, Newfoundland, in the fall of 1989. This site was 1 km west of the park information center beside the Trans Canada Highway, and it had been occupied by a 50-year-old black spruce stand that was clear-cut 12 years previously. Following the clear-cut, the site was dominated by *Kalmia* without any tree regeneration. Fresh leaves of *Kalmia* were collected from a patch of ericaceous heath at Pippy Park in St. John's, Newfoundland, in the fall of 1989 and the summer of 1990.

Preparation of Water Extracts of Kalmia *Leaves and Soil.* Fresh green *Kalmia* leaves (100 g in fresh weight) were soaked in 1 liter of distilled water for 24 hrs at 4°C. The extract was then filtered through Whatman No. 1 filter paper and stored at − 20°C. The water extract so obtained had a pH of 5.2 and an osmotic potential of −1.5 bar. This solution was treated as 100% (v/v) of water extract of leaves for bioassay.

A 2-liter volume of the organic soil (H layer and the top part of A layer classified according to Pritchett, 1979) of fresh soil samples was collected from the field and placed into a plastic cylinder (15 cm diameter × 40 cm height), the bottom of which had 25 small holes that allowed drainage. Leaching was conducted for two days in a cold room (5–8°C) and was achieved by releasing 2 liters of distilled water at a slow rate from a vessel over the cylinder. The leachate was collected and passed through Whatman No. 1 filter paper and stored at −20°C. The soil leachate so obtained had a pH of 5 and an osmotic potential of −1.9 bar. This solution was treated as 100% (v/v) of water extract of soil for bioassay.

Isolation and Identification of Phenolic Acids. One liter of water extract of *Kalmia* leaves (fraction 1) prepared as described above was acidified to pH 3 with 0.2 M HCl and centrifuged at 12,000 g for 10 min. The supernatant was extracted three times with 300 ml of hexane to remove lipids (fraction 2). The

resulting aqueous solution (fraction 3) was extracted four times with 250 ml of ethyl acetate. The combined ethyl acetate extract (fraction 4) was dried over anhydrous sodium sulfate and then evaporated to dryness in vacuum at 39°C.

To obtain preliminary identification of phenolic acids, the organic phase of ethyl acetate extract was dissolved in 25 ml of methanol and analyzed by using two-dimensional thin-layer chromatography (TLC) on cellulose plates developed in benzene–acetic acid–water (6:7:3) as the first direction and 15% aqueous acetic acid as the second direction. The spots were revealed by their color under UV light and by spraying with Folin reagent in presence of NH_3 vapor. The color and R_f values of the spots were then compared with phenolic acid standards (Sigma Chemical Co., St. Louis, Missouri) developed under the same conditions.

Ethyl acetate extract (fraction 4) and fractions collected from TLC plates were further analyzed by using an HPLC (Perkin-Elmer series 4) equipped with a reverse-phase C-18-μBondapak column (7.8 mm × 30 cm, Water). Elution was monitored at 280 nm using a variable wavelength Perkin-Elmer LC-85B spectrophotometric detector. The solvent system was methanol and 5% formic acid in water, and the separation was done under the conditions shown in Table 1. Identification of phenolic compounds was done by comparison of the retention times of unknown peaks with the retention times of phenolic acid standards.

Bioassays. Seeds of black spruce obtained from Mount Pearl Tree Nursery, St. John's, Newfoundland, were stratified in cold running water for three days and subsequently surface sterilized in 30% H_2O_2 for 15 min. The seeds were germinated in sterile Petri dishes (90 mm diameter) with a sheet of Whatman No. 3 filter paper and sterile distilled water. When a radicle of 2 mm emerged, the germinants were carefully transferred into a bioassay Petri dish with Whatman No. 3 filter paper wetted with sterile distilled water or different allelopathic solutions as described in the following experiments.

In experiment 1, water extracts of leaves (10, 25, 50, and 100%, v/v; pH 5.2), water extracts of soil (10, 25, 50, and 100%, v/v; pH 5), and distilled

TABLE 1. HPLC GRADIENT CONDITIONS FOR SEPARATION OF PHENOLIC ACIDS FROM WATER EXTRACT OF *Kalmia* LEAVES

Time (min)	Flow rate (ml/min)	5% HCOOH (pump %)	Methanol (pump %)	Gradient curve
0–9	1.5	90	10	linear
90–20	1.5	60	40	linear
20–25	1.5	60	40	linear
25–35	1.5	0	100	linear

water (control) were sterilized by passing through sterile membrane filters (Millipore, 0.45 μm) and then 5 ml of the treatment solution was applied to a Petri dish lined with a sheet of Whatman No. 3 filter paper. Twenty germinants with radicles of 2 mm were transferred into the Petri dish to expose the treatments.

In experiment 2, water extracts of leaves (10, 25, and 50%, v/v) and distilled water (control) were adjusted to pH 3, 4, or 5 with 0.1 M HCl before they were sterilized by passing through membrane filters (Millipore, 0.45 μm). Five milliliters of the treatment solution was applied to a Petri dish lined with a sheet of Whatman No. 3 filter paper. Twenty germinants with radicles of 2 mm were transferred into the Petri dishes to expose the treatments.

In experiment 3, for bioassay with various fractions of the extracts, organic fractions (equivalent to 500 mg of fresh leaves) were first taken to dryness under nitrogen gas, dissolved in 2 ml of methanol, sterilized by passing through a nylon membrane filter, and then spotted on Whatman No. 3 filter paper in a Petri dish. After methanol was evaporated from the filter paper, 5 ml of sterile distilled water was added onto the filter paper and 20 germinants were transferred into the Petri dish. Aqueous fractions (amount equivalent to 500 mg of fresh leaves) were sterilized using membrane filters and applied to the bioassay Petri dishes directly. Known phenolic compounds were first dissolved in methanol, diluted with distilled water to final concentrations 0.5, 1.0, 2.0, and 5.0 mM, and 5 ml of each treatment solution was applied to bioassay Petri dishes.

All of the bioassay experiments were conducted at 20–23°C with a 16-hr photoperiod for up to 14 days. During the period of incubation, 3 ml of sterile distilled water was added to each Petri dish every three days. The experiment was repeated twice in triplicate Petri dishes for each treatment. At the end of the experiment, the lengths of the primary roots and shoots of at least 15 seedlings were measured to the nearest millimeter. Microscopic observations of primary root development were also carried out during the experiment.

RESULTS

Effects of Water Extracts of Leaves and Soil. Water extract of leaves inhibited the primary root growth of black spruce seedlings, and this inhibitory effect became significant as the extract concentrations increased (Table 2). At 100% leaf extract, the length of primary roots of the seedlings was only half that of the controls. A 10% of water extract of humus inhibited root growth of germinants by 19% as compared to controls exposed to distilled water (Table 2). An increase of the humus-extract concentration from 10 to 100%, however, did not cause further reduction in root growth. Shoot growth of the germinants was not significantly affected by water extracts of leaves or soil at any of the concentrations tested. Microscopic examination showed that the primary roots of

TABLE 2. EFFECT OF WATER EXTRACTS OF *Kalmia* LEAVES AND SOIL ON GROWTH OF BLACK SPRUCE SEEDLINGS[a]

Treatment	Conc. (%)	Length (mm) of Root	Length (mm) of Shoot
Leaf water extract	0	16.0 ± 0.6a	19.5 ± 2.0
	10	13.9 ± 0.3ab	20.3 ± 0.7
	25	13.4 ± 0.8b	19.6 ± 1.2
	50	12.2 ± 1.2b	18.7 ± 0.5
	100	8.2 ± 0.9c	18.1 ± 1.0
Soil water extract	0	16.0 ± 0.6a	19.5 ± 2.0
	10	13.0 ± 1.2b	19.5 ± 1.0
	25	13.1 ± 0.8b	19.7 ± 0.7
	50	13.9 ± 1.0b	21.8 ± 1.0
	100	12.7 ± 1.2b	20.0 ± 1.2

[a] Data are means and standard deviations of 40 seedlings; mean values in a column within a treatment followed by the different letters are significantly different at $P \leq 0.01$ in Tukey's multiple range test.

the seedlings treated with water extracts of leaves and humus did not produce normal radicle growth beyond the point of the root–shoot transition zone and were unable to produce root hairs.

Effects of Extracts at Different pH. Since the soil pH of *Kalmia* sites in central Newfoundland was very acidic (between pH 2.8 and 4.5), the bioassay with water extracts of leaves (10, 25, and 50%) was performed at pH 3, 4, and 5. In the absence of the extracts, acidic pH alone caused a 50% reduction in root growth at pH 3 and 30% at pH 4 as compared to that at pH 5 (Table 3). In the presence of the water extract of leaves, there was a clear trend showing more reduction in root growth as the extract concentration increased and as the pH became more acidic. At pH 3, the germinants were unable to grow in the presence of 50% of the extract, and the root growth was severely inhibited by the extract at concentrations 10 and 25%. At pH 4, about 20, 40, and 50% reductions in root growth were obtained when the germinants were exposed to 10, 25, and 50% of the extract, respectively. At pH 5, about 10, 20, and 45% reductions in root growth were observed when the germinants were exposed to 10, 25, and 50% of the extract, respectively. Shoot growth was inhibited only at pH 3 by 25 and 50% of the extract (Table 3).

Isolation and Identification of Allelopathic Compounds. The water extract of fresh leaves of *Kalmia* was light yellow in color at pH 3 and became dark brown at pH above 5. Approximately 90% of the water extract was recovered after hexane extraction and 82% of the original volume was recovered after

TABLE 3. GROWTH OF BLACK SRUCE SEEDLINGS EXPOSED TO WATER EXTRACT OF *Kalmia* LEAVES AT DIFFERENT pH VALUES[a]

	Length (mm) of root			Length (mm) of shoot		
Conc. (%)	pH 5	pH 4	pH 3	pH 5	pH 4	pH 3
0	18.0 ± 1.3a	16.3 ± 0.9ab	10.8 ± 0.3b	20.0 ± 1.8a	17.5 ± 2.1a	17.5 ± 1.6a
10	16.2 ± 1.5a	12.5 ± 1.0b	3.2 ± 0.5c	19.1 ± 1.8a	19.9 ± 1.1a	17.1 ± 1.7a
25	13.9 ± 1.1a	9.4 ± 1.0b	2.0 ± 0.3c	18.3 ± 0.9a	16.8 ± 2.6a	2.1 ± 1.2b
50	9.5 ± 0.7a	6.5 ± 0.5b	0.5 ± 0.3c	18.7 ± 1.3a	18.2 ± 2.0a	1.0 ± 0.6b

[a]Data are means and standard deviations of 40 replicate seedlings; mean values within a extract concentration treatment followed by the different letters are significantly different at $P \leq 0.01$ in Tukey's multiple range test.

ethyl acetate extraction. When bioassayed on black spruce germinants (Table 4), all fractions exhibited a degree of inhibitory effect on primary root growth; fractions 1, 3, and 4 were more inhibitory than fractions 2 and 5. Shoot growth was slightly inhibited by fractions 1 and 4. The stronger inhibitory effects found in fractions 3 and 4 indicate that the allelopathic compounds in water extract were effectively partitioned into the aqueous phase after hexane extraction and into the organic phase after ethyl acetate extraction. The slightly lower activity in the hexane aqueous phase (fraction 3) and the ethyl acetate extract (fraction 4) as compared to water extract may be due to the loss of some of the water extract to the hexane organic phase and incomplete separation by ethyl acetate.

Because of its high activity, the ethyl acetate extract (fraction 4) was used for further analyses by TLC and HPLC. Two-dimensional TLC of fraction 4 revealed the presence of 18 bands, and eight of them were identified as simple phenolic compounds by comparing with standards (Table 5). The chemical nature of the other bands was not identified. The presence of the eight phenolic compounds was confirmed using HPLC analysis of the fractions collected from TLC plates by comparing their retention times with standards (Table 5). The identified phenolic compounds were *m*-coumaric acid, *p*-coumaric acid, ferulic acid, gentisic acid, *p*-hydroxybenzoic acid, *o*-hydroxyphenylacetic acid, syringic acid, and vanillic acid. HPLC analysis of ethyl acetate extract (fraction 4) detected at least 30 peaks (Figure 1). All eight identified phenolic compounds eluted between 0 and 22 min along with a number of unknown peaks. Bioassay with black spruce germinants showed that the 0- to 22-min fraction contained inhibitory activity and caused 53% inhibition in root growth, while the 23- to 35-min fraction caused only 13% inhibition in root growth.

TABLE 4. EFFECT OF DIFFERENT FRACTIONS OF *Kalmia* LEAF EXTRACT ON GROWTH OF BLACK SPRUCE SEEDLINGS[a]

Fraction	Root growth		Shoot growth	
	Length (mm)	% of control	Length (mm)	% of control
Control	16 ± 0.7a	100	18 ± 2.0	100
Water extract (1)	5 ± 1.4d	31	15 ± 2.6	83
Hexane organic phase (2)	14 ± 2.5ab	88	18 ± 2.8	100
Hexane aqueous phase (3)	9 ± 1.7c	56	19 ± 3.3	105
Ethyl acetate organic phase (4)	7 ± 2.0cd	44	17 ± 3.1	90
Ethyl acetate aqueous phase (5)	13 ± 1.6b	81	18 ± 1.8	100

[a]Data are means and standard deviations of 15 replicate seedlings; mean values in a column followed by the different letters are significantly different at $P \leq 0.05$ in Tukey's multiple range test.

TABLE 5. PHENOLIC ACIDS IDENTIFIED FROM WATER EXTRACT OF *Kalmia* LEAVES BY TLC AND HPLC

Phenolic compound		TLC R_f		HPLC retention time (min)
Common name	Systematic name	Solvent 1	Solvent 2	
Gentisic acid	2,5-Dihydroxybenzoic acid	0.18	0.75	6.1
p-Hydroxybenzoic acid	4-Hydroxybenzoic acid	0.36	0.75	7.4
o-Hydroxyphenylacetic acid	2-Hydroxyphenylacetic acid	0.50	0.86	10.7
Vanillic acid	4-Hydroxy-3-methoxybenzoic acid	0.92	0.77	12.4
Syringic acid	4-Hydroxy-3,5-dimethoxybenzoic acid	0.90	0.77	16.2
p-Coumaric acid	4-Hydroxycinnamic acid	0.42	0.56	18.4
m-Coumaric acid	3-Hydroxycinnamic acid	0.43	0.62	19.9
Ferulic acid	4-Hydroxy-3-methoxycinnamic acid	0.69	0.50	21.7

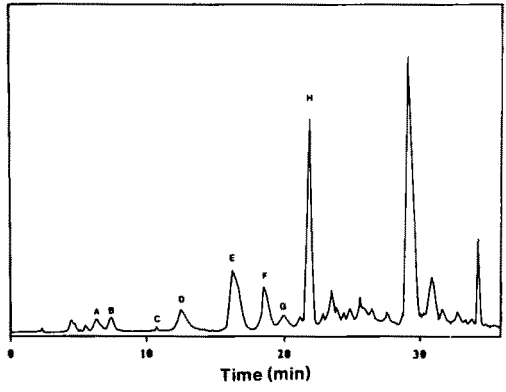

FIG. 1. Separation of phenolic acids from ethyl acetate extract of *Kalmia* leaf water leachate by HPLC. Peak A, gentisic acid, B, *p*-hydroxybenzoic acid; C, *o*-hydroxyphenylacetic acid; D, vanillic acid; E, syringic acid; F, *p*-coumaric acid; G, *m*-coumaric acid; and H, ferulic acid.

Allelopathic Activity of Identified Phenolic Compounds. Allelopathic activities of the identified phenolic compounds were assayed on black spruce at four concentrations. All of the compounds were inhibitory to the growth of black spruce seedlings, particularly to the root growth, and the degree of inhibition varied depending on the individual compound and the concentrations applied

TABLE 6. EFFECT OF PHENOLICS ISOLATED FROM WATER EXTRACT OF *Kalmia* LEAVES ON GROWTH OF BLACK SPRUCE SEEDLINGS[a]

Compound	Conc. (mM)	Root growth		Shoot growth	
		Length (mm)	% of control	Length (mm)	% of control
Control (H_2O)	0	16 ± 2.2a	100	19 ± 1.8a	100
Gentisic acid	0.5	15 ± 2.6a	94	22 ± 3.9a	100
	1	13 ± 3.7a	81	17 ± 3.9ab	89
	2	1 ± 0.3b	6	15 ± 2.7b	79
	5	0c	0	0c	0
p-Hydroxybenzoic acid	0.5	13 ± 3.7ab	81	19 ± 2.8a	100
	1	9 ± 2.9b	55	19 ± 3.0a	100
	2	1 ± 0.3c	6	15 ± 2.4b	79
	5	0d	0	0c	0
o-Hydroxyphenylacetic acid	0.5	1 ± 0.5b	6	16 ± 2.3ab	84
	1	0c	0	13 ± 1.6b	68
	2	0c	0	9 ± 2.1b	47
	5	0c	0	0c	0
Vanillic acid	0.5	14 ± 3.7a	87	16 ± 2.0ab	84
	1	6 ± 1.7b	38	15 ± 1.6b	79
	2	4 ± 1.3b	25	16 ± 2.0ab	84
	5	0c	0	13 ± 2.9b	68
Syringic acid	0.5	17 ± 2.7a	100	20 ± 2.5a	100
	1	9 ± 2.5b	57	18 ± 1.7a	95
	2	7 ± 2.1b	44	17 ± 3.2a	89
	5	0c	0	8 ± 2.0b	42
p-Coumaric acid	0.5	13 ± 2.6a	81	19 ± 3.3a	100
	1	9 ± 2.5a	56	15 ± 3.1ab	79
	2	3 ± 0.7b	19	13 ± 2.2b	68
	5	0c	0	13 ± 3.6b	68
m-Coumaric acid	0.5	16 ± 2.3a	100	19 ± 3.2a	100
	1	13 ± 1.9a	81	19 ± 3.1a	100
	2	12 ± 3.2a	75	17 ± 3.4a	89
	5	4 ± 1.5b	25	17 ± 3.5a	89
Ferulic acid	0.5	15 ± 2.3a	94	17 ± 2.6a	90
	1	12 ± 1.9ab	89	18 ± 3.1a	95
	2	8 ± 2.2b	42	15 ± 3.0a	89
	5	0c	0	0c	0

[a] Data are means and standard deviations of 36 replicate seedlings; mean values in a column followed by the different letters are significantly different at $P \leq 0.01$ in Tukey's multiple range test.

(Table 6). Among the eight phenolic acids, *o*-hydroxyphenylacetic acid was the most toxic and caused severe inhibition (94%) of root growth at the concentration of 0.5 mM, and *m*-coumaric acid was the least toxic compound that caused only 75% of inhibition at the concentration of 5 mM. The toxicity of the other six compounds was between *o*-hydroxyphenylacetic and *m*-coumaric acids and caused 100% inhibition of root growth at 5 mM, 56–94% inhibition at 2 mM, 11–62% inhibition at 1 mM, and 0–19% inhibition at 0.5 mM. Microscopic examination showed that the roots of inhibited seedlings did not produce root hairs. Shoot growth was generally less affected by these compounds than root growth.

DISCUSSION

Water extract of fresh *Kalmia* leaves inhibited growth of black spruce seedlings and the inhibitory effect was directly on the primary root growth of these seedlings rather than on shoot growth. Microscopic examination of the root–shoot transition regions of the inhibited black spruce seedlings showed that they did not produce normal radicle growth beyond the point of the transition zone. Similar results were also reported by Peterson (1965) and Mallik (1987) when they treated black spruce seedlings with water extracts of dry and fresh *Kalmia* leaves and litter. The inhibitory effect of water extract of *Kalmia* leaves on root growth was not caused by a pH effect, although there was a strong interaction effect between the extract and pH. The inhibition also did not appear to be caused by the low osmotic potentials of the bioassay solution since the osmotic potentials were never lower than −2 bar. In most of the bioassays, the leaf leachate concentrations used were lower than 50% and had osmotic potentials always higher than −0.7 bar. Del Moral and Cates (1971) determined the osmotic potentials of a larger number of plant extracts that were assayed for allelopathic activities and found that low potentials even at −2 bar did not cause inhibitory effects. Therefore, inhibition of root growth of black spruce seedlings in the present study was most likely caused by water-soluble phytotoxic substances leached from *Kalmia* leaves.

Isolation of compounds from the water extract of *Kalmia* leaves revealed the presence of ferulic, vanillic, syringic, gentisic, *m*-coumaric, *p*-coumaric, *o*-hydroxyphenyl acetic, and *p*-hydroxybenzoic acids as well as several other unknown compounds. All of these identified phenolic compounds except *m*-coumaric acid have previously been reported to be present in ericaceous plants; they are found in leaves of *Erica scoparia* (Ballester et al., 1977) and *E. australis* (Carballeira, 1980) and in shoots and roots of *Calluna vulgaris* (Jalal et al., 1982). Gentisic, vanillic, syringic, and *p*-hydroxybenzoic acids are benzoic acid derivatives found in many angiosperm species, and the last three are also

present widely in gymnosperms and ferns (Hartley and Whitehead, 1985). Ferulic, *p*-coumaric, and *m*-coumaric acids are cinnamic acid derivatives that occur almost universally in higher plants (Rice, 1984). In addition, some of these compounds, particularly *p*-hydroxybenzoic, vanillic, *p*-coumaric, and ferulic acids, have been found in heathland soils dominated by *Calluna vulgaris* (Jalal and Read, 1983a,b) and other ericaceous plants (Chou and Muller, 1972; Carballeira, 1980). Recently, Oden et al. (1992) identified a germination inhibitor called batatasin III (5-methoxy-3,3-dihydroxy-dihydrostilbene) from air-dried leaves of *Empetrum hermaphorditum*, a mat-forming understory plant in northern Sweden. Phenolic compounds in soil could arise directly from residuals and living tissues of plants. There is also evidence that phenolic acids in the soil originate in part from the decomposition of plant residues and in part from synthesis by soil microorganisms (Rice, 1984).

It is well established that many phenolic acids are toxic to plants. Most of these phenolic acids identified in the present study have been widely reported to have phytotoxic properties and play an important role in allelopathic interactions (Chou and Muller, 1972; Del Moral and Muller, 1970; Fisher, 1980; Rice, 1984). In the present study, all eight phenolic acids significantly inhibited the root growth of black spruce seedlings at concentrations of 1 mM or above. At the concentration 0.5 mM, most of individual phenolic acids had a slight or no inhibition on the root growth. The overall toxicity of these compounds appeared to decrease in the order *o*-hydroxyphenylacetic acid > *p*-hydroxybenzoic > vanillic > *p*-coumaric > gentisic > syringic > ferulic > *m*-coumaric.

The presence of these phenolic compounds in water extract from *Kalmia* leaves may be of particular importance to the allelopathic activity of *Kalmia* on black spruce. Although the estimated concentration of each phenolic acid in the water extract was less than 0.1 mM, these compounds might interact in an additive or synergistic manner. It has been demonstrated in many studies that combinations of allelopathic compounds often exert additive or synergistic effects against the growth of plants (Rice, 1984). For example, Read (1984) tested 11 acids identified from *Calluna* soil (including vanillic, ferulic, syringic, and coumaric acids), singly and in mixture, against *Agrostis tenuis* seedlings and found that the mixture almost completely inhibited the root growth. The mechanisms of action of these phenolic acids on black spruce are not known. According to Rice (1984), phenolic compounds can affect respiration, protein synthesis, specific enzymes, mineral uptake, and membrane permeability. Several workers (Lee, 1977; Lynch, 1980) also suggested that the activity of phenolic acids is manifested maximally only when they are in the undissociated form. Since a decrease of pH leads to an increase in the relative proportions of undissociated to dissociated acids, the potential phytotoxicity increases greatly with increasing acidity. *Kalmia*-dominated soils in Newfoundland frequently have a pH in the range 2.8–4.5 (Mallik, unpublished data), and there is a strong indication that

Kalmia, being an ericaceous plant, may, like other members of the Ericaceae such as *Calluna vulgaris*, acidify the soil (Grubb et al., 1969; Gimingham, 1972). Results presented in this study demonstrate clearly that increased acidity alone can cause an inhibitory effect on the primary root growth of black spruce and the interaction between the acidic pH and *Kalmia* allelopathic compounds can create even more severe inhibitory conditions to the root growth of the seedlings. The inhibition of root growth of black spruce seedlings by water extract of *Kalmia* humus was also observed in this study. It is difficult to determine whether allelopathic compounds in the humus extract are of *Kalmia* plant origin or formed by soil-inhabiting microorganisms. Allelopathic compounds may be produced in any part of the plant, but the highest concentrations are often found in foliage (Fisher, 1980). The chemicals can be released by leaching or by volatilization from the intact leaves into the soil. Microorganisms may also degrade higher plant constituents to allelopathic compounds. In the case of *Kalmia*, it has been suggested that the allelopathic substances are either synthesized in *Kalmia* leaves and then transported to the soil through rainfall or they may be the decomposition products of the leaves (Mallik, 1987). Since the allelopathic compounds of the intact fresh leaves are soluble in water, the results reported here provide support for the above hypothesis.

From a practical viewpoint, allelopathy, as caused by a water extract of *Kalmia* leaves, has the potential to induce regeneration failure of black spruce in Newfoundland. While the potential of the *Kalmia* allelopathic influence exists, other possibilities such as low soil pH and low available nutrient status of soil may also have significant effect on the black spruce growth in *Kalmia*-dominated sites of Newfoundland.

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OVIPOSITION STIMULANTS IN *Barbarea vulgaris* FOR *Pieris rapae* AND *P. napi oleracea:* ISOLATION, IDENTIFICATION AND DIFFERENTIAL ACTIVITY

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Abstract—The closely related butterflies, *Pieris rapae* and *P. napi oleracea*, readily laid eggs on *Barbarea vulgaris* in greenhouse cages. When offered a choice between cabbage and *B. vulgaris*, *P. rapae* showed no preference, but *P. napi oleracea* preferred *B. vulgaris*. Bioassays of extracts of *B. vulgaris* foliage revealed the presence of oviposition deterrent(s) in 1-butanol extracts as well as stimulants in the postbutanol water extracts. However, the deterrent effect was apparently outweighed by the strong stimulatory effect in the whole plants. The postbutanol water extract was preferred over an equivalent cabbage extract by both species, but more significantly in the case of *P. napi oleracea*. The stimulants were isolated by open column chromatography and HPLC, and the activity was associated with three glucosinolates. *P. napi oleracea* was more sensitive than *P. rapae* to the natural concentration of compounds **1** and **3**, whereas both species were strongly stimulated to oviposit by natural concentrations of compound **2**. Compounds **1** and **2** were identified as (2*R*)-glucobarbarin and (2*S*)-glucobarbarin, respectively, and **3** was identified as glucobrassicin, on the basis of their UV, mass, and NMR spectra. When the pure compounds were tested at the same concentrations applied to bean plants, the (2*R*)-glucobarbarin at 0.2 mg/plant was preferred over a standard cabbage extract by both butterfly species. However, at a dose of 0.02 mg/plant, *P. rapae* preferred the cabbage extract whereas *P. napi oleracea* still preferred the (2*R*)-glucobarbarin. No such difference in response of the two species to the same two concentrations of (2*S*)-glucobarbarin was obtained. The results indicate a distinct difference in sensitivity of these butterflies to the epimers of glucobarbarin, and the differences in behavioral responses of the two butterfly species depend to a large extent on the concentration of stimulant present.

Key Words—*Pieris rapae*, *Pieris napi oleracea*, Lepidoptera, Pieridae, *Bar-*

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barea vulgaris, oviposition, stimulants, glucosinolates, glucobarbarin, glucobrassicin.

INTRODUCTION

The cabbage butterflies, *Pieris rapae* and *P. napi oleracea*, have host ranges that overlap but are not identical (Richards, 1940; Chew, 1972a,b). *P. napi oleracea* larvae are found on a greater diversity of plants compared with *P. rapae* larvae (F.S. Chew, personal communication). Because the female adults of *Pieris* species are highly mobile and the larvae are relatively stationary, acceptance or rejection of potential host plants by egg-laying butterflies is the first and key step in determining this host range. Oviposition stimulants and deterrents are known to play an important role in this step (Renwick and Radke, 1988; Renwick et al., 1989).

The significance of a balance between negative and positive chemical input in host selection by herbivorous insects (Dethier, 1982; Miller and Strickler, 1984; Renwick and Radke, 1987) was demonstrated in our recent studies with *P. rapae* and *P. napi oleracea* as a model system (Huang and Renwick, 1993; Huang et al., 1993a,b). Furthermore, distinct differences between *P. rapae* and *P. napi oleracea* in acceptance of potential hosts have been found (Huang and Renwick, 1993). Different preferences can be explained by differential sensitivity of the two species to stimulants and deterrents. For example, *P. rapae* was strongly deterred by the cardenolides erysimoside and erychroside and was only weakly stimulated by the glucosinolates glucoiberin and glucocheirolin in *Erysimum cheiranthoides*, resulting in rejection of the plant (Huang et al., 1993a). In contrast, *P. napi oleracea* was very sensitive to the stimulants but was less sensitive to the deterrents, leading to acceptance of the same plant species (Huang et al., 1993a). Similarly, *Iberis amara* contains both stimulants and deterrents that are involved in regulating oviposition by *P. rapae* and *P. napi oleracea* (Huang et al., 1993b). 2-*O*-β-D-glucosyl cucurbitacin I and 2-*O*-β-D-glucosyl cucurbitacin E from *I. amara* were identified as oviposition deterrents to *P. rapae*, but *P. napi oleracea* was behaviorally insensitive to these compounds (Huang et al., 1993b). Furthermore, the minor glucosinolate (glucoiberin) in *I. amara* was less stimulatory to *P. rapae*, whereas *P. napi oleracea* was stimulated as strongly by this glucosinolate as by the most abundant glucosinolate (sinigrin) (Huang et al., 1993b). In nature, these *Pieris* species do not often share host plants, but some overlap probably occurs (F.S. Chew, personal communication). *Barbarea vulgaris* is a common host of *P. rapae* in early spring, as it is readily available before *Brassica* crops are growing. However, *B. vulgaris* is not a known host of *P. napi oleracea*. The plant is acceptable for oviposition, but the larvae do not survive (F.S. Chew, personal communication).

Preliminary observations with caged butterflies indicated that both species

readily accept *B. vulgaris*. This study was initiated to determine whether the same chemical cues from *B. vulgaris* are used by both species for oviposition and to identify the compounds involved.

METHODS AND MATERIALS

Insects and Plants. *P. rapae* and *P. napi oleracea* butterflies for behavioral assays were obtained from colonies started from field-collected insects each summer and maintained in the laboratory at ca. 22°C under fluorescent lights providing a photoperiod of 16:8 hr light–dark. Oviposition occurred in the greenhouse, with supplementary lighting, at ca. 25°C. *P. rapae* larvae were reared on cabbage (*Brassica oleracea* L. var. Golden Acre) and *P. napi oleracea* on *Conringia orientalis* (hare's ear, Cruciferae) plants. Pupae were separated by sex (Richards, 1940) and kept in screen cylinders until eclosion. *B. vulgaris* plants were collected from the field in the middle of July in Ithaca, New York. Cabbage plants (4–6 weeks old) for extraction were grown in an air-conditioned greenhouse maintained at ca. 25°C. Supplemental light was provided by 400-W multivapor high-intensity discharge lamps.

Extraction of Plant Materials. Fresh foliage was extracted in boiling ethanol for 5 min, cooled, homogenized, and filtered. The ethanolic extract was evaporated to dryness under reduced pressure and lipids were removed with *n*-hexane. The defatted residue was dissolved in water and the aqueous extract partitioned three times with 1-butanol. The butanol extract and the postbutanol water extract were concentrated under reduced pressure at ca. 50°C and stored in the refrigerator.

Thin-Layer Chromatography. Thin-layer chromatography (TLC) on 5 × 10-cm, 0.25-mm-thick, Whatman K6 silica gel plates was employed to monitor separation of the active compounds, with ethyl acetate–methanol–acetic acid–water (4:1:1:0.5) as the solvent system. The plates were dried with a hair-drier immediately after development. Spots were visualized by spraying with 1% ceric sulfate solution followed by heating at 110°C for ca. 15 min. Preparative TLC was carried out on 20 × 20 cm, 0.5-mm-thick, Merck silica gel 60 plates with the same solvent system and the bands were extracted with MeOH.

HPLC of Desulfoglucosinolates. Desulfated samples were prepared to test for the presence of glucosinolates, according to the method of Minchinton et al. (1982). A column was packed in a 12.5-cm Pasteur pipet with 200 mg DEAE Sephadex A-25 in 0.5 mol pyridine-acetate buffer. The column was conditioned with the buffer (6 ml) followed by water (6 ml). After loading the samples (5–10 g leaf equivalents in 0.5–1.0 ml water), the column was eluted with water (10 ml or until the eluate was colorless), and 1 ml 0.25% aqueous solution of

sulfatase (Sigma Chemical Co.) was applied. The column was kept at room temperature overnight and then eluted with 2 ml water. The desulfated products were filtered and analyzed by HPLC on a reversed-phase C_{18} column (25 × 0.46 cm) using a solvent gradient program as follows: 0% CH_3CN in water from 0 to 2 min, 10% at 10 min, 30% at 30 min, and 100% at 35 min. The flow rate was maintained at 1 ml/min. A diode array detector (Hewlett Packard model 1040A) was used to monitor the eluate at 219 nm.

Fractionation of Stimulants. The stimulatory materials in the postbutanol water extract were subjected to preliminary separation by flash column chromatography using 45 × 2-cm reversed-phase columns packed with 30 g 55- to 105-μm preparative C_{18} silica (Millipore Corporation, Milford, Massachusetts 01757). About 200 g leaf equivalents of the extracts were loaded onto each column. Twenty-five fractions (15 ml each) were collected by sequentially eluting the column with 0.5% potassium sulfate (150 ml), water (150 ml), 25% (15 ml), 33% (15 ml), 50% (15 ml), and 100% (30 ml) methanol in water. These fractions were tested for stimulatory activity.

Isolation of Active Compounds. Three glucosinolates, **1–3,** were found in the postbutanol water extract of *B. vulgaris* foliage. Preliminary experiments showed that only those glucosinolate-containing fractions from open-column chromatography of the post-butanol water extract were stimulatory to *P. rapae* and *P. napi oleracea.* TLC and HPLC of desulfoglucosinolates revealed that open-column fractions 1–8 contained only nonglucosinolate constituents. Fractions 9–12 contained minor amounts of glucosinolates **1** and **2** along with some other unknown compounds. These unknown compounds were removed from the fractions by TLC and combined with fractions 1–8 to provide a nonglucosinolate fraction (FNG). The remaining glucosinolates from TLC were combined with fractions 13–19 in which glucosinolates **1** and **2** were the major components to form F1 + 2. Fractions 20–25, which contained many unknown compounds as well as glucosinolate **3,** were combined to form F3. These fractions resulting from combinations (FNG, F1 + 2, and F3) were tested for stimulatory activity in choice bioassays with cabbage postbutanol water extract as a control stimulant.

Glucosinolate **1** was eluted from the open column earlier than the major part of glucosinolate **2,** although some overlap occurred after the first run. Those fractions containing both **1** and **2** were combined, reloaded onto the same column, and the column was eluted with the same solvent system. This procedure was repeated two more times until **1** was isolated from **2** and other compounds.

Fractions containing glucosinolate **2** and other minor nonglucosinolates were combined and separated by HPLC on a semipreparative reversed-phase C_{18} column (50 × 0.8 cm) using a linear water–acetonitrile gradient from 0% CH_3CN at 0 min to 100% CH_3CN at 30 min. Glucosinolate **2** was collected and its purity confirmed by HPLC of desulfated products.

Fraction F3 containing glucosinolate **3** was loaded onto a 25 × 1-cm ion-exchange column packed with 1 g DEAE Sephadex A-25. Ten fractions (20 ml each) were collected by successively eluting the column with H_2O (20 ml), 0.5% (20 ml), 1% (20 ml), 2% (20 ml), 4% (20 ml), and 10% (100 ml) K_2SO_4. Fractions 7 and 8, which contained most of glucosinolate **3**, were combined, dried under reduced pressure, and extracted with MeOH. The MeOH-soluble portion was evaporated to dryness under reduced pressure and separated by HPLC on a Supelcosil LC-ABZ column (25 × 0.46 cm) using a gradient program as follows: 20% CH_3CN in 25 mM KH_2PO_4 at 0 min, 25% at 10 min, and 100% at 15 min. Glucosinolate **3** was collected and its purity checked by HPLC of the desulfoglucosinolate.

Bioassays. Oviposition bioassays were conducted in screen cages (48 × 48 × 48 cm) in a greenhouse as described by Renwick and Radke (1988). Eight pairs of newly emerged butterflies were transferred to each cage in the greenhouse. Each cage was supplied with a vial of 10% sucrose solution containing yellow food coloring and a cotton wick to facilitate feeding. During the preoviposition period, a cabbage plant was placed in each cage. When more than 50 eggs in one day were observed, the plant was removed and the butterflies were used for testing the next day. Treated and control plants were placed in opposite corners of the cage. Positions of the plants were alternated in each cage to control for possible position effects. Bioassays were started at 0930 hr and the eggs laid were counted at 1530 hr. Both *Pieris* species were tested at the same time to minimize the possible effects of differences between plant batches, intensity of sunlight and other factors on oviposition behavior. Treated plants were sprayed with samples dissolved in a methanol–water combination (70 or 90% methanol depending on solubility of the samples). For stimulant assays, control plants were sprayed with cabbage postbutanol water extract (as a standard of stimulant). An extract from the same batch of cabbage plants was used in all stimulant bioassays. In deterrent assays, control plants were sprayed with solvent alone. The solutions were applied in a fine mist with a chromatographic sprayer to both upper and lower leaf surfaces. Deterrent activity was monitored by applying test solution on cabbage plants (ca. 5 weeks old, with a canopy span of ca. 16 cm) grown individually in 10-cm cord pots. Stimulatory effects were tested using neutral (stimulants were not present) bean (*Phaseolis vulgaris* var. Sieva) plants as the oviposition substrate. The bean plants used for bioassays were presented as single plants at the two-leaf stage (ca. 4 weeks old, with a canopy span of ca. 14 cm) in plastic pots (6.25 × 6.25 cm).

Design and Analysis. A replicate consisted of one cage with eight pairs of butterflies, and 8–12 replications were performed for each bioassay. When cabbage postbutanol water extracts were used as controls and when comparisons between bioassays were necessary, the relative stimulatory activities were presented as an oviposition stimulant index (OSI), based on the number of eggs

laid on treated and control plants: OSI = 100 (Treated − Control)/(Treated + Control).

A positive OSI means that the test material is more stimulatory than the cabbage postbutanol water extract, whereas a negative OSI indicates the stimulatory activity is weaker than that of the cabbage postbutanol water extract.

Deterrent activities were compared by calculating an oviposition deterrent index (ODI) from the number of eggs laid on treated and control plants: ODI = 100 (Control − Treated)/(Control + Treated).

The proportion of eggs laid on the treated plant to that on both treated and control plants was calculated, and the data were subjected to arcsine square root transformation. Differences between treatments and controls were analyzed using a one-sample *t* test on the transformed data, under the null hypothesis that the total number of eggs was distributed evenly over treated and control plants. A paired *t* test or a Waller-Duncan *K*-ratio *t* test on the transformed data was used to assess significance of differences between insect species or among treatments.

Identification. Identification of the stimulants was based on their UV, mass, ^{13}C and ^{1}H NMR spectra. UV spectra of isolated compounds were obtained from the diode array detector in water–acetonitrile. The glucosinolates (**1, 2,** and **3**) in the plant were collected as desulfated products (**1a, 2a,** and **3a**) by HPLC on the semipreparative reversed-phase C_{18} column (50 × 0.8 cm) using a water–acetonitrile gradient as follows: 0% CH_3CN at 0 min, 5% CH_3CN at 2 min, 10% CH_3CN at 30 min, and 100% CH_3CN at 40 min. The structures of the isolated desulfoglucosinolates were determined by mass spectrometry and ^{13}C and ^{1}H NMR spectroscopy. ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra in MeOH-d_4 were recorded on a Varian XL-400 instrument. The chemical shifts were indirectly referenced to tetramethylsilane by using the solvent as an internal reference. Positive-ion FAB mass spectra were obtained on a VG ZAB-SE mass spectrometer. Magic bullet (5 : 1 dithiothreitol–dithioerythritol) (Costello, 1990) was used as a matrix (Mass Spectrometry Laboratory, University of Illinois).

RESULTS

In choice bioassays with whole plants, *P. rapae* laid a similar number of eggs on *B. vulgaris* and on its favorite host plant, cabbage (Figure 1). However, *P. napi oleracea* preferred *B. vulgaris* over cabbage when offered the same choice. The preference for *B. vulgaris* was different between insect species ($P < 0.0032$). Both *Pieris* species laid fewer eggs on cabbage plants treated with butanol extract from *B. vulgaris* than on plants treated with solvent alone, indicating the presence of oviposition deterrent(s) in the butanol extract (Figure 1). The deterrence by the extract was similar for both species ($P < 0.3402$) and

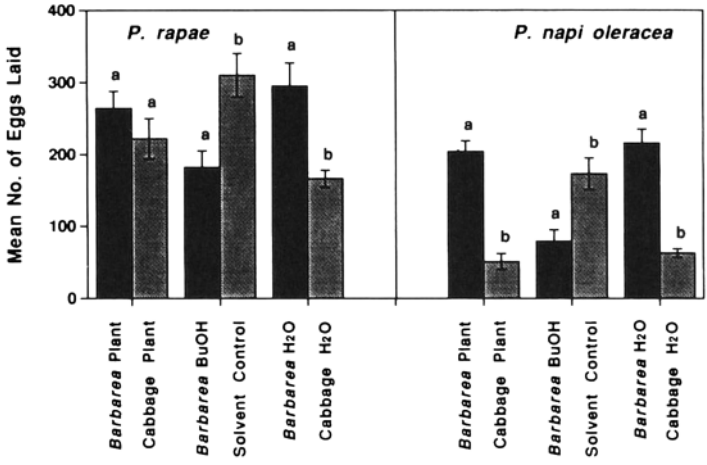


FIG. 1. Oviposition by *P. rapae* and *P. napi oleracea* on: a choice of *B. vulgaris* or cabbage plants, a choice of cabbage plants treated with butanol extract from *B. vulgaris* or with solvent alone (control); and a choice of bean plants treated with postbutanol water extracts from *B. vulgaris* or cabbage (control). Five gram leaf equivalents of each extract were used for each replication; replicated eight times with the exception of postbutanol water extract, which was replicated 12 times. A replication consisted of one bioassay cage with eight pairs of butterflies. Means ($\pm$SE) with the same letters in each series are not significantly different according to a one-sample t test ($P < 0.05$), under the null hypothesis that eggs were distributed evenly over control and treated plants.

was rather weak (oviposition deterrent index: *rapae* 26.4; *napi* 36.6) at the concentration tested. In a choice between bean plants treated with postbutanol water extract of *B. vulgaris* or cabbage (as control, containing stimulants), both *Pieris* species laid many more eggs on the *Barbarea*-treated than on the cabbage-treated plants (Figure 1), but the oviposition stimulant indices (OSI) for *P. rapae* (24.7) and *P. napi oleracea* (52.1) were significantly different ($P < 0.0029$). These results indicate that the *B. vulgaris* extract contained strong stimulants but that the two *Pieris* species were stimulated to different degrees by the active material. Subsequent studies were, therefore, focused on the isolation, identification, and biological activity of the stimulants.

When glucosinolates in the postbutanol water extract from *B. vulgaris* were analyzed by HPLC of their desulfoglucosinolates, three prominent peaks were detected (Figure 2). These were referred to as **1a, 2a,** and **3a,** the desulfated products of compounds **1, 2,** and **3,** respectively. The fraction containing mainly compounds **1** and **2** (F1 + 2) was more stimulatory to both *P. rapae* and *P. napi oleracea* than was the equivalent cabbage postbutanol water extract (Figure 3). The fraction containing compound **3** along with nonglucosinolate compounds

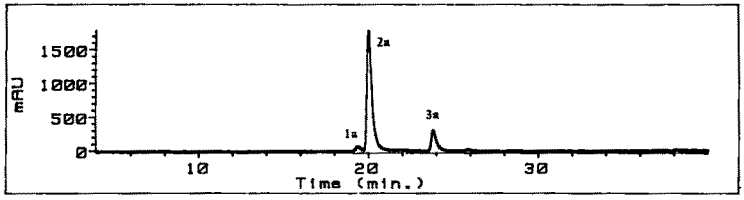


FIG. 2. HPLC separation of desulfoglucosinolates from the postbutanol water extract of *B. vulgaris*. UV monitoring at 219 nm.

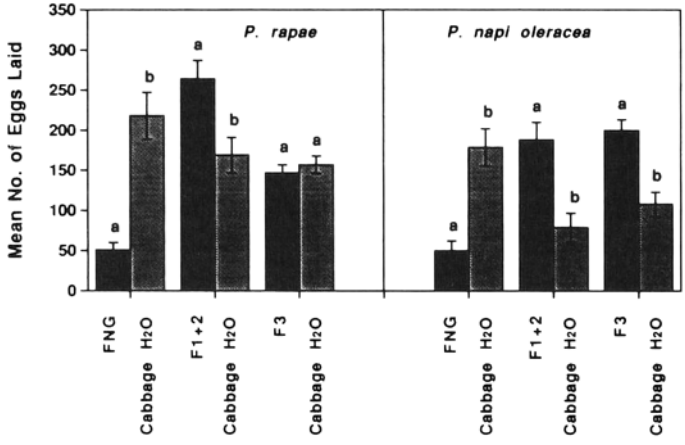


FIG. 3. Oviposition by *P. rapae* and *P. napi oleracea* on bean plants treated with fractions FNG (nonglucosinolates), F1 + 2 (containing glucosinolates **1** and **2**), or F3 (containing glucosinolate **3**) from *B. vulgaris*, with postbutanol water extract from cabbage as controls in all cases. Three gram leaf equivalents were used for each replication; replicated eight times. A replication consisted of one bioassay cage with eight pairs of butterflies. Means (±SE) with the same letters in each series are not significantly different according to a one-sample *t* test ($P < 0.05$), under the null hypothesis that eggs were distributed evenly over control and treated plants.

(F3) was also stimulatory to *P. rapae*, although no significant differences were found between the numbers of eggs laid on bean plants treated with this fraction or with the cabbage extract (Figure 3). However, *P. napi oleracea* was stimulated more by F3 than by the cabbage extract (Figure 3). The fraction containing no glucosinolate (FNG) had little stimulatory activity for either of the species when compared with the cabbage extract (Figure 3). These results suggest that glucosinolates probably play a major role in stimulating oviposition by the two

butterfly species. The glucosinolates were therefore isolated and tested individually in bioassays.

P. rapae and *P. napi oleracea* responded differentially to compound **1** at the natural concentration (ca. 0.03 mg/g fresh leaf material, based on HPLC peak area of desulfoglucosinolate) in *B. vulgaris* (Figure 4, $P < 0.0029$). *P. rapae* was stimulated more by the cabbage postbutanol water extract than by compound **1** (OSI = −23.6), but the reverse was true for *P. napi oleracea* (OSI = 17.4). Compound **2** was the most abundant glucosinolate in foliage of *B. vulgaris*. The concentration of **2** (ca. 1.82 mg/g fresh leaf material) was about 55 times higher than that of **1.** More eggs were laid by both species on bean plants treated with **2** than on those treated with the cabbage extract (Figure 4; OSI: *rapae* 19.1, *napi* 33.2). The number of eggs laid by *P. rapae* on bean plants treated with compound **3** (ca. 0.13 mg/g fresh leaf material) was not significantly larger than that on plants treated with the cabbage extract (OSI = 9.8), but *P. napi oleracea* was stimulated more by compound **3** than by the cabbage extract (Figure 4; OSI = 39.6). Based on these results, we conclude that compounds **1, 2,** and **3** can account for the stimulatory effect of the postbutanol water extract from *B. vulgaris* on the two *Pieris* species.

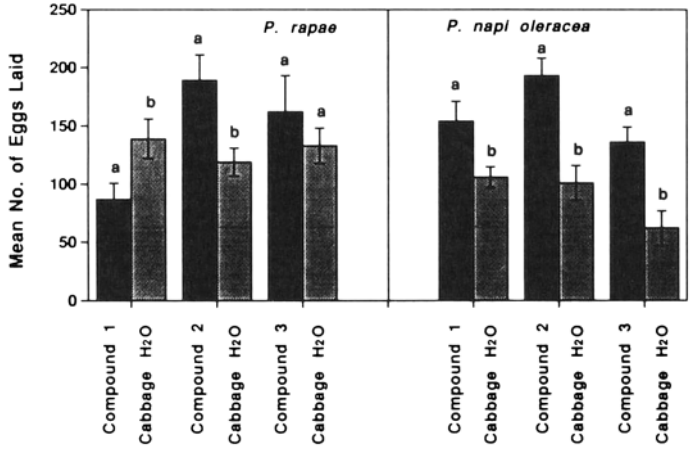
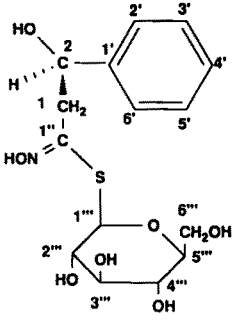
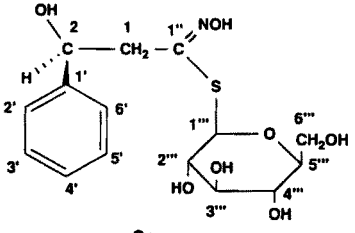


FIG. 4. Oviposition by *P. rapae* and *P. napi oleracea* on bean plants treated with glucosinolates **1, 2,** or **3** from *B. vulgaris*, with postbutanol water extract from cabbage as control in all cases. One gram leaf equivalent of each compound was used for each replication with compounds **1** and **3,** and two gram leaf equivalents were used for compound **2.** Replicated eight times. A replication consisted of one bioassay cage with eight pairs of butterflies. Means (±SE) with the same letters in each series are not significantly different according to a one-sample *t* test ($P < 0.05$), under the null hypothesis that eggs were distributed evenly over control and treated plants.

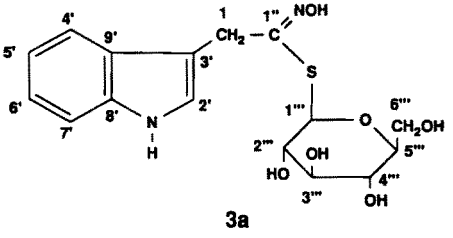
Compounds **1**, **2**, and **3** were identified as (2*R*)-glucobarbarin, (2*S*)-glucobarbarin, and glucobrassicin, respectively, based on UV, mass, ^{13}C, and ^{1}H NMR spectra of their desulfated products **1a**, **2a**, and **3a** (Figure 5). The UV spectra of **1a** and **2a** were identical (λ_{max} in H_2O–CH_3CN:228 nm). Moreover, the FAB mass spectra suggested that the two compounds were isomers [*m/z*: 360 $[M + H]^+$, 342 $[M - OH]^+$, and 177 $[S. Glu - H_2O]^+$]. This conclusion



1a



2a



3a

FIG. 5. Structures of **1a**, **2a**, and **3a**, desulfated products of (2*R*)-glucobarbarin (**1**), (2*S*)-glucobarbarin (**2**), and glucobrassicin (**3**), respectively.

TABLE 1. ^{1}H NMR SPECTRAL DATA[a] OF **1a** AND **2a**

Proton	**1a**	**2a**
1a	2.90 dd (8,15)	2.85 dd (5, 15)
1b	2.98 dd (8, 15)	2.98 dd (8, 15)
2	5.04 t (8)	5.18 dd (5, 8)
2′ and 6′	7.46 d (9)	7.40 d (8)
3′ and 5′	7.27 t (9)	7.32 t (8)
4′	7.18 t (9)	7.22 t (8)
1‴	4.95 d (10)	4.79 d (9.8)
2‴-5‴	3.20-3.40 m	3.25-3.45 m
6‴a	3.50 dd (5, 12)	3.62 dd (5, 11.9)
6‴b	3.69 d (12)	3.88 bd (11.9)

[a]Chemical shifts are given in ppm and *J* values in parentheses are in Hz. d = doublet; dd = double doublet; bd = broad doublet; t = triplet and m = multiplet. Proton designations according to Figure 5.

TABLE 2. ^{13}C NMR SPECTRAL DATA OF **1a, 2a,** AND **3a**

Carbon	**1a**	**2a**	**3a**
1	42.8	43.0	30.3
2	73.1	72.7	
1′	145.2	144.9	
2′	129.3	129.3	123.9
3′	128.4	128.4	111.5
4′	127.3	127.1	119.5[a]
5′	128.4	128.4	119.8[a]
6′	129.3	129.3	122.5
7′			112.3
8′			138.2
9′			128.2
1″	151.9	151.2	154.3
1‴	82.5	83.3	82.8
2‴	74.5	74.3	74.5
3‴	79.6	79.4	79.4
4‴	71.2	71.1	71.2
5‴	82.1	82.1	82.1
6‴	62.6	62.8	62.7

[a]Values may be interchanged. Carbon designations according to Figure 5.

was further supported by ^{1}H and ^{13}C NMR data (Table 1 and 2). These data suggested that **1a** and **2a** were isomers of 2-hydroxyphenethyl desulfoglucosinolate. Since **2** appears to be the predominant glucobarbarin isomer usually found in *B. vulgaris*, and this is known to have the 2*S* configuration (Kjaer and Gmelin, 1957), we concluded that **1** must have the 2*R* configuration. Further support for this assignment was obtained when the optical rotation (measured on a Perkin-Elmer 241 polarimeter) were found to be distinctly different. Compound **1** was more dextrorotatory. Furthermore, our assignments are consistent with the observations of Jensen (1990), who found that when the desulfoglucosinolates were separated by reversed-phase HPLC, the 2*R* epimers of 2-hydroxy-substituted glucosinolates were always eluted earlier than the 2*S* epimers.

The UV maxima of **3a** in H_2O–CH_3CN (220 and 278 nm) were identical to those of desulfoglucobrassicin (Renwick et al., 1992). The identity of **3a** as desulfoglucobrassicin was confirmed by its mass spectral data (FABMS, *m/z*: 369 $[M + H]^+$, 177 $[S.Glu - H_2O]^+$, and 130 $[C_9H_8N]^+$) and ^{13}C NMR data (Table 2) (Sachdev-Gupta et al., 1992).

To determine whether *P. rapae* or *P. napi oleracea* respond differently to the epimeric 2-hydroxyl substituted glucosinolates, (2*R*)glucobarbarin and (2*S*)-glucobarbarin were isolated and compared at the same concentration, with cabbage postbutanol water extract as control. (2*R*)-Glucobarbarin was isolated from seeds of *B. vulgaris* ssp. *X* (collected by J.K. Nielsen in Denmark), by open-column chromatography and HPLC of the postbutanol water extract as described in the Methods and Materials. The seeds of this subspecies were chosen as the source for isolating (2*R*)-glucobarbarin because this was the only glucosinolate present (J.K. Nielsen, personal communication), and the concentration was high (ca. 2.75 mg/g dry seeds). As shown in Figure 6, (2*R*)-glucobarbarin at a dose of 0.2 mg/bean plant was preferred by both *Pieris* species over the cabbage extract at a dose of 0.4 gram leaf equivalents (GLE) per plant (OSI: *rapae* 26.7, *napi* 15.3; between insect species $P < 0.2580$). When the dose of this compound was decreased by a factor of 10 (0.02 mg/plant) and that of cabbage extract maintained unchanged, *P. napi oleracea* was still stimulated more by the compound than by cabbage extract, while *P. rapae* preferred the cabbage extract, and the OSIs were significantly different (11.2 vs. −12.2) (Figure 6). This result is consistent with the bioassay results using a natural concentration (ca. 0.03 mg/plant) of (2*R*)-glucobarbarin (Figure 4, compound **1**). There were no significant differences between insect species or responses to different concentrations (0.2 mg and 0.02 mg/plant) when (2*S*)-glucobarbarin was compared with cabbage extract (Figure 6). The OSIs for this isomer ranged from 3.1 to 19.4. The higher dose (0.2 mg/plant) of this compound used in these bioassays was about 18 times lower than that used in the bioassays with the natural concentration (Figure 4, compound **2**, ca. 1.82 mg/GLE, 2 GLE/plant).

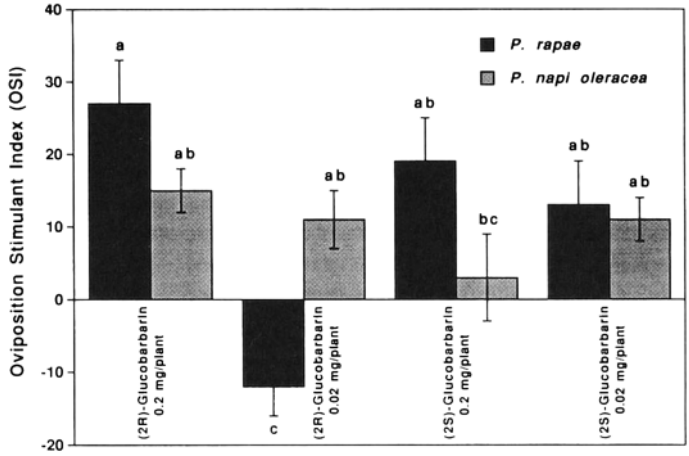


FIG. 6. Oviposition stimulant index (OSI) of isolated (2*R*)-glucobarbarin and (2*S*)-glucobarbarin tested at different doses for *P. rapae* and *P. napi oleracea* on bean plants. Postbutanol water extract from cabbage at a dose of 0.4 gram leaf equivalents/plant was used as control in all cases. OSI = 100 (Treated − Control)/(Treated + Control). Replicated eight times. A replication consisted of one bioassay cage with eight pairs of butterflies. Means (±SE) with the same letters are not significantly different according to a Waller-Duncan *K*-ratio *t* test (*K* = 100).

DISCUSSION

The presence of both deterrents and stimulants affecting oviposition by *P. rapae* and *P. napi oleracea* on *B. vulgaris* has been clearly demonstrated in bioassays of butanol and postbutanol water extracts, respectively. The deterrence of the plant to both species was relatively weak, and the deterrent(s) have yet to be identified. Because the deterrent activity was outweighed by the stimulatory effect of the glucosinolates, the plant species was highly acceptable for ovipositing butterflies of both species. This indicates that a highly acceptable host plant may also contain deterrents, and the final "decision" to accept or reject a potential host by the *Pieris* butterflies is based on a balance between positive and negative inputs from the plant. This finding is consistent with the results of previous studies where oviposition stimulants and deterrents regulated differential acceptance of *E. cheiranthoides* and *I. amara* by the two *Pieris* species (Huang et al., 1993a,b).

Glucobarbarin was first identified by Kjaer and Gmelin (1957, 1958) from seeds of *B. vulgaris* and leaves or inflorescences of *Reseda luteola*. The presence of sinigrin and gluconasturtiin in roots of *B. vulgaris* was also previously reported (reviewed by Kjaer, 1960). More recently, gluconasturtiin, sinigrin, and glu-

coiberverin as well as glucobarbarin have also been found in seeds of the same species (Daxenbichler et al., 1991). Jensen (1990) detected the two epimers (2*S*) and (2*R*) of glucobarbarin as well as gluconasturtiin in the seeds of *B. vulgaris*. In this study we identified (2*S*)-glucobarbarin, (2*R*)-glucobarbarin and glucobrassicin as the major glucosinolates in foliage of *B. vulgaris*, and only traces of gluconasturtiin were detected (based solely on the HPLC retention time of the desulfoglucosinolates). This is the first time that glucobrassicin has been reported as one of the prominent glucosinolates in this plant species.

The (2*S*)-glucobarbarin, (2*R*)-glucobarbarin, and glucobrassicin isolated from *B. vulgaris* foliage in this study were all stimulatory to both *Pieris* species at the natural concentrations, and the activities of these glucosinolates can account for the stimulation of oviposition by the butterflies on this plant. Differential sensitivity of the two species to (2*R*)-glucobarbarin was demonstrated, although responses of the two butterfly species to (2*S*)-glucobarbarin from the plant were similar (Figures 4 and 6). Therefore, a distinct difference in sensitivity to the geometric isomers was suggested. (2*R*)-Glucobarbarin was present at a very low concentration in *B. vulgaris*, and it was less stimulatory to *P. rapae* at the natural concentration compared with cabbage postbutanol water extract (Figure 4). In contrast, *P. napi oleracea* preferred this compound over cabbage postbutanol water extract under the same conditions. Results of bioassays using different doses of (2*R*)-glucobarbarin confirmed the fact that the two *Pieris* species are differentially sensitive to this compound at a low concentration (0.02 mg/plant, Figure 6). This finding might explain why *P. rapae* showed no preference when given a choice between *B. vulgaris* and cabbage plants, but *P. napi oleracea* preferred *B. vulgaris* over cabbage (Figure 1). However, the sensitivity of *P. napi oleracea* to (2*R*)-glucobarbarin did not significantly increase when the concentration was increased 10-fold, whereas *P. rapae* responded much more strongly to the higher concentration (Figure 6).

Different sensitivities of *P. rapae* and *P. napi oleracea* to glucosinolates were previously found when glucoiberin, glucocheirolin, and sinigrin were identified as oviposition stimulants in *E. cheiranthoides* and *I. amara* (Huang et al., 1993a,b). However, different responses of these related butterflies to geometric isomers of a glucosinolate have not been reported previously. Different biological activity of (2*R*)- and (2*S*)-glucobarbarin has been noted in rats. Feeding experiments showed that epimeric 2-hydroxy-substituted glucosinolates including (2*R*)-glucobarbarin and (2*S*)-glucobarbarin had different physiological and toxic effects on rats (reviewed by Jensen, 1990). The two *Pieris* species apparently respond differently to changing concentrations of the major glucosinolates in *B. vulgaris*. *P. napi oleracea* is more sensitive to low concentrations and is less responsive to increases in concentration. Similar differences in the response of the two butterflies to other glucosinolates has recently been found (Huang and Renwick, 1994). This means that the natural concentrations of semiochem-

icals present in a plant are very important in determining the relative acceptability of potential hosts to these closely related pierids.

Previous studies showed that *P. rapae* strongly preferred the indole glucosinolate, glucobrassicin, over sinigrin, which has an aliphatic aglycone (Traynier and Truscott, 1991; Renwick, et al., 1992). In a comparative study using four plant species, Sachdev-Gupta et al. (1992) suggested that *P. rapae* responds preferably to aromatic glucosinolates. Therefore, the strong stimulatory effect of *B. vulgaris*, which contains aromatic and indolic glucosinolates in the foliage, could be expected for *P. rapae*. However, previous studies on other plants have suggested that *P. napi oleracea* is more sensitive to aliphatic, thioalkyl, sulfinyl, and sulfonyl glucosinolates, as in the cases of *E. cheiranthoides* (Huang et al., 1993a) and *I. amara* (Huang et al., 1993b). The preference of *P. napi oleracea* for *B. vulgaris* over cabbage might be explained by its high sensitivity to both isomers of glucobarbarin. Another possible explanation is that the high concentration of glucobrassicin in cabbage extracts could have a slight suppressant effect on *P. napi oleracea* (Huang and Renwick, 1994). The degree of preference of *P. napi oleracea* for *B. vulgaris* also represents an "oviposition mistake" by this species, as the larvae do not generally survive on the plant (F.S. Chew, personal communication). Differences in preferences of the two *Pieris* species may reflect their different habitats, but *B. vulgaris* is generally available to both butterflies. Thus utilization of *B. vulgaris* favors population growth for *P. rapae*, but may be responsible in part for limiting growth of *P. napi oleracea* populations in areas where both species occur.

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CHEMICAL DISCRIMINATION BY TONGUE-FLICKING IN LIZARDS: A REVIEW WITH HYPOTHESES ON ITS ORIGIN AND ITS ECOLOGICAL AND PHYLOGENETIC RELATIONSHIPS

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Abstract—Tongue-flicking is a synapomorphy of squamate reptiles functioning to sample chemicals for vomerolfactory analysis, which became possible in primitive squamates when ducts opened from the vomeronasal organs to the roof of the mouth. Extant iguanian lizards in families that do not use the tongue to sample chemical prey cues prior to attack partially protrude it in two feeding contexts: during capture by lingual prehension and after oral contact with prey. These lizards do not exhibit strike-induced chemosensory searching. Lingual prey prehension is present in iguanian lizards and in *Sphenodon*, the sister taxon of Squamata. During attempts to capture prey, the tongues of primitive squamates inevitably made incidental contact with environmental substrates bearing chemicals deposited by prey, conspecifics, and predators. Such contact presumably induced selection for tongue-flicking and ability to identify biologically important chemicals. Most iguanian lizards are ambush foragers that use immobility as a major antipredatory defense. Because tongue-flicking at an ambush post would not allow chemical search beyond the vicinity of the head and would render them easier for predators and prey to detect, typical iguanians tongue-flick neither while foraging nor to identify predators. They do detect pheromones by tongue-flicking. Scleroglossan lizards are typically active foragers that rely on speed to escape. Being freer to move the tongue, they have evolved lingual sampling allowing detection of chemical cues of conspecifics, predators, and prey, as well as strike-induced chemosensory searching, some can follow pheromone trails by tongue-flicking. Some families have lingual morphology and behavior specialized for chemosensory sampling. In varanids and snakes, the taxa showing the greatest lingual specialization, additional prey-related chemosensory behaviors have evolved. In iguanian and scleroglossan families that have secondarily adopted the foraging mode typical of the other taxon, prey chemical discrimination

involving tongue-flicking and strike-induced chemosensory searching are typical for the foraging mode rather than the taxon. Because foraging mode and state of prey chemical discrimination are stable within squamate families and to a large extent in higher taxa, both features have been retained from the ancestral condition in most families. However, in three cases in which foraging mode has changed from its ancestral state, the state of prey chemical discrimination has also changed, indicating that prey chemical discrimination is adaptively adjusted to foraging mode. Indeed, acquisition of lingually mediated prey chemical discrimination may have made feasible the evolution of active foraging, which in turn appears to have profoundly influenced the further evolution of squamate chemosensory structures and behavior, placing a selective premium on features enhancing the tongue's efficiency as a chemical sampling device. The advent of tongue-flicking to sample prey chemicals and thus detect hidden prey may have allowed generalist (cruise) or ambush foragers, if early squamates were such, to become specialists in active foraging. Alternatively, if the common ancestors of squamates were active foragers, the adoption of ambush foraging would have selected against participation of the tongue in locating prey. Acting jointly, tongue-flicking and active foraging have had momentous consequences for squamate diversification. Specialization for active foraging would appear to have had ramifying effects on antipredatory defenses, body form, territoriality, mating systems, and reproductive physiology.

Key Words—Tongue-flicking, vomerolfaction, Squamata, Scleroglossa, Iguania, lizards, snakes, iguanas, geckos, predatory-prey interaction, olfaction, active foraging, ambush foraging, poststrike elevation in tongue-flicking, strike-induced comosensory searching.

INTRODUCTION

Tongue-flicking is the quintessentially squamate behavior. It is both a defining characteristic as a unique synapomorphy among squamate reptiles and an enabling component of basic functions such as chemical identification of prey and pheromonal mediation of social responses (reviewed by Halpern, 1992). The tongue acts as a chemical sampling device that retrieves chemicals from the external environment. When the tongue is protruded from the mouth, volatile molecules in the air and nonvolatiles on substrates adhere to its moist surface. Molecules thus collected are carried into the oral cavity when the tongue is retracted. By a disputed mechanism (e.g., Gillingham and Clark, 1981; Young, 1990), the molecules pass into vomeronasal ducts that open into the roof of the mouth and lead to the vomeronasal organs. They eventually reach the vomeronasal epithelium, the site of the vomerolfactory receptor cells (Halpern and Kubie, 1980; Graves and Halpern, 1989).

The chemical senses are critically important to squamate reptiles in several ecological contexts, most notably in foraging, avoidance of predators, and social relationships. Although olfaction is highly developed in many taxa (Gabe and

Saint Girons, 1976), its roles have received scant study. Almost nothing is known regarding sensory and behavioral aspects of taste in squamates despite the presence of lingual taste buds in many lacertilian families (Schwenk, 1985, 1988). On the other hand, a substantial body of knowledge has been accumulated regarding the behavioral and ecological importance of vomerolfaction, the sense supported by the vomeronasal system (Cooper and Burghardt, 1990a). Some of this information has been obtained through direct experimentation, either by eliminating vomerolfaction or by excluding the participation of other senses (reviewed by Halpern, 1992). However, much of the information on squamate chemosensory behavior depends heavily on observations of tongue-flicking behavior by animals having all chemosensory systems intact.

In experiments eliminating the vomeronasal sense by section of the vomeronasal nerves or sealing the vomeronasal ducts, chemical discrimination of prey, feeding, and responses to pheromones have been disrupted (Halpern and Frumin, 1979; Halpern and Kubie, 1983; Graves and Halpern, 1990; Cooper and Alberts, 1991). In these experiments functional olfaction and possibly gustation were inadequate to support the behaviors studied. Thus, whatever roles other chemical senses may play in such behaviors, it is clear that the participation of vomerolfaction is required for normal lingually mediated responses to prey chemicals and pheromones. Although such experimental information has been obtained for very few taxa of lizards and snakes, it very likely applies to all taxa that tongue-flick reliably and frequently in contexts requiring chemical discriminations. It is not certain that the chemosensory basis for a given discrimination is vomerolfaction, even if the behavioral criterion used is tongue-flicking behavior, but this is my operating assumption and can be readily disproven.

In this paper I will briefly review our knowledge of lingually mediated chemical discrimination of prey and predators and of pheromonal communication, with an emphasis on the taxonomic distribution of such behaviors in lizard families and in snakes. Examination of this information in relation to squamate ecology and phylogeny leads to several new hypotheses that may shed light on the origin of chemosensory tongue-flicking in squamates, the history of lingual participation in squamate vomerolfaction, and the diversification of squamates.

Due to the large amount of information available and what I believe to be its centrality to squamate evolution, heavy emphasis is placed on the importance of foraging behavior and its relationships to lingual chemical sampling. Roles of foraging mode and phylogeny in determining use of tongue-flicking to detect prey chemicals and the evolution of chemosensory behaviors for food acquisition in squamates are discussed. Scanty data on chemosensory responses to predators and their adaptive roles and on pheromonal communication preclude comparable analysis. Because these topics figure less importantly in the evolutionary and

ecological considerations, methods of study are omitted and findings are discussed in less detail.

RESPONSES TO PREY CHEMICALS: BEHAVIORS, METHODS OF STUDY, AND TAXONOMIC DISTRIBUTION

Detection and Discrimination

The Behaviors. Chemosensory discriminations and behaviors are fundamental features in a wide range of squamate taxa, but there is great diversity in the presence and degree of development of the behaviors at the familial and higher taxonomic levels. Whereas some lizard species do not even tongue-flick to detect or identify prey (e.g., Cooper, 1989a; 1993b), some snakes employ tongue-flicking to scent-trail envenomated prey (e.g., Chiszar and Scudder, 1980). Between these extremes lie many taxa showing various degrees of reliance on tongue-flicking and presumably vomerolfaction in their predatory behavior.

The most basic chemosensory ability likely to be of use in foraging is detection of prey chemicals in the external environment. More useful is the ability to identify prey by lingually sampling chemical cues. Because tongue-flicking may be activated by responses to airborne molecules, it has been hypothesized that olfactory detection of volatiles activates tongue-flicking (Cowles and Phelan, 1958; Duvall, 1981). According to the original hypothesis and its subsequent elaboration, the olfactory detection of unidentified volatile molecules of low information content triggers tongue-flicking for sampling of larger nonvolatile molecules having higher information content. These larger molecules are thought to provide the information needed for precise identification of prey by vomerolfaction (e.g., Cowles and Phelan, 1958; Stoddart, 1980; Burghurdt et al., 1988).

However, because a recent study on turtles reveals comparable responses by the olfactory and vomerolfactory systems to a series of volatile substances (Shoji and Kurihara, 1991), vomerolfaction may contribute to detection by squamates of volatile molecules. Olfactory receptors would routinely be exposed to volatiles in the air by breathing, but volatiles would not readily reach the vomeronasal system of a squamate at rest with its mouth closed. Nevertheless, tongue-flicking might allow sampling of airborne molecules for vomeronasal analysis. This would account for the widespread occurrence of tongue-flicks not directed to any substrates, contact with a substrate being necessary only for sampling nonvolatile chemicals. Experimentation is needed to determine the relative roles of olfaction and vomerolfaction in responses to volatile substances.

Prey chemical discrimination is a more sophisticated response that implies an ability not only to detect chemical prey cues, but to identify prey by che-

mosensory means, responding appropriately to prey chemicals and differentially to prey chemicals and other substances. Some species may detect prey chemicals, yet not identify them.

Methods of Study. Although several techniques can be used to study prey chemical detection and discrimination, a large majority of studies have employed variations of an experimental technique first used extensively by Gordon Burghardt for work with natricine snakes (e.g., Burghardt, 1967, 1970a) and recently used for comparative studies of squamates with an emphasis on lizards (reviewed by Cooper, 1990a).

In this technique, chemical stimuli are presented to squamates on cotton-tipped applicators. Differences in responses elicited by various stimuli allow inference of prey chemical detection and discrimination. The two primary behaviors recorded are number of tongue-flicks emitted and biting attacks on the cotton swabs. If no biting occurs, a trial lasts a predetermined interval, usually 60 sec. If the reptile bites the swab, the trial is terminated at the time of the bite. Data are typically analyzed for three variables: number of tongue-flicks, frequency of biting, and tongue-flick attack score (TFAS).

The interpretation of TFAS requires some explanation. Tongue-flicking indicates chemosensory investigation, and biting represents predatory attack. Because trials are terminated by attack, the number of tongue-flicks possible is reduced, especially for individuals that bite early in the trial interval. TFAS is a composite measure combining the number of tongue-flicks and attack to give a single index of response strength to chemical stimuli. For each individual, TFAS is the number of tongue-flicks emitted in 60 sec if the individual does not attack the swab. If the reptile bites the swab, TFAS is the maximum number of tongue-flicks emitted in response to any stimulus plus 60 minus the latency to bite in seconds. Because a trial begins with the first tongue-flick, TFAS is always greater than the maximum number of tongue-flicks emitted in the absence of biting. This seems appropriate because predatory attack indicates identification of prey chemicals more strongly than any amount of tongue-flicking. For experiments using independent groups designs, TFAS is calculated using the maximum number of tongue-flicks in any trial by any individual for the entire experiment. For experiments using randomized blocks designs, TFAS(R) is calculated using the maximum number of tongue-flicks emitted by the particular individual in any stimulus condition. For additional explanation, see Cooper and Burghardt (1990b).

In experiments using this method, a significant stimulus effect combined with a significant difference between response to prey chemicals and the odorless control implies detection of prey chemical stimuli tested (Cooper and Burghardt, 1990b). Usually the stimuli tested include surface chemicals from one or more types of prey, a pungency control such as cologne for reactions to odoriferous nonprey stimuli, and an odorless control, typically deionized or distilled water,

to measure responses to the general experimental setting. Prey chemical discrimination is inferred when responses to prey chemical stimuli are significantly greater than those to the pungency and odorless controls or to chemical stimuli from other potential foods.

It is more difficult to establish the absence of prey chemical detection and discrimination. One cannot prove a null hypothesis, but by using results of similar experiments to estimate the statistical power (Cooper, 1989a), it is possible to estimate the likelihood of detecting a true difference if it occurs. Given sufficiently large sample sizes and high power, it can be inferred that the absence of significant differences among stimuli is highly improbable if the animals respond in a similar manner to those in previous experiments in which significant differences were found.

Survey of Results. Species that neither detect nor discriminate prey chemicals tongue-flick little or not at all in the experiments (Cooper, 1989a, 1994a,b). They tongue-flick neither while searching for food nor prior to attacking prey (e.g., Gravelle and Simon, 1980; Simon et al., 1981; Cooper et al., 1993a). Although it is possible that some of these species may have the ability to detect and even identify prey chemicals, they do not appear to use the tongue-vomeronasal system either to locate prey or evaluate it prior to attack.

Among species that discriminate prey chemicals from control stimuli, there is considerable variation in response to prey chemicals. Some species direct nearly all tongue-flicks to the swab (e.g., *Eumeces laticeps*; Cooper and Vitt, 1989); others tongue-flick the swab and then tongue-flick elsewhere, often moving about as if searching for prey (e.g., *Podarcis hispanica*, Cooper, 1990b; *P. muralis*, Cooper, 1991a; *Ophisaurus ventralis*, Cooper, unpublished observations). Typically, the initial tongue-flicks consist of extensions of the tongue anteriorly well beyond the snout because the applicator is presented at a distance, usually 1–2 cm. Some individuals then approach the swab so that subsequent tongue-flicks are of short excursion. There is a variable degree (unquantified) of labial licking among individuals and species.

One of the major differences among taxa is the proportion of individuals that attack the swab after tongue-flicking. In lizards this ranges from 0.17 in *Tupinambis rufescens* (Cooper, 1990b) to 0.67 in *E. laticeps* (Cooper and Vitt, 1989) and *Heloderma suspectum* (Cooper, 1989b) to 0.89 in *Gerrhosaurus nigrolineatus* (Cooper, 1992a). The percentage of individuals biting does not necessarily indicate the degree to which prey chemical discrimination is developed in a given taxon because nonchemical aspects of the experiment may affect attack. Probable cases in point are the red tegu, *T. rufescens* (Cooper, 1990b), and the savannah monitor, *Varanus exanthematicus* (Cooper, 1989b), which bit only 0.33 of swabs bearing prey chemicals. The most likely explanation in these two species that are clearly quite responsive to prey chemicals is that the standard size of swabs used was too small, relative to the lizards, to simulate a profitable

prey object. For other species in which high proportions of individuals did not bite, chemical stimuli may serve to identify prey, but other cues may be necessary to induce attack.

The status of our knowledge about prey chemical detection and discrimination is shown in Table 1. There is a strong linkage between prey chemical detection and discrimination such that either both abilities are present or both are lacking. Among the lizard families and snakes studied, prey chemical discrimination is known to occur in nine of the 10 families that detect prey chemicals and may well occur in the remaining family, Amphisbaenidae. Both abilities are absent in the other five families studied. Detection and discrimination are significantly associated at the familial level for lizards plus snakes (Fisher exact test, $P = 0.004$ or 0.0006, two-tailed, depending on the state of discrimination in Amphisbaenidae; Siegel, 1956). Both behaviors are absent in all but one of the iguanian taxa studied, whereas both are present in almost all of the scleroglossan taxa. Influences of feeding ecology and phylogeny on prey chemical detection and discrimination will be discussed below.

The data on prey chemical detection and discrimination in Lacertidae and Amphisbaenidae require further discussion. It was recently reported that juvenile *Lacerta viridis* did not tongue-flick in response to distilled water or cricket chemicals on cotton placed in their cages, but did tongue-flick and attack dead crickets during a 10-min observation period (Goosse and Bels, 1990). Tongue-flicking also occurred during exploration and in response to tap water.

In contrast, I have reported prey chemical discrimination by *Podarcis hispanica* and *P. muralis* (Cooper, 1990b, 1991a). These species were both more difficult to study than are many others because some individuals initially tongue-flicked applicators bearing prey chemicals and then began tongue-flicking away from the applicators as if searching for prey. Other individuals bit the applicators. Some individuals of both species did not complete the experiment either because they fled or would not remain still near the applicator. Several factors may account for the difference in results between my studies and that of Goosse and Bels (1990). First, large adults, as used in my studies, have proven to be much more responsive, perhaps because they are less defensive. Second, as no description of the method of preparation for cricket chemical stimuli was presented by Goosse and Bels (1990), these stimuli might have been ineffective, which appears likely given that tap water, but not distilled water, elicited tongue-flicking. Third, the method of presentation may have affected tongue-flicking. I placed applicators anterior to each lizard and held them above the ground during the 1-minute test interval, whereas Goosse and Bels (1990) placed the cotton on the substrate and withdrew to videotape responses for 10 min.

Goosse and Bels (1990) concluded that visual stimuli elicit attack and that prey chemicals do not. In my experiments, cotton swabs that are moved to a position anterior to a lizard's snout and held slightly above the substrate may

TABLE 1. ROLES OF TONGUE-FLICKING IN PREDATORY BEHAVIOR AMONG LIZARD FAMILIES AND SNAKES[a]

Taxon	Dtct	Disc	PETF	SICS	Sources[b]
Iguania					
Chamaeleonidae	–	–	–	–	1, 2
Corytophanidae	?	?	?	?	
Crotaphytidae	?	?	?	?	
Hoplocercidae	?	?	?	?	
Iguanidae	+	+	+	?	3, 4
Opluridae	?	?	?	?	
Phrynosomatidae	–	–	–	–	1, 2
Polychridae	–	–	?	?	1
Tropiduridae	–	–	–	–	5
Scleroglossa					
Eublepharidae	+	+	+	+	5, 6, 7, 8
Gekkonidae	–	–	?	?	8
Pygopodidae	?	?	?	?	
Xantusiidae	?	?	?	?	
Lacertidae	+	+	+	+	9, 10
Teiidae	+	+	+	+	9, 11
Gymnophthalmidae	?	?	?	?	
Scincidae	+	+	+	+	12, 13, 14, 15
Cordylidae	?	?	?	?	
Gerrhosauridae	+	+	+	+	16
Anguidae	+	+	+	+	11, 17
Xenosauridae	?	?	?	?	
Helodermatidae	+	+	+	+	11, 18
Varanidae	+	+	+	+	18, 19, 20
Dibamidae	?	?	?	?	
Amphisbaenidae	+	?	?	?	21
Serpentes	+	+	+	+	e.g., 22, 23, 24

[a] Citations on prey chemical discrimination and detection are for experiments in which chemical stimuli were presented on cotton-tipped applicators. Dtct—prey chemical detection; Disc—prey chemical discrimination; PETF—poststrike elevation in tongue-flicking; SICS—strike-induced chemosensory searching.

[b] 1-Cooper, 1989a; 2-Cooper, 1994a; 3-Cooper and Alberts, 1990; 4-Cooper and Alberts, 1991; 5-Cooper and DePerno, unpublished data; 6-Dial, 1978; 7-Dial et al., 1989; 8-Cooper, 1994b; 9-Cooper, 1990b; 10-Cooper, 1991a; 11-Cooper, unpublished data; 12-Loop and Scoville, 1972; 13-Burghardt, 1973; 14-Cooper and Vitt, 1989; 15-Cooper, 1992a; 16-Cooper, 1992b; 17-Cooper, 1990d; 18-Cooper, 1989b; 19-Cooper, 1989c; 20-Cooper, 1993; 21-Lopez and Salvador, 1992; 22-Burghardt, 1970a; 23-Chiszar and Scudder, 1980; 24-Cooper et al., 1989.

have some visual properties of potential, unidentified prey items. If an initial tongue-flick leads to sampling of possible or identified prey chemicals, more tongue-flicking or attack may occur. Thus, the finding of Goosse and Bels (1990) that visual cues stimulate attack on a clearly recognizable natural prey item is consistent with my findings that chemical cues affect responses in the absence of visual prey cues. A further indication that prey chemical discrimination occurs in lacertids is that blinded, olfaction-deprived *Acanthodactylus scutellatus* can locate prey (Kahmann, 1939).

The only amphisbaenian studied, *Blanus cinereus*, was reported to detect prey chemicals, but not discriminate them from control stimuli (Lopez and Salvador, 1992). Adult *B. cinereus* tongue-flicked at higher rates to prey chemicals from ants and beetle larvae than to deionized water, but response rates for prey chemicals and cologne did not differ significantly (Lopez and Salvador, 1992). Because cologne elicited high tongue-flicking rates, it may not be a suitable control for prey chemical discrimination in *B. cinereus*. Further study, perhaps using preferred and nonpreferred prey types, will be required to ascertain the presence or absence of prey chemical discrimination.

In summary, the abilities to detect prey chemicals and to discriminate them from other chemicals by tongue-flicking occur in many squamate taxa, especially in scleroglossans. In a given taxon, with the possible exception of Amphisbaenidae, both abilities occur or neither occurs. Despite this similarity, the importance of functions such as initial location of prey or hunting sites and confirmation of sources of unidentifiable visual stimuli may vary among taxa.

SICS and PETF

The Behaviors. Strike-induced chemosensory searching (SICS) is an increase in tongue-flicking rate and accompanying searching movements after prey has been attacked and has either been released or has escaped. Initially, SICS was defined as an increased tongue-flicking rate and searching movements following envenomation and voluntary release of prey by venomous snakes, especially rattlesnakes (Chiszar and Scudder, 1980). Rattlesnakes, other crotalines, and a variety of highly venomous snakes including some elapids have subsequently been found to exhibit SICS (Chiszar and Scudder, 1980; Chiszar et al., 1985; O'Connell et al., 1985). At least in crotaline snakes, voluntary release of prey is followed by a brief resting period. Within a minute or two, the tongue-flicking rate increases dramatically and search for chemical cues from the prey begins. Once these cues are encountered, the subdued prey is located by scent-trailing. This sequence of behaviors is called the strike–release–trail strategy (Chiszar et al., 1983), which is believed to be adaptive in avoiding injury from potentially dangerous prey such as rodents. The pause before initiating a search after envenomation plus the time spent in scent-trailing are sufficient to allow the prey to be incapacitated, greatly reducing risk.

It has recently been found that SICS or something very similar occurs in diverse squamate taxa including nonvenomous snakes and several families of lizards (Table 1). One major difference in the behavior of these squamates from that of rattlesnakes and other highly venomous species is that the nonvenomous snakes and lizards do not voluntarily release prey and thus do not employ the strike–release–trail strategy. However, because attacked or bitten prey sometimes escape, use of chemical cues obtained during the attack can presumably aid in relocation of prey items that are not immediately visible (Cooper, 1989c; Cooper et al., 1989).

In the first two studies of squamates other than highly venomous snakes, experimental removal of bitten prey from the mouth induced a marked increase in tongue-flicking rate and obvious searching movements in the nonvenomous colubrids *Thamnophis sirtalis* and *Elaphe guttata* (Cooper et al., 1989) and in the highly chemosensory varanid and helodermatid lizards (Cooper, 1989c). Although tongue-flicking might help lizards and nonvenomous snakes find escaped prey, it seems likely that only those species that normally follow scent trails to find prey would attempt to scent-trail escaped prey. Scent-trailing of escaped prey has not been studied in any nonvenomous squamates, but might be examined profitably in lizards such as varanids and actively foraging snakes that consume prey susceptible to location by scent-trailing.

Because tongue-flicking and searching movements might vary independently among nonvenomous snakes and lizard families, the two aspects of SICS are best considered separately. This distinction could be especially important for comparative purposes. The increase in tongue-flicking rate after release of prey or its involuntary removal by an experimenter is termed the poststrike elevation in tongue-flicking (PETF) rate. PETF can vary among taxa in magnitude and duration. Unfortunately, I have not quantified the extent of searching movements in most studies of SICS in nonvenomous squamates. Measurement of searching movements is highly desirable for future studies.

Study of SICS and related behaviors in lizards and nonvenomous snakes is a new field of research that has exciting prospects for adding to our understanding of the chemosensory behavior in squamates, especially its importance in foraging and its evolution. Despite the confirmation of both PETF and searching movements in several taxa, numerous aspects of the relationships of these behaviors to SICS and the strike–release–trail strategy remain to be determined. In the absence of relevant field observations and experiments to determine the effectiveness of PETF and searching for relocation of prey, the importance of these behaviors is unknown.

Methods of Study. In venomous snakes that voluntarily release prey after striking, SICS and trailing behaviors may be studied by comparing tongue-flicking, movement, and scent-trailing after envenomation and release with those in baseline and experimental conditions. A representative sample of the basic

methods used by Chiszar and his colleagues in conducting a large series of experiments of this sort can be found in Chiszar and Scudder (1980), Chiszar et al. (1985), and Melcer and Chiszar (1989).

The study of SICS in lizards and nonvenomous snakes is more difficult because for most species it is necessary to remove the prey from the reptile's mouth. This interference inevitably causes some degree of mechanical disturbance. In some species it is possible to pull the prey out of the reptile's mouth without handling the reptile, but in other species that hold the prey tenaciously, the predator must be grasped and forced to relinquish the prey. Although the prey can be retrieved without damaging the squamate, experimental controls must be used to distinguish PETF and SICS from possible effects of removing the prey on subsequent behavior. Additional controls are needed to determine whether removal of prey affects chemosensory behavior.

The basic experimental design (Cooper, 1989c; Cooper et al., 1989, and references on SICS below) includes four experimental conditions. Although independent groups designs are feasible, experiments to date have had randomized blocks designs in which each animal is tested in all conditions. This allows reduction in the sample size by a factor of four because each animal is tested four times and by some further factor because each animal provides its own matched samples in all conditions, reducing the error variance. To prevent possible sequential biases, the order of stimulus conditions is randomized or incompletely counterbalanced.

The primary experimental condition is the strike condition, in which the squamate is allowed to bite its prey. To begin a trial, the experimenter slowly approaches the reptile's home cage, removes the top, and positions the prey item (held by forceps or string) at a fixed distance anterior to the reptile's snout (10 or 15 cm in studies thus far). When the reptile bites the prey, the experimenter removes the prey, using as little force as possible, and then withdraws to record numbers of tongue-flicks per minute and movements. The duration of recording varies from 2 to 60 min as determined by pilot observations for each species.

Procedures for the control conditions are similar to those of the strike condition except that aspects other than biting that might affect chemosensory behavior are examined. In the mechanical disturbance control, the reptile is allowed to approach and examine the prey. However, when the reptile prepares to strike, the prey is rapidly withdrawn and the reptile is pulled or handled in a manner simulating the degree of disturbance needed to remove bitten prey in that species. In the sight condition controlling for effects of seeing prey, the prey is held in open view at the far end of the reptile's cage. The prey is removed as soon as the reptile approaches or after a maximum of 10 sec. The fourth condition is a control for responses to the experimental setting. In this condition

the experimenter introduces hand-held forceps or string, used to hold prey in the other conditions, as in the sight condition.

Although the mechanical disturbance control condition simulates the effects of removal of prey, it does not eliminate the possibility that mechanical stimulation of oral tissues during biting is responsible for any observed changes in chemosensory behavior. To address this issue, I have conducted additional simple experiments with one species each of lizard and nonvenomous snake. In these studies the reptiles were grasped, their oral epithelia were stimulated by cotton swabs bearing either prey chemicals or deionized water, and their tongue-flicking rates observed after release. Both species tongue-flicked at much higher rates following stimulation by prey chemicals, indicating that prey chemical stimuli are the specific cause of increased tongue-flicking rates.

In my first studies of SICS and PETF, I recorded only the presence or absence of movement and did not formally compare even these data among conditions. To allow more precise statistical comparison, I now record number of movements in each minute in all experimental conditions. Records of time spent in motion and distance moved might also be helpful. Statistical demonstration that movement is greater in the strike conditions than in the other conditions is the best criterion in the questionable cases for determining whether a species exhibits SICS or merely PETF.

PETF is demonstrated only if the tongue-flicking rate is significantly higher in the strike condition than in each of the other conditions; the presence of SICS is inferred if (presumptive searching) movements occur during PETF. A more rigorous criterion for SICS would demand significantly greater movement in the strike condition than in any of the other conditions. The duration of PETF is the longest interval over which tongue-flicking is significantly greater in the strike condition than in the other conditions. Because response rates in the mechanical disturbance condition are typically greater than in the other control conditions, duration can usually be assessed by individual comparisons of responses restricted to the strike and mechanical disturbance conditions.

Survey of Results. PETF and SICS occur in a wide variety of squamate taxa, including all families of scleroglossan lizards and snakes studied (Table 1). In snakes, Varanidae, and Helodermatidae (Cooper, 1989c, unpublished data; Chiszar and Scudder, 1980) movement increased greatly during PETF. Much movement was associated with PETF in teiids and, to a lesser degree, in lacertids (Cooper, 1991a, unpublished data). Movements were noticeably less extensive in the remaining families that exhibited PETF.

Among the latter families, no statistical comparisons of movements among conditions were conducted for representatives of Gerrhosauridae (Cooper, 1992a) and Scincidae (Cooper, 1992b). However, the frequency of movement in the strike condition was significantly greater than in the other conditions in the anguid *Elgaria coerulea* (Cooper, unpublished data), which did not appear to

move more extensively than the scincids and gerrhosaurids. In contrast, no significant increase in movement was detected during PETF in a eublepharid gecko, *Eublepharus macularius* (DePerno and Cooper, unpublished data), for which movements were recorded only as present or absent in each minute. Data on number of movements, distance moved, or percent of time spent moving might reveal a significant difference.

PETF and SICS appear to be much less widely distributed in Iguania (Table 1). Although representatives of only four iguanian families have been studied, both PETF and SICS are absent in three of the four (Table 1). PETF is highly developed in the herbivorous iguanid *Dipsosaurus dorsalis* (Cooper and Alberts, 1993), but no data on movements were collected (Cooper and Alberts, unpublished data), suggesting that any such movements were not extensive. This would not be surprising because extensive search would not be required to relocate plant food dropped before ingestion.

For iguanids such as *D. dorsalis*, PETF would presumably facilitate relocation of food in the immediate vicinity. For many small scleroglossan lizards that consume insects and other arthropods, scent-trailing may be important only over very short distances or not at all. PETF presumably helps these lizards find prey nearby, and increased movements may serve more to bring these lizards close enough to detect chemical cues from prey that have moved out of sight a short distance away from the site of attack. For snakes and lizards that consume large, mobile prey that can be profitably scent-trailed, SICS would additionally increase the likelihood of finding prey that had moved further away from the site of attack.

PETF and SICS occur only in taxa that are also capable of prey chemical detection and discrimination. Using the data from Table 1, prey chemical discrimination and PETF both occur in three families, neither occurs in three more families, and in no case does one occur without the other. Thus, prey chemical discrimination and PETF are significantly associated ($P < 0.0007$, Fisher exact test). The association between prey chemical discrimination and SICS is also highly significant, but at a slightly higher alpha ($P = 0.009$) because data on SICS are unavailable for one family that exhibits prey chemical discrimination (Iguanidae). These associations suggest that once the ability to recognize prey chemicals evolves, squamates use it to help locate prey both before the prey has been discovered and after unsuccessful predation attempts.

The duration of PETF increases with specialization of lingual chemosensory behavior and with lingual specialization for chemosensory sampling. It is zero in species lacking prey chemical discrimination. Among forms capable of prey chemical discrimination, it is very brief in three families lacking advanced lingual specializations (McDowell, 1972), lasting only 1 min in the single species tested in Scincidae and Lacertidae (Cooper, 1991a, 1992b) and 2 min in a single gerrhosaurid. In the varanid *Varanus exanthematicus*, which has a highly

specialized, snakelike tongue, it lasted about 30 min (Cooper, 1993). In rattlesnakes and other pit vipers, SICS is much longer-lasting. It may continue for upwards of 2 hr (Chiszar and Scudder, 1980; Chiszar et al., 1983, 1985). Because the envenomated prey becomes immobilized rapidly and is very likely to remain in the vicinity, this extended chemosensory search is probably the product of selection for thorough, continued search until the prey is located.

CHEMOSENSORY RESPONSE TO PREDATORS AND CONSPECIFICS

Detection of Predator Chemicals

Chemosensory detection and identification of predators mediated by lingual sampling or other means may be an important facet of predation avoidance in lizards, but has been largely neglected. The meager knowledge on this topic presented in Table 2 reveals the extent of our ignorance. In only three families have responses been observed suggesting identification of predator chemicals by tongue-flicking. The studies of scincid and lacertid species showed differential tongue-flicking rates in response to chemical stimuli from saurophagous and nonsaurophagous snakes (Thoen et al., 1986; Cooper, 1990c), suggesting

TABLE 2. RESPONSE TO PREDATOR CHEMICALS IN LIZARDS AND SNAKES

Taxon	Behavior	Sources
Eublepharidae	Tail display in response to skin chemicals of a saurophagous snake but not to control stimuli by *Eublepharis macularius* (likely an olfactory response)	Dial et al., 1989; Dial, 1990
Scincidae	Elevated tongue-flicking rate in response to skin chemicals of two species of saurophagous snakes but not to control stimuli by (*Eumeces laticeps*)	Cooper, 1990c
Lacertidae	Higher tongue-flicking rate on substrates labelled by two species of saurophagous snakes than to control substances by *Lacerta vivipara*	Thoen et al., 1986
Varanidae	*Varanus albigularis* eat prey partially covered by skin from nonvenomous, but reject it if the skin is from venomous snakes	Phillips and Alberts, 1992
Serpentes	Pit vipers body-bridge if exposed to chemical cues from saurophagous snakes	Marchisin, 1980;
	Higher tongue-flicking rates by colubrids to skin chemicals of ophiophagous than nonpredatory snakes	Weldon, 1982

a likely role of vomerolfaction. In the varanid *Varanus albigularis*, tongue-flicking inhibited predatory attack on a harmless prey species only if the prey was labeled by chemical stimuli from the skin of venomous snake species that eat varanids. Prey labeled by skin chemicals from snakes that do not eat varanids were attacked even after tongue-flicking (Phillips and Alberts, 1992). In the single study of a eublepharid gecko, defensive elevation of the tail in response to skin chemicals of predatory snakes demonstrated chemical discrimination of prey, but the low rates of tongue-flicking and absence of differential tongue-flicking suggest that the discrimination was probably olfactory rather than vomerolfactory.

Chemosensory response to predators has been studied somewhat more extensively in snakes (reviewed by Halpern, 1992). A predator-specific defensive behavior called body bridging was elicited in crotalines by chemical cues from ophiophagous colubrids (e.g., Marchisin, 1980). In colubrids representing two subfamilies, higher tongue-flicking rates were emitted in response to skin chemicals from ophiophagous snakes than from nonophiophagous snakes (Weldon, 1982).

Despite the paucity of studies, it is clear that a differential response to chemical stimuli from predators occurs in a wide taxonomic range of scleroglossan lizards and snakes. As this behavior is lingually mediated in some snakes and in three of the four lizard families studied, vomerolfaction is a likely candidate as the sense supporting these discriminations. For iguanian lizards, no systematic studies of chemosensory detection of predators have been reported.

Pheromonal Communication

Because pheromonal communication in reptiles is the subject of a thorough recent review (Mason, 1992), it will not be discussed in depth. The major behavioral phenomena and their taxonomic distributions are presented in Table 3. Pheromonal communication is such an active field of research that Table 3 contains several very recent findings on iguanids, eublepharids, gekkonids, scincids, gerrhosaurids, lacertids, and amphisbaenids that were not available when Mason's review was written.

Detection of pheromones by tongue-flicking is widely distributed in squamates. In all squamate taxa listed in Table 3, tongue-flicking behavior is strongly affected by conspecific chemicals, suggesting that vomerolfaction is an important participant in pheromonal communication. As this applies to all listed cases involving responses to potential mates or reproductive competitors, vomerolfaction is very likely the primary sense used to identify reproductive and territory-marking pheromones. Pheromones affecting aggregation and perhaps kin recognition in snakes and lizards (Werner et al., 1987) may be detected by vomerolfaction and/or olfaction (Mason, 1992).

TABLE 3. PHEROMONAL COMMUNICATION IN LIZARD FAMILIES AND SNAKES

Taxon	Behavior	Sources
Iguania		
Iguanidae	Adults increase tongue-flick rates in response to conspecific femoral pore secretions (*Dipsosaurus dorsalis*)	Alberts, 1989
	Both sexes discriminate conspecific pheromones from control stimuli; responses by and to the sexes similar (*D. dorsalis*)	Dussault and Krekorian, 1991
	Self-licking and lower substrate tongue-flick rates by males responding to their own femoral pore secretions than those of other males (*D. dorsalis*)	Alberts, 1992
	Possible kin recognition by juvenile *Iguana iguana*	Werner et al., 1987
Phrynosomatidae	Males increase tongue-flicks in response to conspecific pheromones on substrates (*Sceloporous occidentalis*)	Duvall, 1979, 1981
	Males are tongue-flicked by conspecifics at higher rates on the head, sides, and back than are females (*S. occidentalis*)	Duvall, 1982
	Juveniles increase tongue-flick rate on substrates marked by juveniles (*S. jarrovi*)	Simon et al., 1981
Scleroglossa		
Eublepharidae	Males court other males having tails bearing female pheromones (*Coleonyx variegatus*)	Greenberg, 1943
	Males court females except before females shed, at which time males are aggressive toward females (*Eublepharis macularius*)	Mason and Gutzke, 1990
	On substrates labeled by conspecifics, both sexes increase tongue-flick rates; males discriminate the sex of the labeling lizard (*E. macularius*)	Brillet, 1990
Gekkonidae	On substrates labeled by conspecifics, both sexes increase tongue-flick rates (*Paroedura pictus*)	Brillet, 1990
Scincidae	Both sexes detect skin and cloacal chemicals of both sexes (*Eumeces laticeps*)	Cooper and Vitt, 1984a,b
	Males, but not females, tongue-flick at higher rates to female pheromones (*E. laticeps*)	Cooper and Vitt, 1984a,b

TABLE 3. CONTINUED

Taxon	Behavior	Sources
Scleroglossa		
Scincidae	Males tongue flick at higher rates to breeding than nonbreeding females (*E. laticeps*)	Cooper and Vitt, 1984a
	Both sexes discriminate among conspecifics and heterospecific congeners of opposite sex; pheromones affect reproductive isolation and aggressive behavior (*E. laticeps*, *E. fasciatus*, *E. inexpectatus*)	Cooper and Vitt, 1986b,c; 1987a,b
	Males discriminate male conspecifics from heterospecific congeners (*E. laticeps*)	Cooper and Garstka, 1987a
	Male sexual behavior is stimulated by a neutral lipid female sex pheromone (*E. laticeps*)	Cooper et al., 1986; Cooper and Garstka, 1987b
	Males scent-trail breeding females (*E. laticeps*)	Cooper and Vitt, 1986a, Fitch, 1954; Goin 1957
	Adults *Tiliqua scincoides* tongue-flick at higher rates on substrates labeled by other conspecifics than by themselves or control chemical stimuli	Graves and Halpern, 1991
Gerrhosauridae	Males detect pheromones of both sexes and tongue-flick female pheromones at higher rates (*Gerrhosaurus nigrolineatus*)	Cooper and Trauth, 1992
Lacertidae	Higher tongue-flick rates on substrates labelled by conspecifics than by other lacertids (*Podarcis hispanica*)	Gomez et al., 1993
Amphisbaenidae	Males tongue-flick at higher rate in response to conspecific precloacal pore stimuli than control stimuli and in response to female than male stimuli (*Blanus cinereus*)	Cooper et al., 1994b
	Males tongue-flick at higher rates in response to precloacal pore and skin chemicals derived from other males than to their own (*B. cinereus*)	Lopez, M., Cooper, W., and Salvador, A., unpublished data
Serpentes	Discrimination of sex, reproductive condition, and species; scent-trailing (several species, best studied in natricines)	Reviews by, e.g., Burghardt, 1970a; Mason, 1992

Behavioral responses to pheromones have been studied in relatively few taxa, but some form of pheromonal communication has been discovered in all saurian families and snakes investigated, including representatives of two iguanian families and seven scleroglossan taxa at or above the familial level (Table 3). Its wide taxonomic distribution suggests that pheromonal communication may be plesiomorphic or have developed in early squamates. However, for meaningful evolutionary interpretations, much more information is needed to determine variation in its anatomical and physiological underpinnings, its roles, and the degree of development among taxa.

THE ORIGIN OF CHEMOSENSORY TONGUE-FLICKING

Among living reptiles only squamates exhibit tongue-flicking behavior, suggesting that tongue-flicking is a squamate symplesiomorphy. Tongue-flicking is absent in Chelonia and in living archosaurs. The best taxon for outgroup comparisons is the other lepidosaurian order, Rhynchocephalia, the sister group of Squamata. However, the reliability of such comparisons is uncertain because there is only one extant rhynchocephalian species, the tuatara (*Sphenodon punctatus*). Because the tuatara is predominantly nocturnal and is active at lower temperatures than are typical squamates (Walls, 1983), the degree to which its chemosensory and other behaviors are representative of the typical rhynchocephalian condition is open to question.

The weight of evidence on several features related to the vomeronasal system and chemosensory behavior collectively suggests that the tuatara is an appropriate outgroup and that chemosensory tongue-flicking is a derived squamate character. The features include the phylogenetic conservatism of chemosensory tongue-flicking behaviors in squamates and the phylogenetic distribution of chemosensory morphology and behavior. Data presented in a following section reveal strong conservatism of lingually mediated prey chemical discrimination at the familial level and above in squamate families. Furthermore, well-developed tongue-flicking behavior is present in all squamate families. The universality of tongue-flicking behavior in squamates and its absence in other reptiles, including *Sphenodon*, suggests its widespread absence in rhynchocephalians.

The morphological and related behavioral evidence is stronger. In the tuatara the vomeronasal organ opens through a laterally oriented duct into the medial wall of the choanae and the paraseptal cartilage supports the organ's floor. Both the vomeronasal organs and the paraseptal cartilages of squamates are rotated ninety degrees about the longitudinal axes with respect to their positions in *Sphenodon*, and the vomeronasal ducts extend ventrally to the roof of the oral cavity (Malan, 1946; Gauthier et al., 1988). Only in squamates do the

vomeronasal ducts allow direct access from the mouth to the chemosensory epithelia of the vomeronasal organs (Bertmar, 1981).

The absence of openings from the roof of the mouth leading to the vomeronasal organs implies that tongue-flicking, if it existed in rhynchocephalians, would have evolved for functions other than vomerolfactory sampling. Conversely, the absence of tongue-flicking behavior in the tuatara and its presence in squamates having such openings implies that tongue-flicking very likely evolved to increase the efficiency of access by chemicals from the external environment to the vomeronasal organs. If so, the natural selection favoring chemosensory tongue-flicking became possible only after openings of the vomeronasal ducts into the roof of the mouth appeared, as argued by Schwenk (1986).

Tongue-flicking and its movement pattern have several possible evolutionary antecedents. First, although tongue-flicking enhances the efficiency of access, chemicals from the external environment can reach the vomeronasal epithelia if the chemicals contact the snout in both snakes and lizards (Halpern and Kubie, 1980; Graves and Halpern, 1989). Therefore, selection for vomeronasal sensitivity to chemicals from prey, predators, and conspecifics reaching the mouth may have begun as soon as openings of vomeronasal ducts into the mouth appeared, prior to the advent of tongue-flicking. At least three movement patterns could have contributed to the evolution of tongue-flicking: lingual prey prehension, postswallowing postural adjustments of the tongue, and lingual grooming. Lingual prehension is commonly used by the tuatara to capture prey (Gorniak et al., 1982) and is a symplesiomorphy of iguanian lizards (Schwenk and Throckmorton, 1989). When openings of the vomeronasal ducts into the mouth appeared in early squamates, contact with environmental chemicals during lingual protrusion for attempted prey capture could have led to selection for lingual protrusion in other contexts and for modification of motor patterns for efficient lingual sampling.

Lingual movements during and after swallowing prey often include some lingual extension. In the iguanian families Phrynosomatidae and Agamidae, the tongue is often protruded slightly and sometimes extensively after swallowing, but in a pattern suggesting postural adjustments, cleaning movements, or tamping of food into the esophagus (Cooper, 1994a). Contact with surfaces bearing selectively important chemicals might occur during these protrusions, but is less likely to occur than when attempting lingual prey prehension. This is especially so when a large proportion of the tongue's length is extended outside the mouth because the head is then tilted upward, reducing the likelihood of contact with substrates (Cooper, 1994a). In a wide range of iguanian and scleroglossan families, the tongue is partially protruded from the mouth to lick the labials after eating. Such lingual protrusions related to grooming could have led to incidental contact with selectively important chemicals in the external environment.

Whatever types of lingual movements initially effected retrieval of chemicals from the environment, selection for efficient sampling presumably led subsequently to several changes. These include greater lingual extension, direction of the tongue to substrates likely to bear the chemicals, increased rates of lingual protrusion in contexts appropriate for chemical sampling, and eventually to modifications of the form of the tongue-flick [e.g., in number of oscillations per protrusion in snakes and in chemosensory specialists among lizards as described by Gove (1979)]. Presumably the increasing efficiency of lingual sampling was accompanied by increasing sensitivity of the vomeronasal system to the biologically important classes of compounds sampled. This scenario for the evolution of tongue-flicking behavior is depicted in Figure 1.

EFFECTS OF FORAGING MODE AND PHYLOGENY ON PREY CHEMICAL DISCRIMINATION

Detention and recognition of prey mediated by lingual chemosensory sampling occurs in many lizard families and is closely correlated with an active search for food. Families of ambush foragers appear to lack such prey chemical discrimination, whereas families of active foragers use their tongues and presumably vomerolfaction to detect and identify prey (Cooper, 1989a, 1990a,b). Because active foragers are scleroglossans and most ambush foragers are iguanians, it has been uncertain whether the presence or absence of prey chemical discrimination is adaptively modified with shifts in foraging mode or whether the apparent relationship between foraging mode and prey chemical discrimination is merely the result of phylogenetic inertia (Schwenk, 1993).

Foraging mode in the broad sense used here appears to be stable in a wide range of lacertilian taxa. I conducted a literature review of lizard foraging modes using the taxonomy of Estes et al. (1988), but incorporating the revisions of iguanian families by Frost and Etheridge (1989), of Gekkonoidea by Kluge

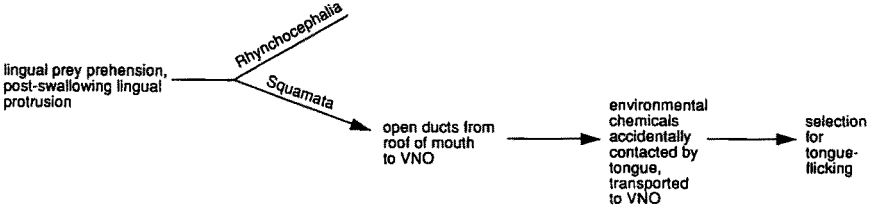

FIG. 1. Prerequisites for selection leading to the origin of tongue-flicking were communication between the mouth and vomeronasal organs, protrusion of at least a small portion of the tongue outside the mouth, and lingual contact with substrates bearing environmental chemicals.

(1987), and of Cordyliformes by Lang (1991). Lacertilian foraging modes are commonly placed in two categories, wide foraging versus ambush (= sit-and-wait) foraging (e.g., Huey and Pianka, 1981; Vitt and Price, 1982). Lizard ecologists have long recognized that variability occurs in each of the categories and have sometimes used the categories in an explicitly relative sense (e.g., Huey and Pianka, 1981). It is generally recognized that both quantitative and qualitative variation occur within each basic foraging mode. However, some investigators emphasize this variation by stating that the two foraging modes are merely opposite extremes of a continuum (e.g., Magnusson et al., 1985), whereas others recognize an intermediate category, cruise foraging, in which lizards search slowly, but while moving (Regal, 1978), and still others emphasize that the distribution of foraging patterns appears to be fundamentally bimodal (McLaughlin, 1989).

Despite the presence of such variation (reviewed by Cooper, in review), it is possible to make a broad distinction between lizards that forage primarily by waiting at ambush posts for prey to come within range (ambushers) and those that search actively, moving a relatively high percentage of the time while searching [active foragers, cruise foragers (Regal, 1978, 1983), and intensive foragers (Anderson, 1993)]. For the broad comparisons to be made here, I recognize two major foraging modes, active foraging and ambush foraging, among insectivorous and carnivorous lizards. Herbivorous lizards may not be active foragers in the same sense as are insectivores, but neither can they be ambushers. Therefore, I recognize herbivorous foraging as a distinct mode (see Cooper, in review for additional discussion). Foraging by omnivores is not discussed.

A partial review of the literature revealed characterizations of foraging mode for 256 species in 94 genera representing 19 families of lizards (Table 4). Assignment of foraging modes and their intrafamilial variations are discussed in Cooper (in review). Among iguanians, all families except Iguanidae consist of ambush foragers. For the species included for these families, only a single agamine chamaeleonid is exceptional in being herbivorous. The family Iguanidae is primarily herbivorous. Among scleroglossans, active foraging is the sole mode reported in five families and the predominant mode in two others. Only in Cordylidae and the closely related families Gekkonidae and Pygopodidae is ambush foraging the sole or major mode. Foraging mode is uncertain in two other scleroglossan families, Gerrhosauridae and Xantusiidae.

Variation in foraging mode appears to be quite limited in most families, but a small proportion of species appear to show mixed or exceptional foraging behaviors. Only in Gekkonidae and Lacertidae are multiple species described as having foraging modes exceptional for the family. Although most gekkonids are ambush foragers, some terrestrial nocturnal species may forage actively. Lacertids typically are active foragers, but some species rely on ambush to some

TABLE 4. TAXONOMIC DISTRIBUTION OF FORAGING MODE IN LIZARD FAMILIES[a]

Taxa	Foraging mode	Genera	Species	Sources[b]
Iguania				
Chamaeleonidae	SW	7	16	4, 15, 21, 50, 52, 64
	H	1	1	4
Corytophanidae	SW	2	2	3, 24
Crotaphytidae	SW	2	2	48, 50, 64
Iguanidae	H	6	9	20, 26, 31, 33, 35, 40, 54, 55, 58
Phrynosomatidae	SW	8	29	1, 2, 13, 45, 48, 50, 64
Polychridae	SW	2	9	32, 37, 64
Tropiduridae	SW	3	4	60, 61, 64
Scleroglossa				
Anguidae	A	3	4	18, 19, 29, 64
Cordylidae	SW	1	1	12
Eublepharidae	A	1	2	30, 50, 62, 64
Gekkonidae	SW	18	52	4, 13, 22, 23, 41, 44, 47,
	A-M	6	8	4, 22, 23, 41, 47
Gerrhosauridae[c]	?	—	—	—
	H	1	1	36
Helodermatidae	A	2	2	9, 10
Lacertidae	A	13	63	4, 5, 6, 14, 25, 38, 43, 49, 50, 56, 57, 59
	SW	3	7	5, 25, 43, 59
Pygopodidae	SW	1	1	39
Scincidae	A	8	15	4, 13, 23, 27, 63, 64
	M	1	1	60, 64
Teiidae	A	3	16	1, 2, 24, 32, 34, 42, 48, 50, 60, 64
Varanidae	A	1	12	4, 7, 8, 16, 46, 47, 51, 53, 65
Xantusiidae	?	2	2	17

[a] In families believed to have more than one foraging mode, genera having both modes are counted twice, but species are counted only once (See text for details). Foraging modes are A = active, H = herbivorous, M = mixed, SW = sit-and-wait (ambush), and ? = poorly known.

[b] 1-Anderson and Karasov, 1981; 2-Anderson and Karasov, 1988; 3-Andrews, 1979; 4-Arnold, 1984; 5-Arnold, 1990; 6-Arnold and Burton, 1978; 7-Auffenberg, 1981; 8-Auffenberg, 1984; 9-Beck, 1990; 10-Beck and Lowe, 1991; 11-Branch, 1988; 12-Broadley, 1978; 13-Cooper, personal observations; 14-Diaz and Carrascal, 1990; 15-Dunham et al., 1988; 16-Edroma and Ssali, 1983; 17-Fellers and Drost, 1991; 18-Fitch, 1935; 19-Fitch, 1989; 20-Gleeson, 1979; 21-Heatwole, 1970; 22-Henle, 1990; 23-Henle, 1991; 24-Hirth, 1963; 25-Huey and Pianka, 1981; 26-Iverson, 1979; 27-James, 1991; 28-Jones, 1983; 29-Karges and Wright, 1987; 30-Kingsbury, 1989; 31-Lazell, 1973; 32-Magnusson et al., 1985; 33-Mautz and Nagy, 1987; 34-McGovern et al., 1984; 35-Minnich and Shoemaker, 1970; 36-Mitchell et al., 1987; 37-Moermond, 1979; 38-Murray and Schramm, 1987; 39-Murray et al., 1991; 40-Nagy, 1973; 41-Odendaal, 1979; 42-Paulissen, 1987; 43-Perry et al., 1990; 44-Peterson, 1990; 45-Peterson and Whitford, 1987; 46-Pianka, 1968; 47-Pianka, 1971; 48-Pietruszka, 1986; 49-Rica, 1982; 50-Shenbrot et al., 1991; 51-Shine, 1986; 52-Shine and Lambeck, 1989; 53-Stebbins and Barwick, 1968; 54-Sylber, 1988; 55-Trillmich and Trillmich, 1986; 56-Van Damme et al., 1990a; 57-Van Damme et al., 1991; 58-VanDevender, 1982; 59-Vernet et al., 1988; 60-Vitt, 1990; 61-Vitt, 1991; 62-Vitt and Congdon, 1978; 63-Vitt and Cooper, 1986; 64-Vitt and Price, 1982; 65-Vogel, 1979.

[c] Foraging mode uncertain for insectivores.

extent. However, as all but one of these species spend a much higher proportion of the time moving than do iguanian ambush foragers, they can be characterized as active foragers for interfamilial comparisons.

There is a great need for data on quantitative aspects of foraging movements in a wide diversity of lizard taxa. However, enough data are available to characterize the predominant foraging mode of most of the major lizard families. Although there is some variation in foraging behavior within families, the basic foraging mode appears to be fixed in most lizard families and quite stable within most others, with some apparent exceptions for foraging mode in Lacertidae and Gekkonidae. Quantitative data are completely lacking for Anguidae and for *Sphenodon*, the outgroup for Squamata.

Information on lingually mediated prey chemical detection or discrimination is available for a much smaller sample of lizards species and for fewer families than data on foraging mode (Cooper, in review). However, for the available experimental and observational data, the presence or absence of prey chemical discrimination is fixed within lizard families (Cooper, in review). In Iguania, all insectivorous families lack the ability; only in the herbivorous Iguanidae is it known to occur (Table 1). In Scleroglossa, prey chemical discrimination is present in all families studied except Gekkonidae and possibly Amphisbaenidae (which detects prey chemicals) (Table 1). Snakes, which are also scleroglossans, also identify prey chemical by tongue-flicking (Chiszar et al., 1986a,b; Cooper, 1990a, 1991b, 1992a). Much more information on the distribution and taxonomic stability of prey chemical discrimination is needed.

Nevertheless, enough information is already available to detect a broad relationship between foraging mode and prey chemical discrimination. In a recent paper I presented evidence that foraging mode affects the use of prey chemical discrimination independently of phylogeny and that phylogeny also influences prey chemical discrimination (Cooper, in review). In particular, when active foraging appeared first in primitive scleroglossans, prey chemical discrimination by tongue-flicking also made its debut. In Gekkonidae, a shift to active foraging was accompanied by loss of prey chemical discrimination. Among iguanians, lizards in all but one of the families studied are ambush foragers that do not detect prey by chemosensory means. In Iguanidae, however, the diet has changed to herbivory, necessitating changes in the method of search for food. The only iguanid species studied, *Dipsosaurus dorsalis*, exhibits food chemical discrimination (Cooper and Alberts, 1990), indicating another shift in chemosensory behavior corresponding to a change in foraging mode.

The phylogeny in Figure 2 contains data on foraging mode and prey chemical discrimination in families for which information on both is available. The polytomy indicates uncertain relationships among iguanian families. One obvious conclusion is that both foraging mode and prey chemical discrimination are quite stable even in some suprafamilial taxa. In most families, then, retention of the

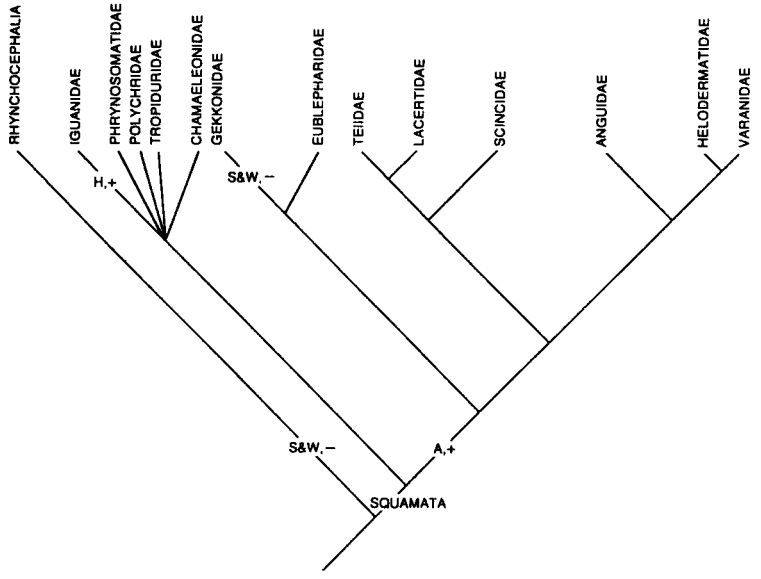

FIG. 2. Cladogram of Lepidosauria for taxa in which states of both foraging mode and prey chemical discrimination are known. States of variables are shown only for the outgroup (Rhynchocephalia) and where changes from the ancestral condition have occurred. Foraging modes: A, active; H, herbivorous, S&W, ambush (= sit and wait). Prey chemical discrimination: +, present; −, absent.

ancestral condition accounts for the type of foraging mode and presence or absence of prey chemical discrimination.

A more interesting conclusion is that changes in prey chemical discrimination are linked to changes in foraging mode. In all three cases in which foraging mode changed from its ancestral condition, prey chemical discrimination also changed to the state typical for the new foraging mode. I have argued that this perfect correlation between changes in foraging mode and changes in prey chemical discrimination shows that prey chemical discrimination is adaptively matched to foraging mode (Cooper, 1994c).

A different line of evidence for the importance of chemosensory behavior to active foragers is morphological. The effect of active foraging on selection for prey chemical discrimination is reflected by the evolution of structural modifications of the tongue to enhance chemosensory sampling. Elongation and development of pronounced forking of the tip are most obvious in the most actively foraging families of scleroglossans, such as teiids and varanids, whereas

the tongues of iguanians are more rounded, less elongated, and lack deep forking (McDowell, 1972).

EVOLUTION OF LINGUAL CHEMOSENSORY ROLES

Detection of Food

Foraging mode profoundly affects many aspects of lizard natural history, having consequences for diet, defensive behavior, reproduction, and other ecologically important features (Vitt and Congdon, 1978; Huey and Pianka, 1981; Huey and Bennett, 1986). As discussed, foraging mode appears to have a strong, direct, determining influence on the use of the tongue for detection of food. It has been predicted that active foragers have more acute olfactory senses than ambush foragers (Regal, 1978). Although this hypothesis has not been tested directly, the findings that lizards belonging to families of active foragers detect chemical prey cues by tongue-flicking, whereas those belonging to families of ambush foragers do not (Cooper, 1990b, 1993c,d), are consistent with it.

Food chemicals are detected by lingually assisted vomerolfaction in Iguanidae, (Cooper and Alberts, 1990, 1991), but not in other iguanian families studied, including Polychridae, Phrynosomatidae, Chamaeleonidae, and Tropiduridae (Cooper, 1989a, this paper). Because most iguanians other than iguanids are ambush foragers (Vitt and Price, 1982), frequent tongue-flicking would increase the likelihood of being detected and avoided by prey. As they are typically motionless while foraging, maintaining visual vigilance in a well-known territory (Stamps, 1977, 1983) patrolled by occasional movements (Tinkle, 1967), they would benefit little from information gained by repeated tongue-flicking in the same place between movements. The difference between iguanids and other iguanians is also explicable by their diets and foraging behavior. Whereas most iguanians are carnivores, iguanids are herbivores. As iguanids evolved herbivorous diets, they presumably altered their foraging mode to a more active movement between food patches. Chemosensory tongue-flicking presumably allows assessment of the potential nutritional value of immobile objects. Freedom from the necessity to avoid detection by prey, and perhaps to some extent by predators, may account in part for their ability to evolve high tongue-flicking rates and detect and identify food vomodors (Krekorian, 1989; Cooper and Alberts, 1990; Cooper and Burghardt, 1990a).

In contrast to iguanians, lizards of most scleroglossan families are active foragers (Evans, 1961; Vitt and Price, 1982) that escape predators by rapidly running away (Vitt and Price, 1982); they are thus free to evolve lingual movements. Even if tongue-flicking were to cause detection by prey, their smaller prey would be less likely to escape. Prey chemicals are widely detected by tongue-flicking among these lizards (reviewed in Cooper, 1990a, 1992a, this

paper). The same is true of snakes (e.g., Burghardt, 1967; Arnold, 1981; Cooper, 1990a), in which this ability was presumably derived from scleroglossan, perhaps varanoid, lizards (McDowell, 1972; Schwenk, 1988).

Data necessary for understanding the evolution of PETF and SICS are only now becoming available. That SICS does not exist in the few species of ambush foragers studied, such as phrynosomatids and agamines, is not surprising. These lizards lack the prey chemical discrimination needed to conduct a chemosensory search, and adoption of SICS would demand that they temporarily switch to active search while attempting to relocate escaped prey. As actively foraging squamates typically use the tongue to gather chemical information about prey (Evans, 1961; Cooper, 1990a,b), the evolution of SICS in these reptiles would appear to require little more than acquisition of neural mechanisms linking the activation of foraging and the accompanying tongue-flicking.

The absence of any clear-cut PETF in iguanian families lacking SICS hints that at least some iguanians that do not use the tongue to identify prey may lack the ability to do so. It is not known whether PETF is a precursor of SICS, but it is doubtful that PETF without searching movements would help locate prey. In this regard, information on relationships between SICS and PETF are needed for more iguanian families and for lineages in which transitions in foraging mode have occurred.

I have hypothesized (Cooper, 1993a) that SICS occurs in species capable of prey chemical discrimination and is absent in those that are not (or that do not use tongue-flicking for this purpose). The data show that both prey chemical discrimination by tongue-flicking and SICS exist in representatives of seven major squamate taxa, including one iguanian taxon and six scleroglossan taxa, and that both chemosensory behaviors are absent in two iguanian taxa (Table 5). As predicted, there is a significant association between prey chemical discrimination and SICS (Fisher exact test, $P < 0.05$, one-tailed). Insufficient data are available to suggest whether or not the behaviors and their association evolved independently as adaptations in the taxa involved.

An additional context in which some squamates, including varanid lizards and snakes, employ tongue-flicking to obtain food is scent-trailing of prey (Burghardt, 1970a; Auffenberg, 1981; Webb and Shine, 1992). As small lizards are predators of invertebrates and small vertebrates that may be highly mobile but do not leave long trails, it might be expected that scent-trailing would not contribute much to their ability to locate food. Most actively foraging scleroglossan lizards appear to chemically detect prey in situ or nearly so. For example, helodermatids can locate buried eggs by chemical cues (Bogert and Del Campo, 1956). Only varanid lizards have been strongly suspected of locating prey by scent-trailing for any distance [*Varanus komodoensis* (Auffenberg, 1981)], and even that case has not been verified experimentally.

Assuming that varanids locate prey by scent-trailing, this ability very likely

TABLE 5. DISTRIBUTION OF PREY CHEMICAL DISCRIMINATION AND STRIKE-INDUCED CHEMOSENSORY SEARCHING (SICS) IN MAJOR LIZARD TAXA IN WHICH BOTH ARE PRESENT OR ABSENT[a]

Taxa	Prey Chemical Discrimination	SICS	Sources
Iguania			
Chamaeleonidae	–	–	Cooper, 1989a, 1993
Iguanidae	+	+	Cooper and Alberts, 1989; Cooper, unpublished data
Phrynosomatidae	–	–	Cooper, 1989a, 1993
Scleroglossa			
Scincidae	+	+	Cooper and Vitt, 1989; Cooper, 1992b
Cordylidae	+	+	Cooper, 1992a
Lacertidae	+	+	Cooper, 1990b, 1991
Anguidae	+	+	Cooper, 1990d, unpublished data
Varanidae	+	+	Cooper, 1989b,c
Serpentes	+	+	Burghardt, 1970; Chiszar and Scudder, 1980

[a]Taxa are described in Estes et al. (1988) and Frost and Etheridge (1989).

has evolved in conjunction with changes in prey types, foraging behavior, and metabolic capacity required to capture such prey. Some large varanid species consume large vertebrate prey (Auffenberg, 1981) that may be highly mobile and occur at relatively low density compared to arthropods. The large size of varanids is a prerequisite for foraging over wide areas; their great aerobic scope in comparison with other lizards (Bennett, 1973; Gleeson, 1981; Bickler and Anderson, 1986) could be a response to natural selection for foraging over wide areas. The ability to follow scent-trails for a long distance may have evolved to allow capture of prey unlikely to be encountered visually or by lingual sampling restricted to the close physical proximity of the prey. A first step toward scent-trailing might have been area-concentrated lingual search upon detection of prey chemicals or after escape of prey detected by any means. Area-concentrated chemosensory search has been reported in *V. bengalensis* (Auffenberg, 1984).

Little information is available for other lizards, but there is some information suggesting that area-concentrated search may be widespread in scleroglossans. Snakes initiate a series of rapid short-excursion tongue-flicks immediately upon lingually contacting conspecific pheromones on a substrate, a behavior termed the trail-contact response (e.g., Brown and MacLean, 1983). A similar response occurs in the scincid lizard *Eumeces laticeps* (observations during the study by Cooper and Vitt, 1986a), which lacks lingual specialization for chemical sampling such as a forked tongue, and in the helodermatid *Hel-*

oderma suspectum (Cooper, unpublished observations). A similar response to prey chemicals can be envisioned to lead to area-concentrated search for prey not readily located by scent-trailing over a distance, yet likely to be found in a restricted area near its scent, and to scent-trailing of prey that deposit directional trails that can be followed. For either type of prey, prolonged PETF would increase the probability of locating the localized scent or the chemical trail of escaped prey.

Beyond the detection of prey followed by scent-trailing that occurs in non-venomous snakes (e.g., Burghardt, 1970a; Webb and Shine, 1992), some highly venomous snakes have evolved a strike–release–trail strategy to avoid injury by dangerous prey (Chiszar and Scudder, 1980; Radcliffe et al., 1980; O'Connell et al., 1985). After striking and envenomating dangerous prey items such as large rodents, rattlesnakes release the prey and remain relatively immobile for a minute or two, after which they begin to tongue-flick rapidly and search for the chemical trail left by the departed prey. When the trail has been located, the snake follows it to the by then immobilized prey. Small, harmless prey such as neonatal rodents are not released prior to ingestion.

Development of the strike–release–trail strategy presumably required the prior attainment of potent venom and presumably occurred in snakes already possessing SICS and scent-trailing abilities. In this scenario all that was lacking was voluntary release of the envenomated prey. Interestingly, SICS and voluntary release by venomous snakes occurs in both active foragers (O'Connell et al., 1985) and ambush foragers (Chiszar and Scudder, 1980; Chiszar et al., 1985). Thus, in at least one lineage of snakes that has (secondarily?) adopted ambush foraging, prey chemical discrimination by lingual sampling has been retained and used in a new context.

There is no evidence regarding antecedents of voluntary release following envenomation or greatly increased duration of SICS, but it may be speculated that two factors have operated. First, large, dangerous prey by their physical exertions are more likely to escape the grasp of venomous snakes when bitten. This would induce selection favoring individuals in which tongue-flicking lasts until the scent-trail has been located. Because the probability that the envenomated prey will die nearby is very high, long-lasting SICS may have evolved to increase the probability of relocation. Second, because large prey are dangerous, one may imagine that voluntary release may often have been stimulated by the prey's defensive behaviors before the advent of consistent voluntary release. Selection on tendency to voluntarily release such prey presumably led to fixation of voluntary release because it greatly decreases the likelihood of injury without, in the presence of already highly developed SICS and potent venom, greatly reducing the likelihood of obtaining a meal. Escape by prey due to retaliation would presumably select for enhancement of preexisting SICS, perhaps through prolongation of PETF and refinement of scent-trailing, but only

retaliation selects for voluntary release. Thus, the strike–release–trail strategy may be conceived as voluntary release superimposed on SICS.

Detection of Predators

Published data reveal detection of predators by tongue-flicking only in those taxa in which both foraging mode and antipredatory defense are compatible with frequent tongue-flicking and locomotion. Among diurnal lizards, this suggests the tongue may be important for predator detection by prey primarily in active foragers that rely on speed for escape. Because such species also use lingual chemical sampling to detect and identify prey, lingually mediated detection of predator chemicals and prey chemical discrimination are closely associated, both occurring in all five scleroglossan taxa listed in Table 2 for which chemosensory detection of predators has been demonstrated.

Detection of predators by tongue-flicking is unknown in iguanian lizards, but no studies of this possible function have been reported. Because lizards not moving as frequently as do active foragers would have less to gain by detecting nonvolatile chemicals associated with predators, it seems highly unlikely to occur in ambush foragers that move and tongue-flick little while foraging. Ambush foragers tend to be sedentary, territorial species (Stamps, 1977; Vitt and Price, 1982) that escape predators by fleeing to known shelters rather than by leaving the area (Tinkle, 1967). On the other hand, detection of either ambushing or actively foraging predators by chemical cues at shelter sites might be adaptive in species occupying burrows or crevices.

Tongue-flicking to detect predators while waiting in ambush would disrupt the crypsis used to avoid detection by them (Vitt and Price, 1982) and would increase the probability of being detected and avoided by prey. Maintenance of crypsis presumably explains the close association in these lizards between tongue-flicking and movement: Most tongue-flicks are emitted immediately after movement (Simon et al., 1981; Cooper et al., 1994a). Thus, if it occurs during foraging, detection of predator (prey, or conspecific) chemicals by tongue-flicking must be accomplished by iguanian ambush foragers primarily after movement to new ambushing sites.

Detection of predator chemicals might alert a territorial species to past or current presence of either ambushers or actively foraging predators. As the major predators of ambushing lizards are expected to be active foragers (Huey and Pianka, 1981), chemical detection of predators may now allow any major increase in ability to avoid predation. One reason for this is that chemical signs of an active forager likely indicate that the forager has already passed through and out of the immediate vicinity. Furthermore, iguanians have well-developed visual acuity on which they rely for detection of prey and predators (Evans, 1961; Underwood, 1970). An iguanian would be very likely to detect movements of an actively foraging predator before moving to a new ambushing site.

In contrast to its effect in iguanian ambush foragers, the foraging mode of most scleroglossan lizards encourages the widespread use of tongue-flicking to detect predators. The eublepharid gecko (Dial et al., 1989) reported to detect predator vomodors (Cooper and Burghardt, 1990a) is a nocturnal active forager unlikely to be detected visually by predator or prey when tongue-flicking. The lacertid (Thoen et al., 1986; Van Damme et al., 1990b), the skink (Cooper, 1990c), and the varanid (Phillips and Alberts, 1992) are active foragers in families that identify chemical prey stimuli (Cooper, 1989b, 1990c; Cooper and Vitt, 1989) and tongue-flick at high rates while forging (Bissinger and Simon, 1979; Cooper and Vitt, 1989).

For actively foraging scleroglossan lizards, many of the major predators are ambushers (Huey and Pianka, 1981) that might be detected by chemical cues before being detected visually. Because even actively foraging predators would be much more likely to detect the motion of an actively foraging lizard's body than of its tongue, there may be little or no selection to prevent lingual sampling.

The relationship between foraging mode and detection of predator chemicals by tongue-flicking is poorly known in snakes, but differs from that in lizards. Crotaline snakes detect chemicals from ophiophagous snakes despite being ambushers (Bogert, 1941; Weldon, 1982). If their own major predators are ambush foragers that detect prey by chemical senses, ambush foragers are unlikely to be detected by using chemical cues to detect predators. Furthermore, many ambushing snakes have greater opportunity to detect scent-trails of predators because they move more extensively than typical lizards, especially during migration and movements between ambush sites, which may be identified by the presence of chemical prey cues (Duvall et al., 1990).

In iguanian lineages that secondarily adopted active foraging or otherwise abandoned relative immobility, selection would have favored evolution of tongue-flicking for detection of food while foraging, as in Iguanidae. In terrestrial active foragers there might have been simultaneous selection for ability to detect predators by tongue-flicking. Whether iguanids have this ability is unknown, but there is some reason to suspect that they may not. Their large size and occupation of exposed perches render them conspicuous, which may have a permissive effect on tongue-flicking, but their prolonged immobility in comparison with many actively foraging scleroglossans would appear to offer relatively little opportunity for detection of predators that would not be detected by the visual vigilance of these lizards. Furthermore, lingual examination of plant food is much more spatially restricted than search for chemical prey cues in widely foraging scleroglossans. These factors suggest that there would be relatively little opportunity or selective advantage for chemical detection of predators by iguanids, especially in larger species. However, the presence of well-developed prey chemical discrimination and pheromonal communication in Iguanidae plus

the possibility of avoiding trees bearing scents of hidden predators indicate that experimental testing is in order.

Pheromonal Communication

The wide phylogenetic distribution of lingual participation in pheromonal communication suggests that touching the tongue to the skin of conspecifics or environmental substrates such as rocks to detect pheromones is subject to less stringent selective constraints than tongue-flicking behavior associated with detection of food and predators or that selective benefits of pheromonal communication outweigh any negative selection. Iguanians engaged in social behaviors are unlikely to forage simultaneously. In any case, social movements would render them conspicuous to predators and prey alike, abolishing any disadvantage of tongue-flicking to obtain pheromonal information.

The form of tongue-flicking by the broad-headed skink in response to chemical stimuli on cotton-tipped applicators appears to be the same whether the chemical cues are derived from predators (Cooper, 1990c), conspecifics (e.g., Cooper and Vitt, 1984a,b), or prey (Cooper and Vitt, 1989). Once the tongue can be brought into contact with external surfaces, perhaps initially during failed predation attempts, selection for all three main uses of the tongue–vomeronasal system is possible. This is consistent with the wide importance of tongue-flicking in pheromonal communication. Even ambushers can and do touch their tongues to environmental substrates (e.g., Gravelle and Simon, 1980; Greenberg, 1985), often immediately after their occasional movements (Greenberg, 1985). They also tongue-flick the skins of conspecifics during social encounters (Duvall, 1982). Any disadvantage of tongue-flicking to detect pheromonally marked substrates by ambush foragers can be greatly reduced by performing such behavior immediately at the end of translational movement to a new ambush post. It may thus be predicted that in iguanians that hunt by ambush, tongue-flicking to detect substrates pheromonally marked by conspecifics occurs primarily or exclusively at the conclusion of locomotion and perhaps immediately prior to it. Many ambush-foraging iguanians perform species-typical social displays at this time, presumably in part for the same reason.

Responses to pheromones are widespread in vertebrates, as illustrated by papers on pheromones in fish, amphibians, reptiles, and mammals in a recent symposium volume (Duvall et al., 1986). Pheromonal communication seems to be important in Testudines and Crocodilia (reviewed by Mason, 1992), reptilian taxa that do not tongue-flick. Pheromonal communication is unknown in the sole extant rhynchocephalian (Mason, 1992). As both iguanians and scleroglossans respond to pheromones, sensitivity to pheromones must have been present in very early lizards. If pheromones existed prior to tongue-flicking, adoption of lingual sampling by primitive squamates presumably had conse-

quences for pheromonal structure and properties. Maximum effective molecular weight and information content may have increased. An attendant decrease in minimum effective volatility may have permitted more prolonged marking of substrates.

Tongue-flicking conspecifics for pheromone sampling might have developed as a result of incidental lingual contacts with conspecifics made while attempting to capture prey or after swallowing prey in close proximity to conspecifics, perhaps temporally contiguous with social encounters (Figure 1). A second possibility is that lingual extension evolved initially for prey capture or detection could readily have led, in the vicinity of conspecifics, to contact with environmental substrates bearing conspecific chemicals. In either case, it is but a short sequence from tongue-flicking skins of conspecifics, which may bear pheromones produced by integumentary or cloacal glands (Cooper et al., 1986; Mason, 1992), to tongue-flicking pheromone-bearing excrement or other deposits as they are produced or in the vicinity of the producers, to tongue-flicking visually marked pheromone deposits (Duvall, 1979; Alberts, 1989) in the absence of conspecifics. Because iguanians other than the primarily herbivorous iguanids do not tongue-flick regularly while foraging, such tongue-flicking presumably was not required for the evolution of scent-marking (assuming its primitive absence) and lingual response to the pheromonal deposits as in phrynosomatids such as *Sceloporus occidentalis* (Duvall, 1979), but could have been important in iguanids (*Dipsosaurus dorsalis*, Alberts, 1989). As previously discussed, pheromone deposits may be detected by ambush foragers primarily when tongue-flicking immediately after movement to new ambushing sites or in the case of territorial males, during territorial patrolling.

After lingual sampling of conspecific chemical cues from the skin and/or environmental deposits was established, contacts with pheromones on visually unmarked substrates might have been important to the evolution of scent-trailing of conspecifics by some scleroglossan lizards and snakes [found, e.g., in skinks (Cooper and Vitt, 1986a); varanids (Auffenberg, 1981); and snakes (Ford, 1986)], in which the tongue is frequently protruded during active foraging.

When squamates are placed in novel surroundings in the laboratory, they tongue-flick at higher rates than in their home cages (e.g., Gravelle and Simon, 1980; Greenberg, 1985), indicating chemosensory exploration of the new setting. Among scleroglossans and snakes, the elevated tongue-flicking rate could allow assessment of the presence of prey, predators, and conspecifics. Among iguanians, the most probable function of tongue-flicking in novel environments is detection of the presence of conspecifics, which would be useful in species having territorial or hierarchical social systems. The presence of conspecifics alone may indicate suitable food and predator conditions (Stamps, 1987). An untested possibility is that lizards that do not tongue-flick prey before attacking, including many iguanians presumably constrained by ambush foraging from

tongue-flicking while foraging, may be able to identify chemical prey cues on substrates and use such cues to assess the quality of unfamiliar environments.

Sequential Evolution of Chemosensory Roles

It is unclear to what extent tongue-flicking behavior and chemosensory capacity may have evolved initially for one chemosensory function and later have undergone sequential modification for use in other roles. If primitive squamates resembled many scleroglossan lizards and snakes in foraging mode and antipredatory defenses, there might have been simultaneous selection for responsiveness to chemical stimuli derived from prey, predators, and conspecifics (Figure 3). Because the presumed behavioral sources for selection leading to tongue-flicking (i.e., partial extension of the tongue during prey capture and lingual protrusion after swallowing prey) are closely associated with feeding, it would have been simplest for the initial selection to have operated primarily on the ability to detect and identify prey chemicals. Once begun, such selection would induce an increased frequency of lingual environmental sampling, thereby improving the opportunity for contact with chemical cues associated with predators and conspecifics. This in turn would enhance the opportunities to evolve selective responses to chemical predator stimuli and pheromones. Selection for lingual sampling of prey, predator, and conspecific cues very likely proceeded simultaneously.

Outgroup comparison suggests that the primitive foraging mode for squa-

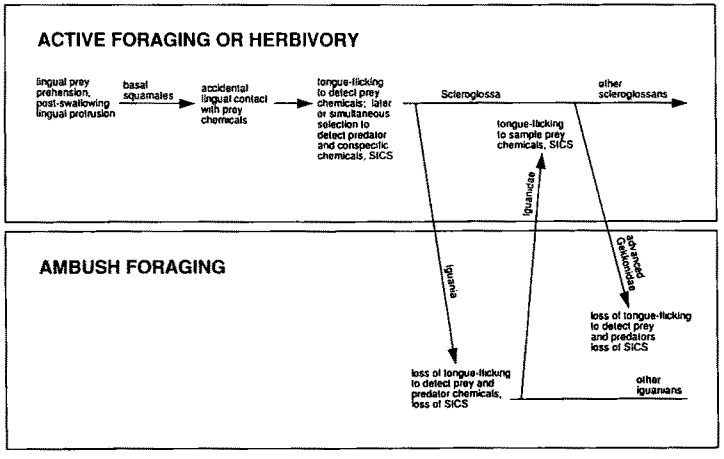

FIG. 3. Probable pattern of evolution of chemosensory roles of tongue-flicking in squamate reptiles if basal squamates were active foragers. Data for "advanced Gekkonidae" limited to gekkonines.

mates was ambush (Walls, 1981), in which case the general evolutionary sequence in Figure 3 seems improbable. However, considerable uncertainty remains regarding the primitive condition of foraging mode in squamates. No quantitative information on foraging movements by *Sphenodon* has been published. Even if *Sphenodon* is a typical ambush forager, there is no guarantee that this species has the same foraging mode as did extinct rhynchocephalians, in particular the line leading to squamates.

Regal (1978) speculated that primitive lizards were cruise foragers from which specialized ambush and active foragers subsequently evolved. Regal's (1978) hypothesis contradicts my current assessment based on outgroup comparison, but in modified form remains viable due to the above uncertainties. If the basal squamates were cruise foragers, here considered to be active foragers, specialization for ambush foraging occurred in Iguania. Scleroglossans having relatively unspecialized lingual features, such as eublepharid geckos and skinks, forage actively, but more slowly and extensively than lingually specialized teiids and varanids. This, too, is consistent with Regal's hypothesis.

A different evolutionary sequence transpired in the preferred scenario in which primitive squamates were ambush foragers using crypsis and immobility as primary defenses (Figure 4). In primitive squamates, tongue-flicking might have evolved initially to sample pheromones. This is consistent with the wide distribution of pheromonal communication in iguanians and scleroglossans. The basal squamate stock may have resembled iguanians more closely than sclero-

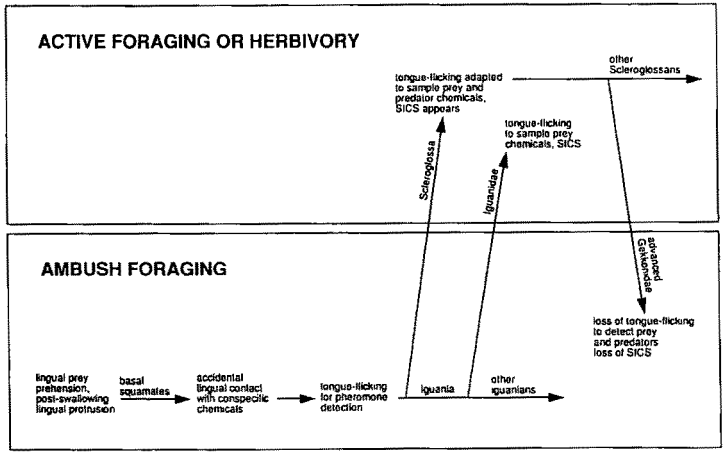

FIG. 4. Probable pattern of evolution of chemosensory roles of tongue-flicking in squamate reptiles if basal squamates were ambush foragers. Data for "advanced Gekkonidae" limited to gekkonines.

glossans because scleroglossans appear to have many derived features (Estes et al., 1988). This similarity reinforces the hypothesis that basal squamates may have been ambush foragers, favoring primary selection of tongue-flicking for detection of pheromones.

Thus, despite great uncertainty, I believe that the most parsimonious hypothesis suggests that natural selection for detection of pheromones may have been the initial selective stimulus for evolution of chemosensory tongue-flicking. This hypothesis is consistent with both the absence of prey chemical discrimination and the probable absence of predator chemical discrimination in most iguanian families and with the widespread presence of pheromonal communication depending on tongue-flicking in both iguanian and scleroglossan families.

In the scenario having the initial selection for pheromonal detection, lingual sampling for detection of prey and predator chemicals would have evolved when the ancestral scleroglossan stock adopted active foraging (Figure 4). The selective milieu favoring active foraging would also have favored tongue-flicking for chemosensory sampling of the area being searched for chemical prey cues. Chemosensory tongue-flicking and active foraging would provide the basis for the most fundamental taxonomic division of squamates, that between Iguania and Scleroglossa. Responsiveness to lingually sampled prey (and predator) chemicals may be expected: (1) to have evolved in other taxa switching from ambush to active foraging, and (2) to have been reduced or lost in scleroglossan groups that reverted to ambush foraging.

The Gekkonoidea, a large scleroglossan clade including a family apparently close to the branch between Iguania and Scleroglossa (Estes et al., 1988; Kluge, 1987), provides a clear example. In the most primitive geckos, the eublepharids, tongue-flicking plays important roles in detecting vomodors of prey [*Coleonyx* (Dial, 1978; Dial et al., 1989); *Eublepharis macularius* (Cooper, unpublished data)] and conspecifics (Greenberg, 1943; Mason and Gutzke, 1989; Brillet, 1990). Predators are also detected by the chemical senses, but the importance of vomerolfaction and/or tongue-flicking has not been established (Dial et al., 1989). As these are typical scleroglossan abilities that occur in the most primitive autarchoglossans, the advent of active foraging is associated with the expected chemosensory adaptations. Similar changes occur after abandonment of ambush foraging in the iguanian family Iguanidae. Another large and more derived gekkonoid family, Gekkonidae, consists largely of geckos that are arboreal ambush foragers that do not tongue-flick prey (Cooper, 1993c). Furthermore, limited information suggests that the use of pheromonal communication in social encounters may be vestigial in gekkonids (Brillet, 1991) relative to that in eublepharids, perhaps rendered less important by the highly developed acoustic communication in gekkonids.

In the foregoing discussion, sequential acquisition of lingual chemosensory roles has been considered to have occurred independently in the major lizard

taxa, yet specializations in one group may have affected the evolution of foraging in the other. Indeed, joint evolution of lingual structure, lingual sampling of prey chemicals and active foraging may well have given competitive impetus to specialization in ambush foraging in other lineages and to evolution of subspecializations among active foragers. It may also have produced unexpected effects on related behaviors. For example, as lingual prey prehension is limited to iguanians (Schwenk and Throckmorton, 1989), one consequence of evolutionary modification of the tongue for sampling chemicals from the external environment may have been loss of lingual prehension of prey by scleroglossans.

Lingual morphology has long been central to squamate taxonomy (e.g., Camp, 1923; Estes et al., 1988; Schwenk, 1988), suggesting that natural selection on the tongue has produced variations associated with distinct suites of adaptive function. It now seems quite likely that contact with prey chemicals during lingual prehension or perhaps during slight lingual protrusions after swallowing was a fundamental event in the evolution of squamate reptiles. In taxa and selective settings for which active foraging was favored, such contact allowed natural selection favoring vomerolfactory detection of hidden prey. Detection of hidden prey by lingually mediated prey chemical discrimination may have made active foraging feasible by allowing capture of more, if smaller, prey items. Despite the greater energetic expenditure by extant active foragers, their frequent capture of small prey yields greater net energy intake than in ambush foragers that eat larger, exposed prey (Anderson and Karasov, 1981, 1988). Active foraging by relatively primitive scleroglossans appears to have set the stage for further evolution of specialized chemosensory behavior and structure, as exhibited by several taxa within Autarchoglossa, which consists of numerous families of scleroglossan lizards excluding Gekkonoidea, and perhaps Amphisbaenidae and Dibamidae (Estes et al., 1988).

Variation in lingual structure among squamate taxa attests to the importance of relationships of tongue-flicking to phylogeny, foraging mode, and evolution of chemosensory roles in foraging. Typical iguanian ambush foragers have fleshy, unforked tongues, but in actively foraging scleroglossans a major evolutionary trend has led to highly specialized tongues capable of efficient chemical sampling. There is a progression of lingual structure from a generalized fleshy type found in geckos and skinks, through increasing elongation and forking (Figure 5) in several families, to the highly specialized form of varanids and snakes (McDowell, 1972), which lack lingual taste buds (Schwenk, 1985). Advanced forms of tongue-flicking behavior involving multiple oscillations outside the mouth occur only in Scleroglossa, primarily in snakes (Gove, 1979). In taxa having specialized lingual morphology, use of the tongue is more pronounced in social, predatory, and antipredatory behaviors, the most frequent use being detection of food. Advanced chemosensory features of foraging such as scent-trailing by varanids and snakes and the strike–release–trail strategy in venomous

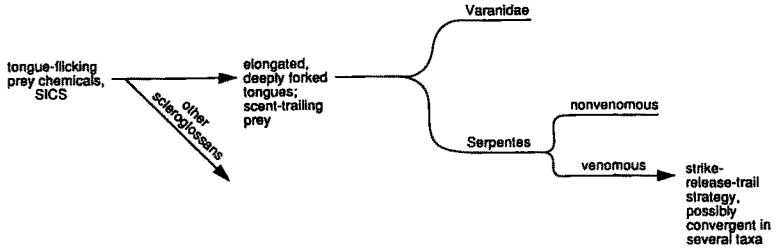

FIG. 5. The evolution of advanced chemosensory behaviors in squamates having tongues and vomeronasal organs highly developed for chemical sampling by tongue-flicking.

snakes evolved in taxa having the most highly specialized lingual morphology for chemical sampling (Figure 5). These observations suggest that foraging behavior has been the most important determinant of evolutionary trends in lingual structure and that foraging behavior and chemosensory tongue-flicking have evolved conjointly in squamates.

For serpents, tongue-flicking and most of its basic chemosensory roles reflect their scleroglossan ancestry. This is undoubtedly true for detection of prey chemicals and pheromones, PETF, and SICS; it is quite likely so for scent-trailing of prey and conspecifics, and probably for detection of predators as well. Lingual morphology (McDowell, 1972), the percentage of sensory cells in the vomeronasal epithelium (Gabe and Saint Girons, 1976), and behavioral responses all suggest that the lingually mediated chemosensory abilities have been highly developed in snakes, but the basic tasks to which vomerolfaction is applied have for the most part not changed since the origin of snakes.

Nevertheless, several changes have occurred, most notably the evolution of the strike–release–trail strategy (Figure 5) in highly venomous viperids (Chiszar and Scudder, 1980; Chiszar et al., 1985) and similar behavior in elapids (O'Connell et al., 1985). As in lizards, the use of tongue-flicking to detect chemical prey cues is related to foraging mode. Ambush foragers such as rattlesnakes typically do not tongue-flick prey before striking (Chiszar and Scudder, 1980) and show no sign of prey chemical discrimination when presented chemical prey stimuli on cotton swabs (Chiszar and Scudder, 1980; Cooper, unpublished observations). Rattlesnakes locate patches containing prey by tongue-flicking (Duvall et al., 1990), but then forage by ambush within those patches. Such behaviors reveal a greater flexibility of lingual use in prey-related chemosensory tongue-flicking and feeding behavior than is known for any lizard. In actively foraging snakes, such as many natricine colubrids, chemosensory tongue-flicking serves both to detect prey and to release attack (e.g., Burghardt, 1970a,b; Arnold, 1981), as in many lizards (e.g., Cooper, 1989b, 1990a; Cooper and Vitt, 1989). Phylogenetic relationships within Serpentes and the distribution

of chemosensory abilities based on vomerolfaction are too poorly known to reconstruct the history of tongue-flicking and its chemosensory uses, but it seems highly probable that active foraging and associated chemosensory behaviors were primitive.

Although lingual sampling of prey chemicals may not have been the initial selective stimulus for the evolution of tongue-flicking, tongue-flicking to detect prey may well have been a crucial factor, perhaps the precipitating and central organizing factor making the evolution of active foraging and therefore Scleroglossa feasible. Subsequent evolutionary refinements of the tongue for chemosensory sampling and the advent of specialized chemosensory foraging behaviors have been major developments in the phylogeny of Scleroglossa that are reflected at the familial level and in higher taxa.

Foraging mode affects antipredatory defenses and appears to be closely associated with utilization of space, social behavior, reproduction, and body form. Because active foragers rely on flight rather than crypsis to avoid predators, their body shape tends to be more elongated and streamlined than that of ambush foragers (Vitt and Congdon, 1978). Streamlining, in turn, affects reproduction by limiting the volume and mass of the clutches (Vitt and Congdon, 1978). Data presented by Stamps (1977) suggest that ambush foragers are much more likely to defend territories than are active foragers, which may defend specific sites or occupy home ranges without defense of any particular space against conspecifics. The degree of polygyny is greater among territorial species, which may account for the greater sexual dimorphism in territorial species (Stamps, 1983). If these generalizations are correct, a shift in foraging mode by lizards had ramifications far beyond feeding behavior and leading to a basic ecobehavioral reorganization.

Other Ecological Factors

Ecological considerations have been limited here largely to foraging mode and defense, but other ecological factors may strongly affect the utility of the tongue–vomeronasal system for identification of prey and predators and for pheromonal communication, and therefore the evolution of chemosensory tongue-flicking roles. Fossoriality and nocturnality have obvious implications, both appearing to place a premium on chemosensory sampling abilities. As there are no fossorial iguanians, it is tempting to speculate that chemosensory tongue-flicking in litter and in and under objects during foraging may be a facilitating, in some cases perhaps even a necessary, precursor to evolution of fossoriality in squamates. Pygopodids could be exceptional.

The effects of nocturnality are not entirely clear. In agreement with expectation, the most primitive geckos, the eublepharids, are nocturnal active foragers that identify prey based on lingually sampled chemicals. However, the large

gekkonid radiation consists mainly of nocturnal species, at least some of which do not tongue-flick while foraging or before attacking prey. Gekkonids have numerous adaptations for vision under low light intensity (Underwood, 1970) and well-developed hearing (Baird, 1970), which permit them to forage visually and aurally, but appear to eliminate the necessity of tongue-flicking to locate exposed prey. The importance of chemical cues to nocturnal lizards, even those not using tongue-flicking to locate prey, is indicated by the presence of highly developed olfactory and vomeronasal organs (Gabe and Saint Girons, 1976).

Arboreality would appear to minimize the importance of chemical cues for species feeding on trunks, stems, and leaves, which may explain the reduction of vomeronasal organs in anoles and the successful arboreal adaptations of other iguanians lacking prey chemical discrimination, especially the chameleons. Among scleroglossans, the relative unimportance of prey chemical stimuli on tree surfaces is consistent with the loss of lingually mediated prey chemical discrimination in the arboreally specialized gekkonids. Some scincid lizards are arboreal or semiarboreal, yet these skinks have retained prey chemical discrimination and are active foragers (e.g., *Eumeces*) or have become herbivorous (e.g., *Corucia*). In *Eumeces* and presumably other insectivorous arboreal skinks, the lizards enter holes and crevices in trees and eat prey that are largely restricted to such hidden places where reduced light would place a premium on chemosensory detection of prey (Vitt and Cooper, 1986).

Even for ecological factors having seemingly obvious implications, interpretation must be cautious. Development of other sensory capacities may lead to adaptations that obviate the need for greater reliance on chemical cues. Ambush foraging dependent on night vision in geckos may be a case in point. Fossorial lizards that detect prey moving on the surface by seismic vibrations (Hetherington, 1989) might (or might not) have reduced needs for chemical prey detection. Finally, phylogenetic or contextual constraints on lingual chemosensory sampling might limit evolutionary possibilities.

CAVEATS

In attempting to reconstruct the evolutionary history of lingually mediated chemosensory and foraging behavior and to consider their effects on other aspects of squamate evolutionary biology, I have had to make lots of guesses, which I have attempted to identify as such. In addition to any logical shortcomings in my synthesis of published information, there are numerous gaps and potential errors in the data. Further information is needed on the distribution of chemosensory behaviors. Even for prey chemical discrimination, data are lacking for some families and are qualitative, indicating presence or absence of ability, but not providing any quantitative information such as the extent of reliance or

sensitivity. Data are also needed for many more taxa within families to ascertain the degree of stability of prey chemical discrimination.

Although foraging modes have been identified qualitatively for many species, quantitative information has been published for relatively few. For example, there are no quantitative data for *Sphenodon* or Anguidae. Active or mixed foraging by *Sphenodon* would affect the interpretations of initial squamate foraging and subsequent evolution of specializations in chemosensory behavior and foraging mode. Ambushing by anguids would suggest that once a certain level of chemosensory evolution for prey chemical discrimination has occurred, the ability is retained regardless of foraging mode, as appears to be the case in crotaline snakes. A particularly fruitful approach may be to study quantitative variation in both chemosensory behavior and foraging mode in families showing substantial variation in foraging behavior, especially Lacertidae and Gekkonidae.

My goal has been to try to make evolutionary sense of the mass of data that has accumulated on lingually mediated chemosensory behavior in lizards. Although some major patterns seem clear, much uncertainty remains. Some hypotheses undoubtedly will be disproven or require modification. I look forward to participating in the process of revision and hope that the admittedly speculative hypotheses will help stimulate research clarifying the evolutionary patterns, some of which can now be discerned only hazily.

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ELECTROPHYSIOLOGICAL AND FIELD ACTIVITY OF HALOGENATED ANALOGS OF (*E*,*E*)-8,10-DODECADIEN-1-OL, THE MAIN PHEROMONE COMPONENT, IN CODLING MOTH (*Cydia pomonella* L.)

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Abstract—Pest monitoring and control of the codling moth, *Cydia pomonella* L., have been developed using the main pheromone component of this species, (*E*,*E*)-8,10-dodecadienol (codlemone). However, the activity of codlemone is not satisfactory for pest control by mating disruption. Thus, we have synthesized halogenated analogs of codlemone to see if they could be used as new agents for pest control of the codling moth. Their biological activity was measured by electrophysiological techniques. In EAG screening, codlemone was the most active compound. F(10,11)-codlemone [(*E*,*E*)-10,11-difluoro-8,10-dodecadienol] and Cl-codlemone [(*E*,*E*)-11-chloro-8,10-undecadienol] elicited significant EAG responses, F(10,11)-codlemone triggering responses not significantly different from responses to codlemone. EAG cross-adaptation experiments and single sensillum recordings revealed that these compounds were detected by the same receptor neuron type as codlemone. No competitive inhibition with codlemone was observed from nonactive compounds. In field trapping, F(10,11)-codlemone and Cl-codlemone were more attractive to male codling moths than codlemone itself. Possible explanations of this activity are discussed.

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INTRODUCTION

The codling moth, *Cydia pomonella* L. (Lepidoptera, Tortricidae), is considered all over the world as the most important pest on apple orchards. The main pheromone component of *C. pomonella* was identified as (8*E*,10*E*)-dodecadien-1-ol (codlemone) (Roelofs et al., 1971; Beroza et al., 1974). Beside codlemone, 10 minor compounds were detected in the pheromone: decan-1-ol (10:OH), dodecan-1-ol (12:OH), tetradecan-1-ol (14:OH), hexadecan-1-ol (16:OH), octadecan-1-ol (18:OH), (8*E*)-dodecen-1-ol (*E*8–12:OH), (9*E*)-dodecen-1-ol (*E*9–12:OH), (8*E*,10*Z*)-dodecadien-1-ol (*E*8,*Z*10–12:OH), (8*Z*,10*E*)-dodecadien-1-ol (*Z*8,*E*10–12:OH), (8*E*,10*E*)-dodecadien-1-al (*E*8,*E*10–12:Ald) (Einhorn et al., 1984, Arn et al., 1985). The behavioral activity of 12:Ac, the second component of the sex pheromone, is controversial. Its synergistic effects with codlemone in wind-tunnel (Einhorn et al., 1986) and in field-trapping experiments (Causse et al., 1988) were not confirmed later on in the wind tunnel (Preiss and Priesner, 1988). In contrast, (8*E*,10*E*)-dodecadienyl acetate (*E*8,*E*10–12:Ac), which is not produced by the females, strongly inhibits the attractivity of codlemone in the field (George et al., 1975; Einhorn et al., 1986).

Pest monitoring and control of the codling moth was developed using codlemone. Although the attractivity of codlemone was considered as satisfactory for pest monitoring, its activity was found to be less reliable for pest control by mating disruption (Audemard et al., 1982). One of the major concerns in the utilization of synthetic pheromone is to prevent their chemical degradation and/or isomerization in the field. This is particularly true for conjugated dienes such as codlemone (Ideses and Shani, 1988). A recent study concerning the use of codlemone dispensers demonstrated that after 150 days, 61% of the pheromone loss was due to chemical decomposition (McDonough et al., 1992). The use of codlemone analogs having a better chemical stability in the field could be of considerable interest.

Olfactory neurons involved in the detection of sex pheromones in Lepidoptera are very specific and sensitive. Modifications in the carbon chain length, in insaturations (number, position, and geometry), in enantiomeric composition, or in the terminal functionality of pheromone compounds reduce their electrophysiological activity by several log units (Priesner, 1979; Liljefors et al., 1984, 1985, 1987; Renou et al., 1991). In contrast, some of the halogenated analogs resulting from isosteric replacements in the molecule were found to retain electrophysiological activity depending on the position and extent of the halogena-

tion (Camps et al., 1984; Prestwich et al., 1988; McLean et al., 1989; Masnyk et al., 1989; Svatos et al., 1990; Jönsson et al., 1991). These compounds exhibit a variety of behavioral activities including attractivity, synergism, or inhibition (Briggs et al., 1986; Prestwich et al., 1986; Masnyk et al., 1989; Bengtsson et al., 1990; Dickens et al., 1991). Thus, we have synthesized halogenated analogs of codlemone. Their electrophysiological activity was measured on males and compared to that of codlemone using electroantennography and single sensillum recording techniques. Their attractivity to males was evaluated in field-trapping experiments.

METHODS AND MATERIALS

Insects. Male codling moths were obtained from laboratory-reared stocks. Eggs were collected and deposited on a semiartificial medium (Guennelon et al., 1981). All the development, from first-instar larvae to emerging adults, was done inside the same medium and lasted seven to eight weeks at 20–21°C and 75% relative humidity. Adults were collected daily. Electrophysiological experiments were conducted the first or second day after emergence.

Stimulus Compounds. Codlemone, four fluorinated analogs, seven chlorinated analogs, and seven nonhalogenated analogs of codlemone were tested (Figure 1). Steric purity of all compounds was >99% as checked by gas chromatography and mass spectrometry. No contamination by codlemone was found in any sample.

Fluorocodlemones were prepared replacing two hydrogen atoms by two fluorine atoms in the two carbons of one or both double bonds (Tellier et al., 1989) and the three hydrogen atoms of the terminal methyl group by three fluorine atoms (Tellier and Sauvêtre, 1992). Chlorinated analogs were also synthesized replacing the terminal methyl group by a chlorine atom (Hammoud, 1988; Tellier et al., 1993). These replacements were designed because they do not have notable steric consequences (Schlosser, 1978; Jönsson et al., 1991). They probably do not modify substantially the original spatial environment of the key sites of the pheromone molecules involved in the pheromone reception process.

Olfactory Stimulation. Stimulus compounds were diluted in hexane. Each compound was deposited on a filter paper inserted into a Pasteur pipet. The outlet of the main branch of a branched glass tube was positioned 1 cm in front of the antenna. A purge stream of humidified pure air (1.0 liter/min, 100% relative humidity) was continuously blown on the antenna through a lateral branch. To stimulate the olfactory receptors, a Pasteur pipet was introduced in the main branch. Stimulations were achieved by blowing a puff of air (1 sec, 0.5 liter/min) through the pipet with a timer controlled solenoid valve.

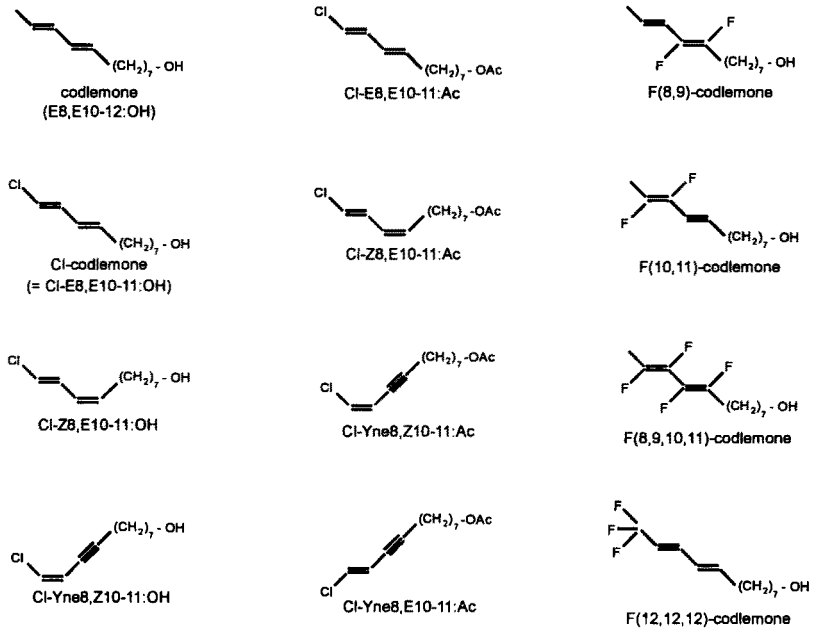

FIG. 1. Halogenated compounds tested. Short names designate codlemone analogs in the text.

Compounds were tested at random for EAG and SSR screening experiments, at a dose of 1 μg. For EAG dose–response experiments, responses to graded doses (in a logarithmic scale from 0.1 to 10,000 ng) of codlemone and four halogenated analogs were measured. F(10,11)-codlemone and Cl-codlemone were selected because of their strong activity in EAG screening and F(12,12,12)-codlemone and F(8,9,10,11)-codlemone because they were then tested for competition experiments with codlemone. Competition experiments between two EAG-inactive fluorinated analogs and codlemone were conducted in order to evaluate the affinity of these analogs for the codlemone binding sites. The effects of increasing amounts, from 1 ng to 10 μg, of F(12,12,12)-codlemone and F(8,9,10,11)-codlemone on the EAG responses to 500 ng of codlemone were measured. In order to study if codlemone and its two most active halogenated analogs, F(10,11)-codlemone and Cl-codlemone, were detected by the same receptor sites, the level of cross-adaptation between these compounds was measured. The adaptation stimulus consisted in repetitive stimulations with 20 μg of the adaptation compound. Responses recorded before and after adaptation were compared. Pure air was tested as a control in all screening and dose–

response experiments, and the interstimuli intervals lasted at least 1 min to avoid sensory adaptation.

Electrophysiology. Electroantennography (EAG) and single sensillum recording (SSR) procedures have been previously described (Lucas and Renou, 1989). EAGs were recorded from males restrained in a styrofoam block. The apex of the antenna was cut off and the cut tip was inserted into the recording electrode. The neck of the insect was impaled with the reference electrode. Both electrodes were connected to a Neurolog NL102 preamplifier through chloridized silver wires. The signal was filtered (DC, 300 Hz) and amplified (×200).

SSRs were conducted according to the tip recording method (Kaissling and Thorson, 1980) on nonisolated antennae. Briefly, the antenna was restrained by narrow sticky bands. The last segments were removed and the reference electrode covered its cut end. The tips of several sensilla were cut off using sharpened forceps and the recording electrode was slipped over the cut end of one hair. The signal was filtered (150–5000 Hz) and amplified (×1000).

Both EAG and SSR signals were recorded on a PC-AT-compatible microcomputer via a DASH 16 (Metrabyte) analog-to-digital conversion board. Acquisition and analysis of EAG and SSR signals were performed by specific programs written in the Asyst Software (Macmillan Software Company). EAGs were sampled at 1 kHz during 15 sec, starting 5 sec before the onset of the stimulation. SSRs were digitized at 10 kHz during 1.5 or 3 sec after the onset of the stimulation. Action potentials emitted in response to pheromone stimulation were counted during the second following the onset of the stimulus.

Field Trapping. Field-trapping experiments were conducted in 1990 from May 17 to September 14 in a 40-year-old Guyot pear orchard (3 ha) located at Ile de la Barthelasse, near Avignon (southeast of France). Codlemone and analogs were deposited in hexane solution, without antioxidant, on rubber septa (SNL, Bondoufle, France) at the dose of 1 mg/dispenser. Traps (INRA Delta trap) were hung 1.5 m above ground and 40 m apart in the orchard. Five compounds were tested with two traps per compound. Every two weeks the rubber septa were changed and the positions of the traps were rerandomized. Traps were checked three times a week with a total of 48 readings of the traps during the complete trapping period. Each reading of a trap was considered as a replicate for statistical analysis. Three different factors were considered for the statistical analysis: (1) age of the dispenser, (2) position of the trap, and (3) pheromone compound tested.

Relative volatilities of codlemone, its fluorinated analogs, and Cl-codlemone were estimated by the calculation of Kovats indices (Kovats, 1958) for a better interpretation of the field-trapping results. Indices were calculated from retention times obtained on a Girdel 32 gas chromatograph equipped with an apolar fused silica capillary column (25 m, 0.32 mm ID, WCOT, CPSil-8CB, from Chrompack) programmed from 140°C to 240°C at 5°C/min.

RESULTS

EAG Screenings. A first EAG screening was made with codlemone and 11 halogenated analogs (Figure 2A). The most active compound was codlemone, which elicited EAGs over 4 mV. Among halogenated analogs, F(10,11)-codlemone, Cl-codlemone, F(8,9)-codlemone, and Cl-*E*8,*E*10–11 : Ac elicited significant responses when compared to response to pure air (one-way ANOVA, Newman-Keuls test, $P < 0.05$). Responses to codlemone and F(10,11)-codlemone were not significantly different.

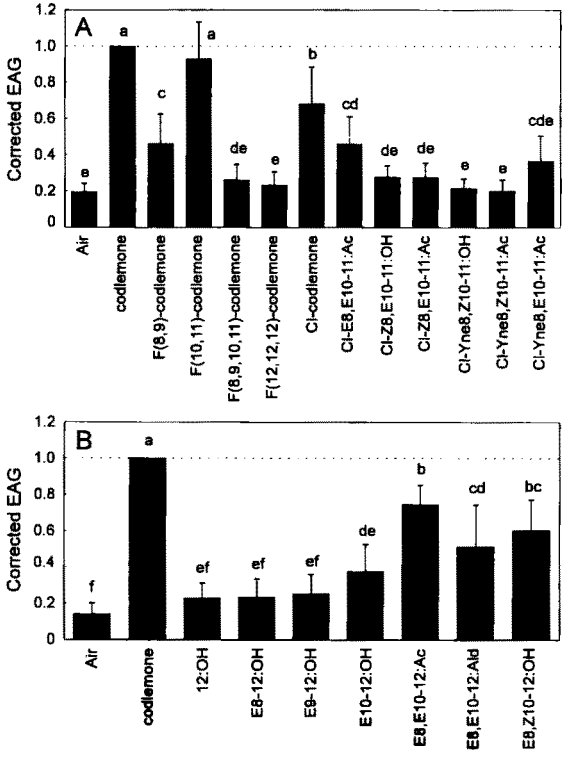

FIG. 2. Mean response profiles of male *Cydia pomonella* to codlemone and a series of 11 of its halogenated analogs (A) and to codlemone and seven of its nonhalogenated analogs (B). Responses to codlemone analogs were expressed as proportions of response to codlemone. All compounds were tested at a dose of 1 μg and with pure air as a control. Mean of the responses of 13 males (A) and nine males (B). Error bars indicate standard deviation at $\alpha = 5\%$. Bars associated with the same letter are not significantly different at $P < 0.05$ (one-way ANOVA, Newman-Keuls test).

In a second EAG screening, codlemone and seven nonhalogenated analogs were tested in the same conditions for structure–activity comparisons (Figure 2B). Once again codlemone elicited the best responses. *E*8,*E*10–12:Ac, *E*8,*E*10–12:Ald, *E*8,*Z*10–12:OH and *E*10–12:OH had significant EAG activity when compared to the control but their responses were inferior to responses to codlemone (one-way ANOVA, Newman-Keuls test, $P < 0.05$). Other compounds with one or no insaturation had no EAG activity.

EAG Dose–Response Curves. Codlemone and F(10,11)-codlemone were the two most active compounds (Figure 3). Their detection threshold was between 1 and 10 ng of product deposited on the filter paper. Responses elicited by different doses of these two compounds are not different (two-ways ANOVA, Newman-Keuls test, $P < 0.05$). However, at the upper dose used (10 μg), an olfactory saturation could be observed for F(10,11)-codlemone but not for codlemone. Cl-codlemone, the second best active halogenated analog, presented an EAG activity parallel to the two former compounds with a one-log-unit shift in concentration. F(12,12,12)-codlemone and F(8,9,10,11)-codlemone had no significant EAG activity when compared to pure air, even at the highest dose tested (10 μg).

Competition Experiments. EAG responses to binary blends of codlemone with increasing amounts of F(12,12,12)-codlemone or F(8,9,10,11)-codlemone were compared to responses to pure codlemone obtained on each male before and after testing the blends (Figure 4). EAG responses did not depend upon the amount of F(12,12,12)-codlemone or F(8,9,10,11)-codlemone added to codlemone (one-way ANOVA, Newman-Keuls test, $P < 0.05$).

Cross-Adaptation Experiments. After repetitive stimulations by 20 μg codlemone (adaptation stimulus), response to 1 μg codlemone, F(10,11)-codlemone, and Cl-codlemone were decreased to 30–43% of their preadaptation values

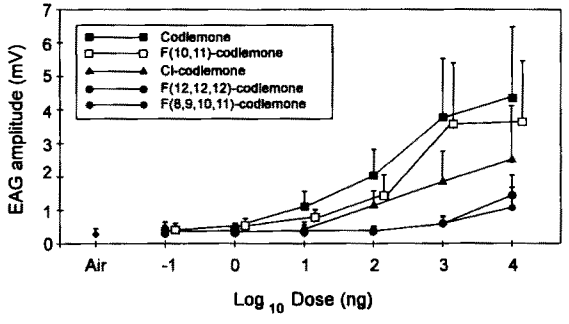

FIG. 3. Average EAG responses to stimulations with graded doses of codlemone, F(10,11)-codlemone, Cl-codlemone, F(12,12,12)-codlemone, and F(8,9,10,11)-codlemone. Each curve was obtained from six males. Error bars indicate confidence intervals.

(Table 1). The adaptation level resulting from repetitive stimulations by the two halogenated analogs was found to be weaker. Responses were between 43 and 50% after adaptation by F(10,11)-codlemone and between 49 and 68% after adaptation by Cl-codlemone (Table 1). The cross-adaptation between these three compounds was in favor of their detection by the same cell type but had to be confirmed by single sensillum recordings.

Single Cell Activity. Spontaneous activities were analyzed on five sensilla trichodea. They clearly revealed the presence of only one class of action poten-

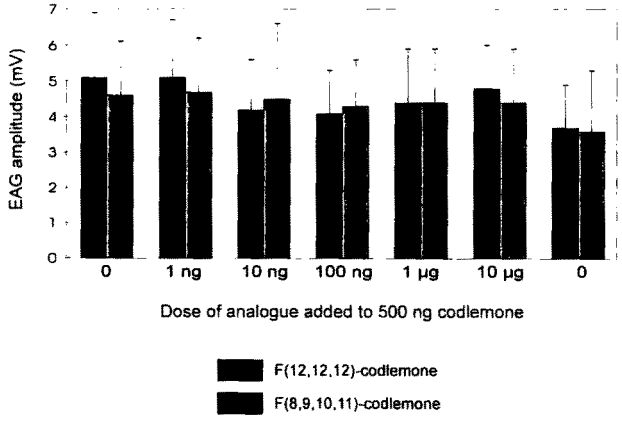

FIG. 4. EAG responses to codlemone alone or in mixture with increasing amounts of F(12,12,12)-codlemone (N = 7) or F(8,9,10,11)-codlemone (N = 8). Error bars indicate standard deviation.

TABLE 1. RESULTS OF CROSS-ADAPTATION EXPERIMENTS BETWEEN CODLEMONE, F(10,11)-CODLEMONE, AND Cl-CODLEMONE[a]

	Postadaptation responses recorded after adaptation by:		
	Codlemone (20 µg)	F(10,11)-codlemone (20 µg)	Cl-codlemone (20 µg)
Codlemone (1 µg)	30.9 ± 11.9	50.4 ± 21.5	68.7 ± 20.6
F(10,11)-codlemone (1 µg)	36.9 ± 19.4	39.5 ± 15.6	49.9 ± 29.6
Cl-codlemone (1 µg)	43.0 ± 22.8	43.3 ± 14.4	57.2 ± 20.9

[a] The adaptation consisted in repetitive stimulations by 20 µg of the adaptation compound. For each male, postadaptation responses were expressed as the percentage of the response recorded to the same stimulus before adaptation. Each value represents the average of ± standard deviation postadaptation responses of six males.

tial amplitude in three sensilla. Their mean frequency amounted to 4.4 ± 3.9 spikes/sec. Smaller impulses were recorded from the other two sensilla and could correspond to the activity of other receptor cells cited by Preiss and Priesner (1988). The frequency of discharge of small action potentials was never increased by olfactory stimulations.

Codlemone, its fluorinated analogs and Cl-codlemone were screened at the dose of 1 μg on five sensilla. Responses were reproducible from one sensilla to the other (Figures 5 and 6). All sensilla were very responsive to codlemone, F(10,11)-codlemone, and to a lesser extent to Cl-codlemone. Stimulations with codlemone, F(10,11)-codlemone, and Cl-codlemone activated the emission of high-amplitude action potentials. The order of activity of the compounds tested paralleled their EAG activity.

Field Trapping. The attractivity of codlemone and four analogs, F(8,9)-codlemone, F(10,11)-codlemone, F(8,9,10,11)-codlemone and Cl-codlemone, was evaluated in field trapping experiments (Table 2). The position of the traps and the age of the pheromone dispensers were not found to interfere with the level of trapping obtained with each component (three-ways ANOVA, Newman-Keuls test, $P < 0.05$). Attractivities of the five different formulations were significantly different. Traps baited with Cl-codlemone and F(10,11)-codlemone caught, respectively, 73% and 43% more males in the field than codlemone. In return, very few males were caught with F(8,9,10,11)-codlemone.

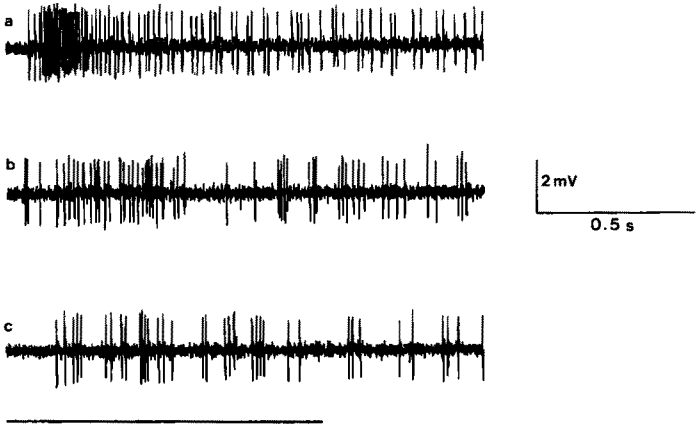

FIG. 5. Samples of responses of a sensillum trichodeum to 1 μg of codlemone (a), 1 μg of F10,11-codlemone (b) and 1 μg of Cl-codlemone (c). The bar below the recordings indicates the duration of the olfactory stimulation (1 sec).

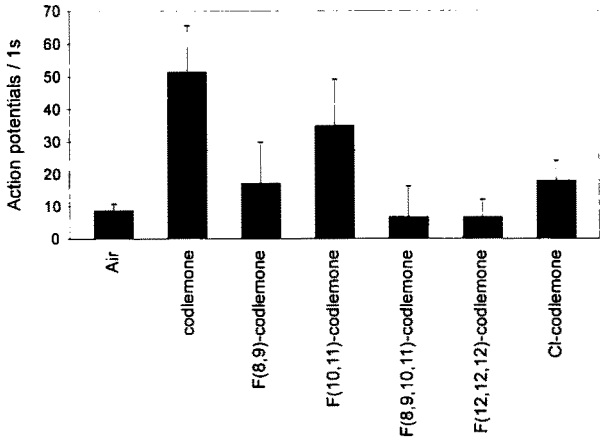

FIG. 6. Mean response profile of receptor neurons present in five sensilla trichodea to codlemone, its fluorinated analogs and Cl-codlemone [$N = 4$ for F(8,9)-codlemone, F(8,9,10,11)-codlemone, and F(12,12,12)-codlemone]. All compounds were tested at a dose of 1 μg with pure air as a control. Error bars indicate standard deviation.

TABLE 2. MEAN NUMBER OF MALE CODLING MOTHS CAPTURED PER TRAP DURING THE COMPLETE TRAPPING SEASON

Cl-codlemone	143	a
F(10,11)-codlemone	118.5	b
Codlemone	82.5	c
F(8,9)-codlemone	69.5	d
F(8,9,10,11)-codlemone	15.5	e

[a] Mean of two replicates with a total of 48 readings per trap. Means were associated with different letters, indicating that the five different formulations are significantly different (three-way ANOVA, Newman-Keuls test, $P < 0.05$).

DISCUSSION

Among a series of 11 monochloro, di-, tri-, and tetrafluoro analogs of codlemone, four compounds had a significant EAG activity on male codling moths: F(10,11)-codlemone, Cl-codlemone, F(8,9)-codlemone, and Cl-*E*8,*E*10-11:Ac. The dienic system is critical to the activity of codlemone as shown by the reduction or loss of EAG activity after saturation of one or the two double bonds. However, reduction of the C10–11 double bond caused a complete loss of activity, as shown by the absence of response to *E*8–12:OH, whereas *E*10–12:OH was still active. In a wind tunnel, *E*8–12:OH is almost ineffective to

attract males, while *E*10–12:OH shows a weak attractivity (Preiss and Priesner, 1988). Fluorination of the carbon atoms of the double bonds had various effects on the electrophysiological activity. The EAG and SSR activities of codlemone and F(10,11)-codlemone were similar, whereas F(8,9)-codlemone elicited very weak responses. The complete fluorination of the dienic system, F(8,9,10,11)-codlemone, caused a complete loss of electrophysiological activity.

Trifluorination of the terminal CH_3 group abolished the activity of the compound. Studies carried out on other species demonstrate that the biological activity of pheromone analogs is negatively correlated with the number of replacements of hydrogens by fluorine atoms in the terminal methyl group. The replacement of the terminal CH_3 group by a CH_2F group slightly reduces the EAG activity of *E*11–14:Ald in *Choristoneura occidentalis* (McLean et al., 1989) and has no effect on the behavioral activity of *E*11–14:Ac and *Z*11–14:Ac in *Ostrinia nubilalis* (Schwarz et al., 1990). *Z*9–12:Ac and its difluoro analog are equipotent on the behavior of *Eupoecilia ambiguella*, while the trifluoro analog does not mimic *Z*9–12:Ac but attracts males of species, which normally respond to a *Z*9–14:Ac-containing sex pheromone (Bengtsson et al., 1990). The trifluoromethyl analog of *Z*5–10:Ac in *Agrotis segetum* is about 100-fold less active in SSR than the natural *Z*5–10:Ac (Wu et al., 1993). In *Cydia medicaginis*, the EAG activity decreases with the number of fluorine atoms introduced in the terminal methyl group of *E*8,*E*10–12:Ac (Svatos et al., 1990). The loss of electrophysiological activity of F(12,12,12)-codlemone cannot be due to a reduction of its absorption and transport in vivo since the CF_3 group is among the most lipophilic substituents (Filler, 1986). No competitive inhibition with codlemone was observed from F(12,12,12)-codlemone. Thus, the absence of activity of this compound is probably due to its reduced binding to the receptor sites resulting from the low affinity of fluorinated hydrocarbons for the receptor sites as proposed by Prestwich et al. (1990).

The replacement of the terminal methyl group by a chlorine atom did not abolish the activity of the analogs as was the case after the introduction of three fluorine atoms. Cl-codlemone was between 10 and 100 times less active than codlemone but a part of this difference of activity may be explained by the difference of volatility between codlemone and its chlorinated analog. In the same way, the acetate analog of Cl-codlemone (Cl-*E*8,*E*10–11:Ac) partly conserved the EAG activity of the acetate analog of codlemone (*E*8,*E*10–12:Ac), a compound known to inhibit the attractivity of codlemone in field trapping (Einhorn et al., 1986). The replacement of a CH_3 by a chlorine atom does not have important steric consequences, but the methyl group has a higher hydrophobicity than the chlorine atom (Jönsson et al., 1991). This reduction of hydrophobicity may account for the reduced electrophysiological activity of Cl-codlemone. Response to other chloro-analogs, which deviated more from the natural pheromone structure, were not found to be significant.

EAG cross-adaptation experiments and SSR revealed that F(10,11)-codlemone and Cl-codlemone were detected by the same receptor cell type as codlemone. The higher levels of male catches with Cl-codlemone and F(10,11)-codlemone than with codlemone could not be expected on the basis of the relative electrophysiological activities of these three compounds. Such a shift between electrophysiological and behavioral activities has already been noticed on *Agrotis segetum* with fluoro-pheromones (Wu et al., 1993). The volatility of fluorinated compounds increases with the degree of fluorination (Prestwich et al., 1990; Wu et al., 1993). However, volatility ratios between di-, tri-, and tetrafluoro analogs and their parent molecules, Z5–10:Ac and Z9–12:Ac, are less than 2 (Bengtsson et al., 1990; Wu et al., 1993). Moreover, the estimation of relative volatilities of codlemone and its halogenated analogs by the calculation of their Kovats indices [codlemone = 1552, F(10,11)-codlemone = 1567, F(8,9)-codlemone = 1555, F(8,9,10,11)-codlemone = 1510, F(12,12,12)-codlemone = 1485 and Cl-codlemone = 1678] shows that codlemone and F(10,11)-codlemone have very similar volatilities. Thus, the higher attractivity of F(10,11)-codlemone in the field cannot be attributed to differences in volatilities between these two compounds. Pheromone compounds bearing a conjugated diene system such as codlemone are not stable in the field (Ideses and Shani, 1988). In general, conjugated double bonds are both photoisomerized to the equilibrium of four possible geometric isomers and oxidized to give endoperoxides that are then converted to furans. The fluorination of the diene system prevents the oxidation of the conjugated double bonds (Dubuffet et al., 1988) and probably also reduces its isomerization, providing a higher chemical stability to the compound.

In contrast to F(10,11)-codlemone, the replacement of the terminal CH_3 by a chlorine atom apparently did not stabilize the molecule since this compound was not found to be stable at room temperature leading to polymers. Moreover, the volatility of Cl-codlemone is inferior to that of codlemone (Kovats indices = 1678 for Cl-codlemone vs 1552 for codlemone). The substitution of the terminal methyl group by a chlorine atom in Z5–12:Ac reduces its volatility by a factor of 5.2 (Jönsson et al., 1991). Other physical properties of Cl-codlemone are probably involved, contributing to its strong biological activity in the field.

The higher activity in the field of Cl-codlemone and F(10,11)-codlemone than that of codlemone, the natural pheromone compound, will have to be confirmed by other tests. Moreover, new behavioral experiments will be necessary to explain the differences in the order of electrophysiological and field activities of these compounds. If their field activity is confirmed, Cl-codlemone and F(10,11)-codlemone could be used as new agents for pest control. This constitutes an attractive approach towards the development of new pest-control agents.

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AGGREGATION PHEROMONE OF PALMETTO WEEVIL, *Rhynchophorus cruentatus* (F.) (COLEOPTERA: CURCULIONIDAE)

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Abstract—5-Methyl-4-octanol is the major aggregation pheromone of the palmetto weevil, *Rhynchophorus cruentatus* (F.). The pheromone (cruentol) was identified by coupled gas chromatographic–electroantennographic (GC-EAD) analysis of male-produced volatiles, coupled GC-mass spectrometry (MS) in electron impact and chemical ionization mode, and coupled GC-high resolution MS. In laboratory and field assays, a diastereomeric mixture of synthetic cruentol greatly enhanced attraction of weevils to cabbage palmetto, *Sabal palmetto* (Walter), stem tissue, indicating that cruentol and host volatiles are synergistically attractive. An attractive lure in combination with efficient traps should facilitate development of semiochemical-based management for *R. cruentatus*.

Key Words—Coleoptera, Curculionidae, *Rhynchophorus cruentatus*, palmetto weevil, *S. palmetto*, aggregation pheromone, 5-methyl-4-octanol, cruentol.

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INTRODUCTION

Rhynchophorus cruentatus (F.), the palmetto weevil, is the only species of palm weevils found in the continental United States (Wattanapongsiri, 1966). This large (24- to 33-mm-long) beetle ranges from the Florida Keys through the coastal regions of South Carolina and Texas (Wattanapongsiri, 1966). Unlike several of its congeners, *R. cruentatus* is not considered a major pest of palms. However, this species will attack transplanted or otherwise stressed ornamental palms (Giblin-Davis and Howard, 1988, 1989). In Florida, *R. cruentatus* is sympatric with the native cabbage palmetto, *Sabal palmetto* (Walter) (Woodruff, 1967), a palm often used as mature specimens in landscaping due to its low cost, natural abundance, and high transplanting survivorship.

Volatiles emanating from moribund palms are attractive to *R. cruentatus* males and females (Chittenden, 1902; Wattanapongsiri, 1966; Weissling et al., 1992). Females lay eggs in leaf bases or directly in the wounds of dying host palms, and immature stages develop in the crown and stem region. Recent studies suggest that *R. cruentatus* males produce an aggregation pheromone that plays a substantial role in weevil colonization of susceptible trees (Weissling et al., 1993). This paper addresses the identification and the electrophysiological and behavioral activity of the aggregation pheromone of *R. cruentatus*.

METHODS AND MATERIALS

Volatile Collections. Weevils were field-collected (Weissling, et al., 1992) or laboratory-reared (Giblin-Davis et al., 1989) and maintained on *S. palmetto* tissue until three days before volatile collections. Groups of 30–40 weevils, separated by sex, were placed in modified 9-liter Nalgene desiccators with or without sugarcane (500 g). A charcoal-filtered airstream (1.5 cm^3/min) was maintained through the desiccators for seven days, collecting insect and plant volatiles on 10 g of Porapak Q packed in Pyrex glass tubing (Oehlschlager et al., 1988, 1992). Volatiles were eluted from the Porapak Q with distilled pentane and concentrated by distillation.

Instrumental Methods. Porapak Q extracts were analyzed by gas chromatographic–electroantennographic detection (GC-EAD) (Arn et al., 1975), using a Hewlett Packard (HP) 5885B equipped with a DB-5-coated, fused silica column (30 m × 0.25 mm ID) (J&W Scientific, Folsom, California) (linear flow velocity: 35 cm/sec; injector and detector temperature: 220°C; temperature programming: 70°C (1 min), 10°C/min to 240°C). A coupled HP 5985B GC-mass spectrometer (MS) equipped with a SP-1000-coated, fused silica column (30 m × 0.25 mm ID) (Supelco Inc., Bellefonte, Pennsylvania) was used for GC-MS in both electron impact (EI) (70 eV) and chemical ionization (CI) mode. A Kratos MS80RFA fitted with a DB-5-coated, fused silica column (30 m ×

0.25 mm ID) (J&W Scientific) was used for coupled GC-high resolution MS in EI mode.

Pheromone Synthesis. Racemic 5-methyl-4-octanol (Pinazzi et al., 1976, 1977) was synthesized by reacting butanal at 0°C in diethyl ether with the Grignard reagent of 2-bromopentane to give the corresponding alcohol as a diastereoisomeric mixture. Analytical data were as follows: MS (EI), *m/z* (percent relative abundance): 101 (13), 83 (28), 73 (100), 55 (83), 43 (34); ^{1}H NMR (relative to TMS): δ 0.95 (9H, m); δ 1.23 (4H, m); δ 1.45 (4H, m); δ 1.5 (1H, br s, D_2O exchangeable); δ 3.48 (1H, m). ^{13}C NMR [diaspereoisomeric] ($CDCl_3$, ppm); 75.91, 75.04, 38.70, 38.07, 36.81, 35.73, 34.26, 20.54, 20.51, 19.51, 19.37, 15.33, 14.23, 14.38, 14.21, 13.65; analytical calculation for $C_9H_{20}O$: C, 74.92; H, 13.98; found: C, 74.71; H, 13.96. The alcohol was purified by flash chromatography (SiO_2, 60% hexanes/diethylether) to afford 5-methyl-4-octanol (90% yield) as a colorless liquid (98% purity).

Laboratory Assay. Weevil response to synthetic pheromone and host-palm volatiles was tested in a binary choice, Y-tube olfactometer (Weissling et al., 1993) with a humidified airstream at 250 ml/min (27°C). For each experiment, 60–100 weevils of each sex were individually tested for 5 min. A positive response was recorded if a weevil walked at least 3 cm into one arm of the Y-tube. Experiments 1–3 tested pheromone released at three rates versus humidified clean air. For the medium (120 $\pm$ 10.6 μg/hr) and high (1300 $\pm$ 194 μg/hr) release rates, one and four 40-μl hematocrit tubes (Fisher Scientific, Pittsburgh, Pennsylvania), respectively, loaded with 10 μl of pheromone were placed in 300-μl plastic centrifuge tubes with two 3-mm-diam. holes drilled 5 mm below the sealed top. For the low release rate (0.18 $\pm$0.02 μg/hr), one 5-μl microcap tube loaded with 5 μl of pheromone was placed in a 300-μl centrifuge tube. Release rates for the high and medium treatments were determined gravimetrically, whereas release of the low rate treatment was determined volumetrically ($N = 6$). Experiments 4 and 5 tested pheromone (1300 μg/hr) alone and pheromone combined with *S. palmetto* tissue (50 g, 24–72 hr old) versus palm tissue. A final experiment tested pheromone (1300 μg/hr) combined with palm versus pheromone alone.

Field Experiments. Four field experiments were conducted in a 300-ha pasture interspersed with *S. palmetto* and saw palmetto, *Serenoa repens* (Bartr.) 12 km south of La Belle, Florida, using complete randomized blocks with traps at 20-m intervals and blocks 300 m apart. Two types of traps were used. "Live traps" were constructed from 19-liter black plastic buckets covered with tops made from polyvinyl chloride tubes (5 cm long, 2.4 cm inside diameter) glued together longitudinally (Weissling et al., 1992). These traps allow weevils in but prevent escape and provide harborage for sustenance. "Lethal traps" (Weissling et al., 1993), similar in exterior appearance to live traps, were also used. However, weevils entering these traps cannot escape and drown in soapy

water. In experiments 1 and 2, live traps were suspended 1.5 m above the ground from *S. palmetto* trees, whereas in experiments 3 and 4, lethal traps were placed on the ground. Ten microliters of synthetic pheromone loaded into 40-μl capillary tubes were placed four per 300-μl microcentrifuge tube as described above. Unless otherwise indicated, this served as the standard release device for all tests. Centrifuge tubes were suspended with a copper wire inside traps 10 cm from the top. Release rates of pheromone were determined gravimetrically ($N = 4$) at 24-hr intervals for seven days.

The first four-treatment, four-replicate experiment tested an unbaited trap (control), the pheromone alone, fresh *S. palmetto* bud and distal stem tissue (2.5 kg) alone, and pheromone and palm combined. All traps without palm tissue in this and subsequent experiments contained four water-moistened sponges. Tests were conducted from March 24 to April 21, 1992. Captured weevils were removed from traps weekly, and clean traps were baited and rerandomized within each block. To determine if the weevil population in the study site was too low for extended testing (indicated by high recapture of the same insects), captured weevils were marked by scratching a number on the metathoracic sternal plate with a dissecting probe and were released in the middle of the study site.

The second experiment compared pheromone released from the standard device versus pheromone released at 10 times this rate. For the high release rate, 80 μl of pheromone was added to 300 μl microcentrifuge tubes packed with cotton. Each release rate of pheromone was tested alone and combined with 2.5 kg *S. palmetto* tissue. Traps were placed in the field as described above (five blocks) from April 20 to 27, 1992.

In the third, five-replicate experiment, the influence of semiochemicals released by trapped weevils on other weevils was removed by using lethal traps. Treatments were identical to those in the first experiment. Tests were conducted from June 5 to 12, and 12 to 19, 1992. Traps were cleaned, rebaited, and rerandomized between test periods.

In the fourth, four-replicate experiment, lethal traps were baited with live *R. cruentatus* males to determine if male-produced volatiles enhanced attraction to synthetic pheromone. Treatments included traps baited with 1.5 kg of palm tissue plus 10 *R. cruentatus* males, 1.5 kg of palm tissue plus pheromone, and 1.5 kg palm tissue plus both 10 *R. cruentatus* males and pheromone. Tests were conducted from July 2 to 9, and 9 to 16, 1992.

Statistical Analyses: Data from laboratory assays were converted to percent attractancy [(number of insects entering a sample arm/total number responding) $\times$ 100], and analyzed by the Kruskal-Wallis test (SAS Institute, 1985). Data from field tests were square root transformed ($x + 0.01$) and analyzed by analysis of variance using PROC ANOVA (SAS Institute, 1985). Treatment effects were estimated both over time and within time periods. Least significant differ-

ence tests (SAS Institute, 1985) were used for mean separation where significant ($P < 0.05$) statistical differences occurred.

RESULTS

Volatile Collections. Analysis (GC-EAD) of volatiles from fed and unfed male weevils revealed a strongly EAD-active compound (Figure 1). Mass spectra of the candidate pheromone (Figure 2) suggested a methyl-branched, secondary alcohol with a molecular weight of 144 (M − 1 in the CI spectrum of Figure 2). The high-resolution mass spectrum revealed that fragment *m/z* 73 contained one oxygen, indicating that the hydroxyl group was bonded to either C-2, C-3, or C-4. Based on mass spectrometric data and retention index cal-

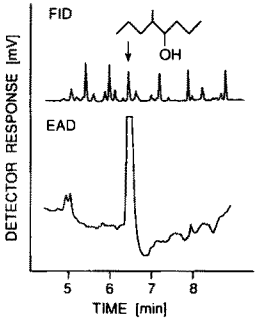

FIG. 1. Flame ionization detector (FID) and electroantennographic detector (EAD) responses to volatiles collected for seven days from unfed *R. cruentatus* males. The antennal recording (EAD) was carried out with a *R. cruentatus* male antenna. Gas chromatographic conditions: linear flow velocity: 35 cm/sec, injector and detector temperatures: 220°C, temperature programming: 70°C (1 min), 10°C/min to 240°C; DB-5 column (30 m × 0.25 mm ID).

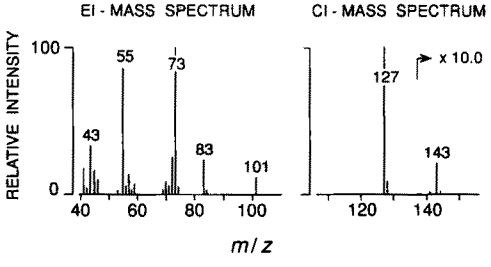

FIG. 2. Mass spectra of 5-methyl-4-octanol.

culations with authentic methyl-branched secondary octanols and nonanols, we hypothesized that the candidate pheromone was 5-methyl-4-octanol. Identical mass spectra, identical Kovats retention indices on two columns with different retention characteristics (DB-5: 1060, DC-23: 1388) and similar GC-EAD responses to equivalent amounts of synthetic 5-methyl-4-octanol and the male-produced compound confirmed the structural assignment.

Laboratory Assay. More male and female *R. cruentatus* were attracted to synthetic 5-methyl-4-octanol (all rates) than to the clean air control (Figure 3, experiments 1–3). Attraction of males to pheromone (1300 μg/hr) exceeded that to palm tissue (Figure 3, experiment 4). The combination of pheromone (1300 μg/hr) and palm tissue was more attractive to males and females than palm alone and more attractive to females than pheromone alone (Figure 3, experiments 5 and 6).

Field Experiments. Racemic 5-methyl-4-octanol was released from the standard device at the rate of 0.41 ± 0.03 mg/day (range: 0.2–0.9 mg/day). Marked weevils comprised 1.5% of total capture (N = 852 weevils) in live traps from March 24 to April 27, 1992, indicating a large population in the study site area.

Treatment effects in experiment 1 were estimated separately for each collection date because of a significant treatment × date interaction (male: F = 5.8; df = 9, 25; $P < 0.01$; females: F = 3.6; df = 9, 25; $P < 0.01$). 5-Methyl-4-octanol was effective at increasing capture of *R. cruentatus* males and females in live traps baited with host-palm tissue (Figure 4). More weevils were caught

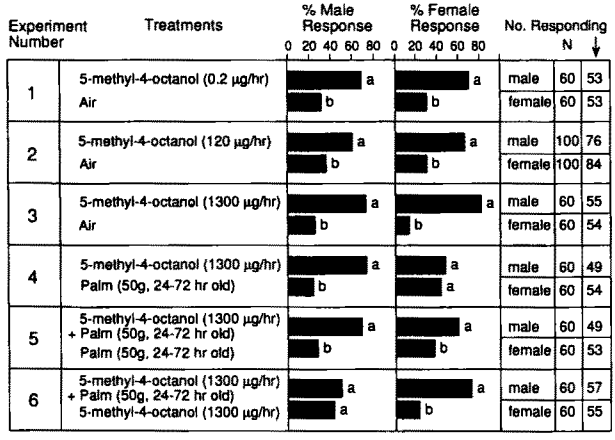

FIG. 3. Response of individual male and female *R. cruentatus* to various Y-tube olfactometer treatments. Weevil responses within a gender, followed by the same letter are not significantly different ($P < 0.05$; Kruskal-Wallis test).

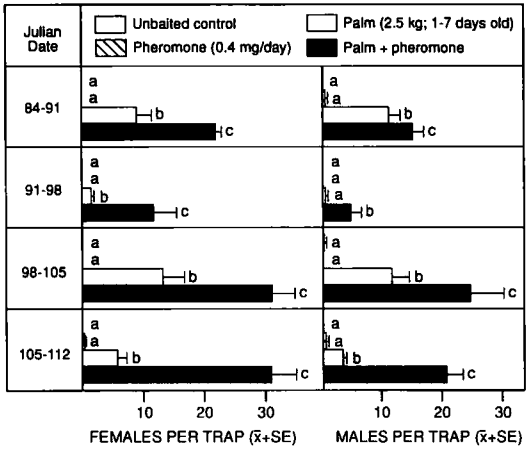

FIG. 4. Mean (+SEM) capture of *R. cruentatus* in live traps baited with *S. palmetto* tissue, (±)-5-methyl-4-octanol, or both combined, La Belle, Florida, 1992. Means within a sex and julian date grouping followed by the same letter are not significantly different ($P < 0.05$; least significant difference).

in traps baited with palm and pheromone than with any other treatment each week. In addition, more weevils were caught in traps baited with palm tissue than in unbaited traps or in traps baited with pheromone during weeks 1, 3, and 4 (Figure 4).

Low (0.4 mg/day) and high (4 mg/day) release of pheromone equally enhanced attraction of weevils to palm tissue (means ± SEM, males; low rate pheromone: 0 ± 0, high rate pheromone: 0 ± 0; palm plus low rate pheromone: 23.0 ± 8.3, palm plus high rate pheromone: 13.0 ± 1.0; females, low rate pheromone: 0.3 ± 0.3, females, high rate pheromone: 0.3 ± 0.3; palm plus low rate pheromone: 33.5 ± 11.8; palm plus high rate pheromone: 22.0 ± 4.0). Significantly more weevils were caught in traps baited with palm tissue and pheromone than in traps baited with pheromone alone (males: $F = 29.7$; $df = 3, 9$; $P < 0.01$, females: $F = 26.6$; $df = 3, 9$; $P < 0.01$).

Weevils captured in lethal traps were killed before contributing to trap attraction. Ten to 15 times more weevils were captured in traps baited with palm plus pheromone than with any of the other treatments (Figure 5). In addition, more females were caught in traps baited with pheromone than in unbaited control traps (Figure 5).

The addition of live conspecific males to lethal traps baited with palm tissue and pheromone did not enhance weevil capture over traps baited only with palm plus pheromone. More males and females were caught in traps baited with palm

tissue plus both 10 conspecific males and pheromone than in traps baited with palm plus 10 males (Figure 6). Additionally, more weevils were caught in traps baited with palm plus pheromone than in traps baited with palm plus 10 males (Figure 6).

DISCUSSION

Following the demonstration of a male-produced aggregation pheromone in *R. cruentatus* (Weissling et al., 1993), we have identified the compound as 5-methyl-4-octanol, and propose the trivial name "cruentol." Cruentol and palm

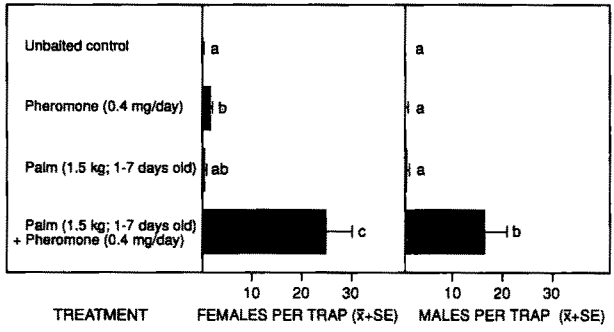

FIG. 5. Mean (+SEM) capture of *R. cruentatus* in lethal traps baited with *S. palmetto* tissue, (±)-5-methyl-4-octanol, or both combined, La Belle, Florida, June 5–19, 1992. Means within a sex followed by the same letter are not significantly different ($P < 0.05$; least significant difference), treatment effects; males: $F = 25.9$; $df = 3, 12$; $P < 0.01$; females: $F = 38.0$; $df = 3, 12$; $P < 0.01$.

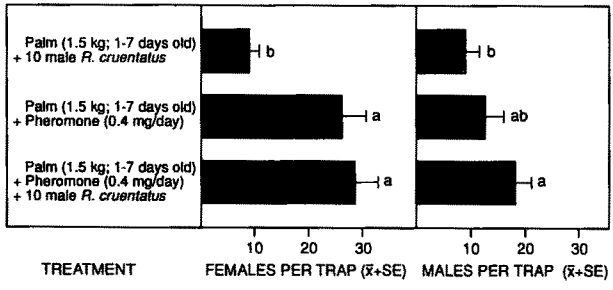

FIG. 6. Mean (+SEM) capture of *R. cruentatus* in lethal traps baited with *S. palmetto* tissue in combination with either (±)-5-methyl-4-octanol, 10 *R. cruentatus* males, or both, La Belle, Florida, July 2–16, 1992. Means within a sex followed by the same letter are not significantly different ($P < 0.05$; least significant difference), treatment effects; males: $F = 6.0$; $df = 2, 6$; $P < 0.04$; females: $F = 6.9$; $df = 2, 6$; $P < 0.03$.

tissue together were as attractive as cruentol, palm, and 10 conspecific males together (Figure 6), suggesting that cruentol is the only pheromone essential for optimal attraction.

Male-produced pheromones have been identified from the curculionid genera *Pissodes* (Booth et al., 1983; Phillips et al., 1984), *Sitophilus* (Phillips et al., 1985; Schmuff et al., 1984), *Sitona* (Blight et al., 1984), *Metamasius* (Perez et al., 1994), and *Rhynchophorus*. In the Rhynchophorinae, aggregation pheromones recently have been identified from the American palm weevil, *R. palmarum* (L.) (Rochat et al., 1991; Oehlschlager et al., 1992), the African palm weevil, *R. phoenicis* (F.) (Gries et al., 1993), and the Asian palm weevils, *R. vulneratus* (Panz.), *R. ferrugineus* (Oliv.) (Hallett et al., 1993), and *R. bilineatus* (Mont.) (Oehlschlager et al., 1994). Unlike their Asian congeners, which produce methyl-branched, secondary nonanols, and unlike *R. palmarum*, which produces a methyl-branched heptenol, *R. cruentatus* and *R. phoenicis* produce methyl-branched, secondary octanols as aggregation pheromones.

In olfactometer tests, cruentol at all release rates was more attractive to walking weevils than the clean air control. It was also more attractive to males than palm tissue (Figure 3). In field tests, however, very few weevils were caught in traps baited only with cruentol, even when cruentol was released at 4 mg/day (experiment 2). We have demonstrated that neither palm tissue or cruentol are efficient trap baits when used alone in lethal traps and that the palm tissue and cruentol grouping is very synergistic (Figure 5). As cruentol by itself did not attract weevils into traps but into the general study area (T.J.W. and R.M.G.-D., personal observation), we hypothesize that the pheromone functions mainly as a long-range attractant, whereas host-plant volatiles are required for weevils to orient to and enter traps. Synergistic combinations of plant- and weevil-produced volatiles have also been demonstrated in *Pissodes nemorensis* Germar (= *P. approximateus*, synonymized by Phillips et al., 1987) (Booth et al., 1983), *Sitona lineatus* L. (Blight and Wadhams, 1987), *R. palmarum* (Oehlschlager et al., 1992), *R. phoenicis* (Gries et al., 1993), *R. vulneratus* and *R. ferrugineous* (Hallett et al., 1993), and *R. bilineatus* (Oehlschlager et al., 1994).

The identification of cruentol as a male-produced aggregation pheromone in *R. cruentatus* is an important step towards understanding the biology and chemical ecology of this insect and furthers the development of management programs. While a stereoisomeric mixture of synthetic cruentol combined with palm tissue attracted large numbers of weevils, optimal attraction may require only one stereoisomer in combination with synergistic host volatiles. In addition, several early fermentation products of palm sap found to be attractive with cruentol (Giblin-Davis et al., 1994) can be used in place of palm tissue to further simplify trapping.

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INHIBITION OF POTATO CYST NEMATODE HATCH BY LIGNANS FROM *Bupleurum salicifolium* (UNBELLIFERAE).

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Abstract—A series of lignans from *Bupleurum salicifolium* Soland (Umbelliferae) were tested for nematostatic activity on the cysts and freed second-stage juveniles of the potato cyst nematodes *Globodera rostochiensis* and *G. pallida*. None of the six lignans tested—bursehernin, matairesinol, syringaresinol, the novel product buplerol, guayarol, and a derivative, nortrachelogenin triacetate—showed nematicidal activity in an in vitro analysis with second-stage juveniles, but significant differences were noted when the lignans were assayed for nematostatic activity as cyst hatching inhibitors. Bursehernin and matairesinol showed the greatest activity, at concentrations of 50 ppm. This is the first known instance of a natural product inhibiting the hatch of the nematode *G. pallida*. The HID (hatching inhibiting dose) of bursehernin was estimated, and some conclusions were drawn about the structure-activity relationships of the lignans under study.

Key Words—Potato cyst nematodes, *Globodera rostochiensis*, *G. pallida*, hatching inhibitors, lignans; Umbelliferae, *Bupleurum salicifolium*.

INTRODUCTION

Globodera rostochiensis and *G. pallida*, two cyst-forming nematodes, are major potato pests (Winslow and Willis, 1972). They are highly specialized pathogens characterized by a narrow host range in the solanaceous plants (including potatoes, tomatoes, aubergines, and tobacco). The potato cyst nematodes have co-

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evolved with their major host, the potato, in their original habitat, the American Andes (Stone, 1985), where they show broad genetic heterogeneity. Successful survival strategies by this parasite involve mechanisms that permit the activation of the infective unit only when the probability of finding a suitable host is high. Eggs from *Globodera* spp. can remain viable for up to 20 years in the soil (Winslow and Willis, 1972) and are induced to hatch by exudates from the potato roots (Atkinson et al., 1987).

The discovery of molecules regulating some stage of the life-cycle of the parasite could provide control strategies other than the search for resistant phenotypes, which is expensive and short-term (given the high variability of the populations even within Europe), and the use of nematicides, which are among the least environmentally acceptable of all the crop-protection chemicals in use. These substances may also be used for research into the processes of hatching and diapause of cyst nematodes (Forrest and Farrer, 1983).

Triffitt (1930) and Ellenby (1945) found that mustard root diffusate had a neutralizing effect on the hatching of *G. rostochiensis*, and Mägi (1970) reported that root diffusates from several Umbelliferae (*Archangelica litoralis*, *Heracleum sibiricum*, *Anthriscus silvestris*, *Pimpinella saxifraga*, and *P. major*) inhibited *G. rostochiensis* hatch by 36–56% compared to controls. Asparagusic acid (1,2-dithiolane-4-carboxylic acid) from *Asparagus* spp. (Liliaceae) has been identified as a hatching inhibitor of the cysts of *G. rostochiensis* and *Heterodera glycines* at a dose of 50 ppm and 25°C (Takasugi et al., 1975).

When *G. rostochiensis* and the tomato brown root rot complex (gray sterile fungus when cultured, GSF) were both present in soil, it was observed that the population of nematode pathogens remained low (Graham, 1966). Glyn (1966) found that this fungus produced exudates in malt extract broth culture medium that significantly inhibited the hatch of *G. rostochiensis* (ca. 25% of the control). The same principle as McParland (1970) observed inhibiting the hatch of *G. rostochiensis* from exudates of the fungus *Rhizoctonia solani* is probably involved.

Bupleurum salicifolium is endemic to the western Canary Islands from Gran Canaria to El Hierro. It is frequently found on cliffs up to 1000 m above sea level (Bramwell and Bramwell, 1974). The plant is rich in shikimic pathway derivatives (lignans, coumarins, flavonols, etc.). Other species of the genus have been studied, and Muckensturm et al. (1982) recorded the biological activity of a phenylpropanoid isolated from *B. fruticosum* that proved to be a strong antifeedant for the insect *Mythimna unipunctata*.

METHODS AND MATERIALS

Extraction and Characterization of Lignans from B. salicifolium.

Leaves of wild specimens of *B. salicifolium* (3.2 kg) collected in the Barranco Rio Badajoz, Güimar (Tenerife) were extracted in a Soxhlet with EtOH. After reduction of the ethanol extract by distillation in vacuo, the extract was

treated with petroleum ether and benzene, leaving a dark semisolid residue (112.8 g), which was chromatographed repeatedly on silica gel and Sephadex LH-20, yielding the following products: the triterpene betulin (0.4 g); coumarins 6,7,8-trimethoxy coumarin (6 mg), herniarin (5 mg), and escopoletin (4 mg); a polyacetylene (150 mg); and the lignans bursehernin **(1)** (93 mg), matairesinol dimethyl ether (500 mg), buplerol **(5)** (43 mg), matairesinol **(2)** (47.4 mg), nortrachelogenin (31.8 mg), guayarol **(3)** (10 mg), and syringaresinol **(4)** (15 mg).

Buplerol and guayarol were described for the first time by González et al. (1990a). All the compounds isolated were fully characterized using spectroscopic techniques (UV, IR, ^{1}H NMR, ^{13}C NMR, HMBC, HMQC, and MS) (González et al., 1990b,c).

Nortrachelogenin triacetate **(6)** was obtained from nortrachelogenin in the usual way. The structures of the lignans are shown in Figure 1.

Selection of Lignans. To investigate the influence of the lactone system on hatching, a furofuranic type lignan, **4,** was chosen. Compound **6** was used to study the effect of hydroxy sterification of the aromatic group and the effect of different groups in the B aromatic ring was studied using lignans **1, 2, 3,** and **5.**

Namatodes. The potato cyst nematodes were physiologically similar, collected from potato fields on Tenerife and cultured in cultivar Désiré under glasshouse conditions (20°C and 16 hr daylight). On June 20, 1991, the cysts were

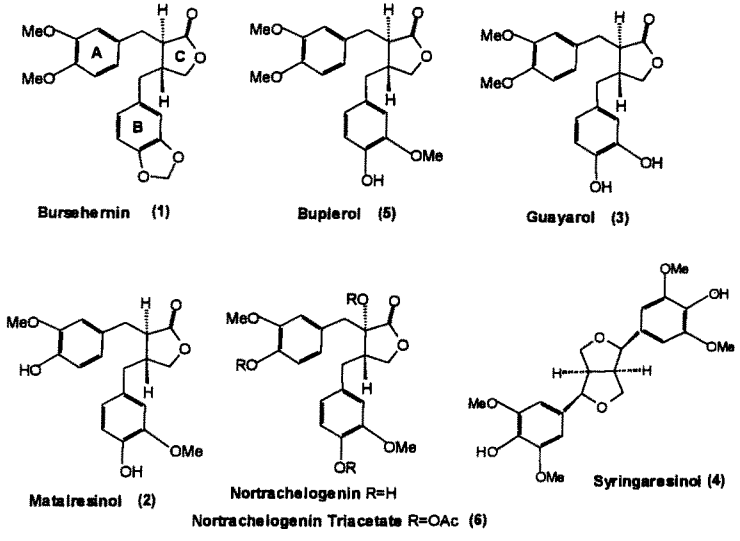

FIG. 1. Structures of the lignans studied.

recovered from the substrate (1:3 sand/loan mixture) using a Schuiling centrifuge (Hietbrink and Ritter, 1982) and stored in the dark at 4°C until used.

A 50:50 mixture of *G. pallida* pathotype Pa2/3 and *G. rostochiensis* pathotype Rol (Phillips and Trudgill, 1983) was used in all experiments. The cysts were not in their dormancy phase.

Bioassays for nematostatic Activity against Globodera *spp.* To test the nematicidal activity of the lignans, batches of up to 1000 cysts were soaked overnight in distilled water and induced to hatch using a 10 mM $ZnSO_4$ solution (Robinson and Neal, 1959; Clarke and Shepherd, 1966). Aliquots (100 μl) containing approximately 50 two-day-old juveniles (second-stage juveniles, J^2) were placed in a water solution containing 250 ppm of the lignan and appropriate controls were set. After 24 hr, percent mortality was recorded.

Nematostatic activity was investigated by studying the effect of the lignans on cyst hatching. Batches of 10 cysts were soaked in distilled water for two days, and the water was then replaced by the test solution containing 50 ppm of the lignans and 10 mM $ZnSO_4$ as hatching agent. The total volume of the assay was 2 cc.

Hatched juveniles were counted regularly, the cysts washed thoroughly with distilled water and the test solution replaced with fresh stock. Each treatment was applied to four replicates (except in the case of **4**, when only 2 replicates were available) and the controls, one (C1) with distilled water plus 0.5% EtOH and the other (C2) with the hatching agent plus 0.5% EtOH, the solvent used with the lignans.

The experimental design was a randomized complete block and the results were analyzed by a two-way ANOVA. To avoid zero values, one unit was added to all the replicates. After examination of the data, a log transformation was needed to normalize the data. The mean number of hatched juveniles at the end of the experiment was compared using Duncan's multiple range test at 5% significance level.

RESULTS AND DISCUSSION

Nematicidal Effect of Lignans. No nematicidal effect was apparent for doses of up to 250 ppm (Table 1).

Nematostatic Effect of Lignans. The chronological hatch of cysts influenced by the different lignans is shown in Figure 2. The analysis of variance showed highly significant differences ($P < 0.01$) between treatments, time, and the interaction treatments × time.

Duncan's multiple range test clustered the means in nonsignificant groups (Figure 3). After 14 days, all the treatments hatched more juveniles than were hatched in distilled water (C1). The number of juveniles hatched when lignans

TABLE 1. PERCENT MORTALITY OF TWO-DAY-OLD *Globodera* sp. SECOND STAGE JUVENILES AFTER 24-h EXPOSURE TO 250 ppm OF LIGNANS

Lignan added	Mortality (%)
None	30.6
Matairesinol (2)	20
Bursehemin (1)	23.1
Nortrachelogenin triacetate (6)	24.2
Guayarol (3)	25.9
Syringaresinol (4)	31.3
Buplerol (5)	34.5

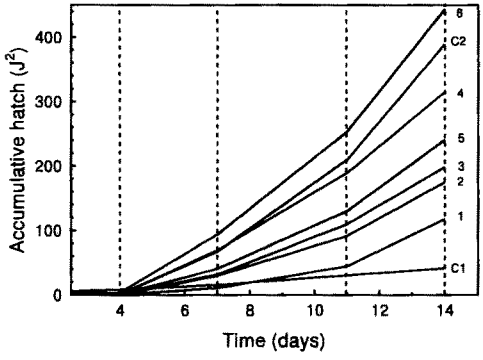

FIG. 2. Chronological hatch of potato cyst nematodes (50:50 *Globodera pallida–G. rostochiensis*) as influenced by lignans.

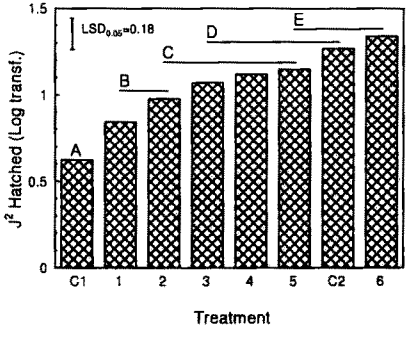

FIG. 3. Hatch (log transformation) of potato cyst nematodes clustered in nonsignificant groups by Duncan's multiple range test.

1 or **2** were present was significantly different from the control (C2) containing 10 mM $ZnSO_4$ as hatching agent, the hatch being reduced by 69.96% **(1)** and 55.14% **(2)**. The behavior of lignan **1** also differed significantly from that of molecules **3** and **5,** which have a similar structure.

The HID_{50} was estimated for lignan 1 using the best fitted curve ($y = -17.032 * \ln x + 97.668$) (Figure 4). Only 16.42 ppm of the lignan was needed to reduce hatch by 50% over a two week period.

Examination of the juveniles hatched during the experiment showed that there were no differences in the effect of lignans on *G. pallida* or *G. rostochiensis*, and it would seem that the inhibitory hatching activity of the lignans tested is nonspecific. This is the first time that a natural product has been shown to affect *G. pallida* hatching behavior.

Structure–Activity Relationship in Lignans. The presence of a methylene-dioxy group in the dibenzyl-butyrolactone skeleton aromatic ring B would seem to play a part in the nematostatic activity of the products tested. Lignans **3** and **5** have the same skeleton as **1** except in this ring where the methylene-dioxy group is replaced by a methoxy and a hydroxyl in **5** or two hydroxyls in **3**. These changes significantly reduced nematostatic activity.

In compounds with no methylene-dioxy group, activity increased according to the number of free hydroxy groups. Thus lignans **2** and **3,** which have two free OH groups, showed more nematostatic activity than **5,** which has only one, while **6,** which has no free OH, displayed the least nematostatic activity of all, significantly different from that of **2** and **3.**

Apart from the absence of free hydroxyls, lignan **6** also has a relatively voluminous acetate group at position 2 in the lactone ring, which may partly figure as a possible steric bulk between this compound and the hypothetical

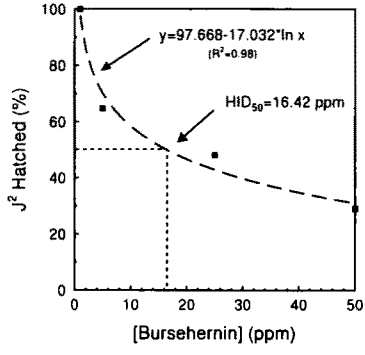

FIG. 4. Reduction in potato cyst nematode hatch by bursehernin and hatching inhibiting dose (HID_{50}).

receptor on the nematode eggshell suggested by Atkinson and Taylor (1980, 1983).

Four of the above lignans have also been isolated in considerable quantities from the ethanol root extracts of *B. salicifolium* (27.5 g): 4,5-matairesinol (**2**) (13.1 mg), matairesinol dimethyl ether (15 mg), bursehernin (**1**) (23 mg), and nortrachelogenin (6.3 mg) (Estévez-Reyes et al., 1992, 1993).

It is possible that some biogenetic compounds from the shikimic pathway, lignans or polyphenols with similar structures, were involved in the inhibitory effect recorded by Mägi (1970) when studying root extracts of umbelliferous plants as cyst nematode hatching inhibitors.

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Journal of Chemical Ecology, Vol. 20, No. 3, 1994

STRATOSPHERIC OZONE DEPLETION AND PLANT-INSECT INTERACTIONS: EFFECTS OF UVB RADIATION ON FOLIAGE QUALITY OF *Citrus jambhiri* FOR *Trichoplusia ni*

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Abstract—Projected decreases in stratospheric ozone may result in increases in shortwave ultraviolet (UVB) irradiation at the earth's surface. Furanocoumarins, phototoxic compounds found in *Citrus jambhiri* foliage, increase in concentration when these plants are grown under enhanced UVB. Survivorship schedules of *Trichoplusia ni* (Lepidoptera: Noctuidae) caterpillars reared on plants in the presence and absence of enhanced UVB regimes differ significantly; larvae develop more slowly in early life when reared on plants exposed to increased UVB. This same developmental pattern is observed when *T. ni* larvae are reared on artificial diets amended with ecologically appropriate amounts of furanocoumarins. Thus, anthropogenically derived changes in stratospheric ozone and concomitant changes in UV light quality at the earth's surface may influence ecological interactions between insects and their host plants by altering secondary metabolism and hence foliage quality for herbivores.

Key Words—*Citrus jambhiri*, *Trichoplusia ni*, Lepidoptera, Noctuidae, bergapten, furanocoumarins, phototoxins, plant-herbivore interactions, psoralen, ultraviolet-B radiation.

INTRODUCTION

Current evidence suggests that anthropogenically derived atmospheric inputs have caused, and will continue to cause, decreases in stratospheric ozone levels

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(International Ozone Trends Panel, 1988). The result of these decreases will be an increase in ultraviolet-B (UVB; 280–315 nm) radiation at the earth's surface (Blumthaler and Ambach, 1990, 1991). Increases in UVB have been shown to impair plant growth (Teramura, 1990), alter competitive interactions between plants (Barnes et al., 1988), and change levels of allelochemicals in plant tissues (McCloud et al., 1992; Larson et al., 1990; and see reviews in Caldwell et al., 1989; Berenbaum, 1988). While a recent study (Quaite et al., 1992) suggests that the direct effects of enhanced UVB on plant DNA may be less harmful than expected, effects on DNA may not be readily generalized to whole plants. Indeed, when whole plant effects are considered, the impact of UVB radiation varies widely both within and between species; there are both resistant and susceptible cultivars in crops such as soybeans and there are differences in UV sensitivity between plant species (Teramura, 1990). Effects of UVB radiation on plant growth have often proven difficult to demonstrate, yet alterations of secondary metabolism are well documented (Caldwell et al., 1989). Despite these findings, there is a remarkable paucity of studies investigating the effects of increased UVB on interactions between plants and their consumers (Panagopolous, 1992; Orth et al., 1990; Yazawa et al., 1992). Collectively, plants and their insect consumers represent approximately 50% of biotic diversity on the planet (Strong et al., 1984). Thus, effects of increased UVB radiation present the possibility for profound alterations of terrestrial ecosystems through effects on interactions between producers and consumers.

Citrus jambhiri (rough lemon) is a rootstock plant used in commercial citriculture. Rootstock selection can have a profound effect on the properties of the scion; also, large numbers of rootstock seedlings must be propagated for grafting. We chose citrus for our model system since few studies have examined the effects of increased UVB irradiation on tropical or semitropical plants. Because natural levels of UVB radiation are higher in the tropics, tropical plants may be less susceptible to UV damage, although, since UVB radiation is less variable in the tropics than in temperate regions (Bowman and Kreuger, 1985), these plants may nonetheless be susceptible to radiation levels at or above the contemporary extremes.

Another reason for selecting *Citrus jambhiri* was that we were interested in examining the effects of UVB not on growth and primary metabolism but rather on secondary metabolism and foliage quality for insect herbivores. Several classes of allelochemicals are ubiquitous in the genus *Citrus*: among these are monoterpene essential oils, the oxygenated triterpene limonoids, coumarins, and furanocoumarins (Waterman and Grundon, 1983; Murray et al., 1982). Furanocoumarins, phototoxic compounds found in the foliage of *C. jambhiri* and other rutaceous plants, are highly toxic to generalist insect herbivores and have been implicated in pathogen resistance in *Citrus* as well as other plants (Martin et al., 1966a,b; Pirone et al., 1960). *Trichoplusia ni* (Lepidoptera: Noctuidae),

the cabbage looper, is a generalist lepidopteran that includes *Citrus* among its hosts (Eichlin and Cunningham, 1978; Tietz, 1972). The susceptibility of *T. ni* to many types of phototoxic and nonphototoxic allelochemicals is documented (Lee, 1991; Ahmad and Pardini, 1990; Bosio et al., 1990, Lee and Berenbaum, 1989; Larson et al., 1988; Wadleigh and Yu, 1988; Prabhaker, 1986; Altieri et al., 1984); so far, limonoids have not been active against *T. ni* (Altieri et al., 1984), while furanocoumarins have proven highly toxic to this generalist herbivore. In *C. jambhiri* leaves, the major furanocoumarins that we have been able to detect are bergapten and psoralen (McCloud et al., 1992).

Here, we present the results of the first study to test directly the consequences of UVB enhancement on plant–insect interactions in a way that approximates stratospheric ozone depletion. In addition, we provide evidence that radiation in the UVB waveband can activate plant-derived photosensitizing agents, resulting in phototoxicity to insects. Although previous work has demonstrated that ultraviolet-A (UVA) radiation can potentiate furanocoumarins (Berenbaum, 1991; Trumble et al., 1991), this study represents the first evidence that UVB radiation applied in a fashion directly relevant to stratospheric ozone depletion can do so.

METHODS AND MATERIALS

We separated the components of this plant–insect interaction by exposing *C. jambhiri* to elevated UVB radiation in the absence of herbivores, by rearing *T. ni* on artificial diets amended with ecologically relevant concentrations of furanocoumarins, and, finally, by rearing *T. ni* to pupation on plants receiving increased UVB irradiation.

Plant Culture and UVB Irradiation. We examined furanocoumarin changes in *C. jambhiri* in the presence of increased UVB irradiation by growing rooted cuttings in the greenhouse under banks of fluorescent UVB bulbs. Cuttings taken from three mature *C. jambhiri* trees were rooted and allowed to grow six weeks before exposure to UVB. Ten cuttings of similar size and degree of apical dominance were selected from each of the three parental groups to yield three clones. Each clone of 10 cuttings was then randomly divided in half such that 15 potted cuttings (five individuals from each of three clones) were assigned to treatment and control groups.

To expose plants to UVB irradiation, we employed the standard irradiation protocols used in studied of simulated stratospheric ozone depletion (e.g., Middleton and Teramura, 1993; Mirecki and Teramura, 1984). Two banks of Westinghouse FS-72 fluorescent UV bulbs were suspended over each half of a 1.67 $\times$ 4.3 m greenhouse bench. Each rack was composed of 14 bulbs placed on 20-cm centers. Half of the lamps (one side of the bench) were wrapped with

type S Mylar film (Gar-Ron Plastics, Baltimore, Maryland) to block UVB. This film does not transmit UVB wavelengths (280–320 nm); its cutoff is near 315 nm. The remaining lamps were wrapped with cellulose acetate (Folex, Inc., Palmyra, New Jersey) film, which has a lower cutoff and allows UVB to pass. UVB bulbs were illuminated for 8 h/day, centered in the 14-hr photoperiod of the experiment. The plants were arranged in a 3 × 5 array under each bench half. Plants were periodically randomized within a bench half and bench sides were periodically switched to avoid position effects.

UVB dose was measured as biologically effective radiation normalized at 300 nm (BE_{300}, see Mirecki and Teramura, 1984; Warner and Caldwell, 1983) and monitored frequently with a UVB radiometer (SED-240, International Light Inc., Newburyport, Massachusetts) calibrated with a portable spectroradiometer (Optronics OL752, Optronics Labs Inc., Orlando, Florida). BE_{300} is a measure of the integrated fluence over the UVB waveband weighted according to Caldwell's generalized plant action spectrum (Caldwell, 1971). We used a moderate (6.4 kJ/day, BE_{300}) daily dose of UVB for our treatment. This dose simulates a very modest increase in UVB flux during the early season leaf flushing period for most citrus-growing regions in the United States. For instance, at 30°N latitude, the UVB flux predicted by a standard radiative transfer model (Green et al., 1980) ranges from 1.4 kJ/day BE_{300} on January 1 to 5.5 kJ/day BE_{300} on April 1. According to this model, UVB flux levels can increase to 9.1 kJ/day at this latitude; however, the model may tend to predict higher flux levels than are actually measured. Booker et al. (1992) found that, in some cases, the model predicted flux levels that were 30% higher than measured levels. Thus, our experimental conditions represent a very conservative increase in UVB fluence, which may more realistically simulate the UVB environment of citrus in the near term.

Since photosynthetic photon flux density can interact with enhanced UVB, aggravating its effects when photosynthetically active radiation levels are low (Caldwell, 1971; Mirecki and Teramura, 1984), we supplemented light levels with an additional bank of metal halide lamps. Eight high intensity discharge metal halide lamps were situated approximately 1.5 m over the banks of fluorescent UVB bulbs to supplement light entering the greenhouse. The metal halide lamps were evenly spaced in a rectangular array over the bench upon which the plants were grown.

The potted cuttings, which were undergoing a vigorous growth flush, grew for 10 days under the light racks before harvest and chemical analysis. At harvest, leaves were divided into those that completed or began expansion during the experimental period (young leaves) and those that were fully expanded and hardened before the experimental period began (old leaves). A randomly selected sample of young leaves and old leaves from each plant was removed and immediately frozen on dry ice for furanocoumarin and soluble protein analysis.

Furanocoumarin and Soluble Protein Assay. Fresh-frozen leaf material was weighed and ground in a mortar under liquid N_2. The leaf powder was then suspended in cold Tris buffer (50 mM, pH 8) and vortexed; an aliquot was taken for soluble protein measurement. Aliquots were centrifuged to remove particulates (14,000*g*, 10 min) and the supernatants were precipitated with an equal volume of cold 10% trichloroacetic acid. The precipitates were than redissolved in 0.1 M NaOH for protein analysis by the Lowry method (Lowry et al., 1951). The remaining leaf suspension was extracted with EtOAc and furanocoumarins were measured by HPLC of the extracts (McCloud et al., 1992).

Artificial Diet Experiments. C. jambhiri foliage contains amounts of furanocoumarins that are relatively low in comparison to other furanocoumarin-containing species (Berenbaum, 1991; McCloud et al., 1992). In order to determine if such small amounts of these potent phototoxins might affect the growth and development of *T. ni*, we reared caterpillars on artificial diets to which furanocoumarins had been added. Furanocoumarin toxicity was assayed in two separate experiments: in the first we exposed the caterpillars to levels of UVB that simulate ambient midsummer radiation in temperate latitudes; in the second, the range of furanocoumarin levels assayed was expanded and supplemental UVB was not provided. Comparison of these two experiments provides an estimate of UVB-potentiated phototoxicity of furanocoumarins. In the experiment in which larvae received supplemental UVB irradiation, caterpillars were irradiated for 2 hr each day under Westinghouse FS-40 bulbs wrapped in cellulose acetate as above. Here, the dose was 3.2 kJ/day BE_{300}. During irradiation, visible light was supplied with fluorescent (Phillips Cool White) bulbs. Fluence levels (photosynthetically active radiation) were typically 250 μmol/m^2/sec.

Artificial diets were prepared according to a standard method modified from Waldbauer et al. (1984). Bergapten, psoralen, or a 1 : 1 mixture of the two were dissolved in a small volume of acetone and added to artificial diet while the diet was still liquid. Furanocoumarin concentrations used in the experiment in which caterpillars received UVB irradiation were 100 and 10 μg/g. In the second experiment, the larvae received furanocoumarin concentrations ranging from 10 to 160 μg/g. Due to limitations in space and chemical availability, some combinations were omitted (see results). Control diets had acetone added alone. Diets were mixed thoroughly, dispensed into 1.5-oz plastic creamer cups, and allowed to solidify. Solid diet were held for two days to allow the acetone to evaporate before neonate *T. ni* were introduced. Caterpillars that had hatched within an 8-hr period were considered a cohort and were placed singly into diet cups. The cups were covered and held in an insectary at 30°C for the duration of larval development.

T. ni on C. jambhiri. Cohorts of *T. ni* larvae were reared from egg hatch to pupation on plants growing under enhanced UVB. Three neonate *T. ni* were introduced onto each of 60 rooted *C. jambhiri* cuttings. Half of the plants were

randomly assigned to receive UVB treatment and half served as controls. Plants were periodically rotated as before, and the cuttings had been growing under control or enhanced UVB light conditions for two weeks before the caterpillars were placed on them. UV fluence levels were monitored and adjusted as in previous experiments. Larval development was tracked until pupation, at which point pupae were brought to the laboratory and held until adult eclosion. Larval development was assessed by censusing infested plants daily and noting the instar of each caterpillar on each date.

RESULTS

Exposure to elevated levels of UVB irradiation significantly increased foliar furanocoumarins (Table 1). Results from the furanocoumarin assays were analyzed as a balanced completely crossed three-way analysis of variance with plant clone, UVB treatment, and leaf age as fixed effects. Because of variability from clone to clone, we included clonal identity in our experimental design in order to increase our ability to see effects due to UVB irradiation. However, clonal origin did not account for a significant portion of the variance in furanocoumarin concentration in this experiment. Furanocoumarin induction was greatest in young leaves, in which we observed an appropriate 2.5-fold rise in psoralen levels, while the concentration of bergapten was slightly less than doubled (Table 1). Soluble leaf protein content was not significantly altered by UVB treatment (Table 1).

TABLE 1. MEAN CONCENTRATIONS (±SE) OF FURANOCOUMARINS AND SOLUBLE PROTEIN IN LEAF TISSUE OF *C. jambhiri*[a]

			P values		
	UV_+	UV_-	UVB	Leaf age	UVB × leaf age
Psoralen					
Young leaves	49.3(9.4)	20.8(3.8)			
Old leaves	13.0(3.1)	8.2(2.0)	0.006	0.0001	0.0506
Bergapten					
Young leaves	29.1(4.6)	16.0(1.9)			
Old leaves	27.8(7.1)	13.5(1.9)	0.01	0.876	0.736
Protein					
Young leaves	9.7(2.4)	7.6(1.1)			
Old leaves	10.3(0.9)	8.7(1.0)	0.132	0.707	0.680

[a] Furanocoumarin amounts are $\mu g\ g^{-1}$, protein amounts are mg/g. *P* values from ANOVA with UV treatment, leaf age, and plant clone (not shown) as main effects.

Larvae reared from egg hatch to pupation in the presence of supplemental UVB suffered complete mortality when their diets contained psoralen and bergapten (Table 2). The same total furanocoumarin amount (100 μg/g) was less toxic when the added furanocoumarin was bergapten alone. However, bergapten did lower survivorship relative to controls at this concentration (Table 2). The low bergapten concentration (10 μg/g), did not. Mean development time of caterpillars on unamended diet was not significantly different from that of caterpillars surviving to pupation on amended diets, not did pupal weights of survivors on the control and furanocoumarin-containing diets differ significantly.

An expanded range of test concentrations of furanocoumarins allowed us to determine the minimum concentrations necessary to affect caterpillar development in the absence of supplemental UVB. Early larval development was significantly retarded by a dose of 80 μg/g of either bergapten or psoralen in artificial diet (Table 3). Caterpillars feeding on diets at this concentration grew to less than half the size of control larvae after five days. In contrast, the bergapten concentration required to extend the larval period relative to controls was twice this amount, 160 μg/g (Table 3). While psoralen is far more toxic to a variety of organisms than bergapten in the presence of UV light (Pathak and Fitzpatrick, 1959; Tuveson et al., 1986), its "dark" toxicity in this experiment was similar to that of bergapten (Table 3). Caterpillars reared from diets containing concentrations of furanocoumarins less than 160 μg/g suffered developmental rate depression in early life but completed development at the same time as controls.

TABLE 2. PERFORMANCE OF *T. ni* EXPOSED TO SIMULATED AMBIENT UVB ON ARTIFICIAL DIETS CONTAINING FURANOCOUMARINS[a]

	50 μg/g each psoralen and bergapten (N = 35)	100 μg/g bergapten (N = 35)	10 μg/g bergapten (N = 35)	Control (N = 36)	P
% 5th instar survivorship	0	54.3	77.1	88.9	0.001
% survivorship to pupation	0	25.7	54.3	52.8	0.001
Days to pupation		12.4	12.7	11.9	0.176
Mean pupal weight		148.6	148.9	148.5	0.999

[a] P values for days to pupation and mean pupal weight (mg) from one-way analysis of variance. P values for survivorship to pupation and 5th instar survivorship from G test for 4 × 2 contingency tables. Fifth instar survivorship and survivorship to pupation of caterpillars receiving 100 μg/g bergapten in diet are significantly different from control (G = 11.05, P = 0.0012, fifth-instar survivorship; G = 5.535, P = 0.0197, survivorship to pupation). Caterpillars were reared under 3.2 kJ/day (UVB, BE_{300}).

TABLE 3. DAYS TO PUPATION (±SE) AND WEIGHTS (mg ± SE) AT FIVE DAYS OF LARVAL LIFE OF *T. ni* (15 CATERPILLARS PER TREATMENT COMBINATION) REARED ON ARTIFICIAL DIETS WITH ADDED FURANOCOUMARINS.[a]

	Weight at 5 days			Days to pupuation		
Dose	Psoralen	Bergapten	Both	Psoralen	Bergapten	Both
10	—	—	35.6 (4.4)ab	—	—	9.35 (0.17)d
20	28.2 (4.0)abcde	30.1 (5.2)abcd	40.3 (4.3)a	9.40 (0.16)cd	9.73 (0.21)bcd	9.47 (0.11)cd
40	25.2 (3.3)bcdef	22.6 (2.5)abcdef	31.8 (4.1)abc	9.71 (0.11)bcd	9.75 (0.11)bcd	9.43 (0.15)bcd
80	15.3 (1.6)cdef	14.1 (0.8)def	18.1 (2.5)cdef	10.61 (0.33)abc	10.35 (0.28)bcd	10.18 (0.24)abcd
100	12.8 (3.2)ef	17.8 (2.4)cdef	—	11.28 (0.63)a	9.67 (0.34)bcd	—
160	9.0 (1.2)f	12.35 (1.7)ef	13.2 (1.2)def	11.38 (0.31)a	10.71 (0.38)ab	10.53 (0.30)abcd
Control		35.8 (4.2)ab			9.46 (0.15)cd	

[a]Doses are μg/g of psoralen, bergapten, or a 1:1 mixture of both added to diet. Means sharing the same letter(s) within each half of the table are not significantly different at $P = 0.05$, Bonferroni's corrected means comparison test.

Change in foliar furanocoumarin levels following exposure to UVB and developmental delay in young larvae reared from furanocoumarin-containing diets provide a context in which to evaluate larval development when the insects are reared on UVB-irradiated plants. We used survivorship analysis (SAS Institute, 1989) to compare mean life expectancies between the two groups. This analysis treats plants as experimental units and compares the distributions of the times elapsed until the loss of all caterpillars from a plant. Survivorship of larvae reared on plants grown under enhanced UVB was significantly lower than that of larvae on plants without supplemental UVB (log rank chi-square = 4.36, $df = 1$, $P = 0.036$, Figure 1). Unexpectedly, the pupal weights of survivors from the UVB-treated plants were greater (163 ± 5 mg, SE, $N = 49$, no UVB; 178 ± 5 mg, SE, $N = 35$, UVB treated; $P = 0.0315$) than pupal weights of survivors from the treated plants.

Development on UVB-irradiated plants paralleled the pattern observed when low concentrations of furanocoumarins were fed to the caterpillars in artificial diets. Caterpillars feeding on plants under enhanced UVB had depressed devel-

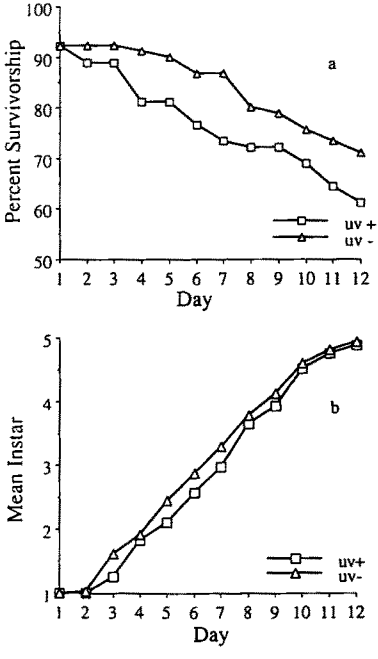

FIG. 1. Survivorship schedules (a) and growth (b) of *Trichoplusia ni* reared on *Citrus jambhiri* plants with and without supplemental UVB radiation (12.1 kJ/day UVB BE_{300}). Caterpillars began to pupate on day 12. $N = 90$ caterpillars in each group.

opment through the first three larval stadia. These larvae reach third instar about 0.5 days after controls (5.76 ± 0.12 days, SE, $N = 66$, UVB-treated; 6.3 ± 0.12 days, SE, $N = 75$, no UVB; $P = 0.0015$, t test). Moreover, we used a repeated-measures analysis of variance on the mean larval instar on each plant to examine the developmental curves (Figure 1b) over the first nine days of larval life. We found that caterpillars feeding on plants under enhanced UVB exhibited a significantly depressed developmental rate through this period ($F_{1,54} = 11.78$, $P = 0.0012$). However, over the entire period of larval and pupal development, surviving caterpillars in both control and treatment groups developed at the same rate. The total preadult period did not differ between the two groups (22.9 ± 0.41 days, SE, $N = 38$, UVB-treated; 22.3 ± 0.20 days, SE, $N = 43$, no UVB; $P = 0.561$, Mann-Whitney U test).

DISCUSSION

Our findings suggest that the combination of furanocoumarins and exposure to UVB radiation is more toxic to *T. ni* than furanocoumarins alone (cf. Tables 1 and 3). Thus, this study provides, to our knowledge, the first evidence for potentiation of phototoxins by UVB wavelengths in a eukaryotic organism. In comparison to controls, caterpillars feeding on diet containing bergapten at a concentration of 100 μg/g fresh weight with exposure to UVB radiation suffered greater mortality and had an extended larval period. This furanocoumarin concentration was not sufficient to extend the developmental period of caterpillars not exposed to UVB. Moreover, inclusion of psoralen with bergapten into the diet of caterpillars that were exposed to UVB radiation resulted in complete mortality in this experiment.

While it is appropriate to compare the relative differences between control and treated caterpillars in the two artificial diet experiments, we cannot address the issue of direct effects of UVB radiation on *T. ni* in the absence of phototoxins. In nature, and in our greenhouse study, *T. ni* live and feed on the undersides of leaves (Jones and Granett, 1982). Since they spend their larval lives in shade, the total UVB exposure of the *T. ni* is therefore likely to be low. Nevertheless, diffuse and backscatter radiation may impinge on caterpillars feeding at the edge of the canopy (Iqbal, 1983). Our artificial experiment conducted without supplemental UVB, then, represents a conservative estimate of the detrimental effects of furanocoumarins in a nutritionally rich artificial diet.

We observed the same distinctive effect, that is, prolongation of early larval development with subsequent recovery in later life, in larvae reared on whole plants under enhanced UVB and larvae reared on artificial diets to which furanocoumarins had been added (Figure 1, Table 3). Delayed early larval development was reproduced by similar furanocoumarin concentrations in our artificial

diet experiments. However, the concentration required to produce this effect in artificial diets was 1.6 to 6.1 times higher than foliar levels measured in these studies. This difference may be due to the fact that artificial diets tend to improve performance relative to natural substrates, and both nutrient deficiency and allelochemical levels can combine to affect larval performance in an additive fashion (Lindroth, 1990; Reese and Field, 1986). Moreover, in other work we have documented foliar furanocoumarin levels in *C. jambhiri* well within the effective range suggested by the artificial diet experiments (McCloud et al., 1992).

Extrapolation of results from the greenhouse to the field can be risky; nevertheless, we suggest that our findings are sufficient for such inference. Young *C. jambhiri* foliage is preferred by *T. ni* (McCloud, unpublished data), presumably because it is less tough and has a higher water content than mature foliage. This preferred foliage, which contains the highest furanocoumarin concentrations in *C. jambhiri*, displays the greatest furanocoumarin induction in response to increased UVB. Under field conditions, insects such as *T. ni* typically suffer exponentially decreasing survivorship in which the probability of death is constant in each instar; percent survivorship may be even lower in earlier developmental stages (Price, 1984). In either case, extension of the early period of larval life could lead to increased mortality when caterpillars are forced to contend with less than ideal conditions in the field (Price et al., 1980). Recent work has shown that extension of the early larval period as a result of host toxicity can interact synergistically with parasitoids to increase larval mortality (Johnson and Gould, 1992). Survivorship under laboratory conditions more nearly parallels physiological curves, and the ecological effects of increased vulnerability in early larval stages can, therefore, be masked. In our artificial diet experiment in which supplemental UVB was not provided, we did not observe markedly enhanced mortality in any caterpillars except those receiving the highest levels of dietary furanocoumarins. Greenhouse conditions may be considered to be intermediate between those in the laboratory and those in the field, especially when larvae are not caged on plants. While differential survivorship may have contributed to the apparent developmental rebound in the greenhouse experiment, both this experiment and the artificial diet experiment without enhanced UVB suggest that low levels of dietary furanocoumarins can have greater effects on the early growth and development of *T. ni* than on later growth.

Another factor that may contribute to the developmental rebound of mature caterpillars may be ontogenetic changes in enzymatic detoxification systems of *T. ni*. Ahmad (1992) noted that activity levels of the antioxidant enzymes superoxide dismutase, catalase, and glutathione reductase increase over the period between third and fifth instar in *T. ni*. This generalist is capable of only low levels of cytochrome P-450-mediated metabolism of furanocoumarins (Ivie et al., 1983; Lee and Berenbaum, 1990). Lee and Berenbaum (1990) provided

evidence for the hypothesis that animals that metabolize photosensitizers slowly depend on antioxidant enzymes for metabolic defense against these compounds. An ontogenetic increase in ability to cope with oxidative stress, then, may have contributed to the patterns we observed.

Given the documented allelochemical changes of plants exposed to increased UVB irradiation, it is reasonable to expect stratospheric ozone depletion to have repercussions for a wide variety of plant–insect interactions, including many interactions between insects and crop plants. In this study, in contrast with other studies of anthropogenically induced alterations in plant quality for herbivores (Fajer et al., 1989; Heinrichs, 1988), we have shown that enhanced UVB can potentially increase, rather than decrease, plant resistance to herbivores. Results from our study may not be easily extrapolated to other systems; nevertheless, our results clearly suggest the potential for field-level effects. As a semitropical tree, *C. jambhiri* experiences high ambient levels of UVB and is presumably more resistant to its damaging effects than are temperate species. Additionally, *C. jambhiri* contains UVB-photoactivated secondary metabolites; such compounds are probably not widely distributed among plants. Effects of elevated UVB are likely to be idiosyncratic and possibly unique to each herbivore–host association, rendering the forecasting of future ecological and economic impacts of elevated UVB a challenging prospect. More studies on a broad diversity of systems will be necessary to develop a general predictive paradigm for this impending global change.

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TOWARD THE CHEMICAL ECOLOGY OF MEDICINAL PLANT USE IN CHIMPANZEES: THE CASE OF *Vernonia amygdalina*, A PLANT USED BY WILD CHIMPANZEES POSSIBLY FOR PARASITE-RELATED DISEASES

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Abstract—The bitter and related constituents have been isolated from *Vernonia amygdalina* (Compositae), a plant ingested by wild chimpanzees pos-

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sibly suffering from parasite-related diseases in the Mahale Mountains National Park, Tanzania. Isolated from the plant were four known sesquiterpene lactones, seven new steroid glucosides, and two aglycones of the glucosides. The sesquiterpene lactones showed significant in vitro antischistosomal, plasmodicidal, and leishmanicidal activities. Antischistosomal activity was also found for the major steroid glucoside, vernonioside B_1. A trend in the glucosides to show significant antischistosomal, plasmodicidal, and amebicidal activities when the sugar moiety was removed, was observed. Vernodalin, judged as the most significant constituent for antiparasitic activities in vitro, was tested for in vivo antischistosomal effect. It was, however, highly toxic to the cercaria-infected mouse. Chimpanzees have been only rarely observed to ingest anything but the pith of the young stem. The occurrence of vernonioside B_1 and its aglycone vernoniol B_1, the major constituents among the steroid-related constituents, were detected at significant levels in the pith. However, vernodalin was abundant only in the leaves and bark. Thus, chimpanzees at Mahale were hypothesized to control parasite-related diseases by ingesting the young pith of this tree containing steroid-related constituents.

Key Words—*Vernonia amygdalina*, medicinal plant, chimpanzee, vernodalin, vernonioside, vernoniol, antischistosomal activity, plasmodicidal activity, amebicidal activity, leishmanicidal activity.

INTRODUCTION

Vernonia amygdalina Del. (Compositae) is widely used as traditional medicine and is commonly found in the savanna woodlands of tropical Africa (Watt and Breyer-Brandwijik, 1962). It has also been suggested to be used medicinally by wild chimpanzees. In 1987, Huffman and Seifu observed an apparently sick female chimpanzee chew and suck out the juice from the young piths of *V. amygdalina* in the Mahale Mountains National Park, Tanzania (Huffman and Seifu, 1989). Within 24 hr her condition greatly improved and she resumed her normal activity. Again in 1991, similar field observations were made, and the presence of parasites and subsequently an unusual drop in parasite levels (eggs/g feces) was detected by fecal analysis (Huffman et al., 1994). The leaf, bark, pith, and root of this species is extremely bitter and differs greatly from other chimpanzee food items in this respect. This species is rarely recorded to be ingested by chimpanzees at Mahale (Nishida and Uehara, 1983).

We have hitherto investigated the bitter and related constituents of this species and isolated two classes of bitter constituents. One class obtained from an ethyl acetate-soluble part of acetone (or methanol) extract included the germacrane and elemane type sesquiterpene lactones, vernodalin (**1**) and vernodalol (**4**), and vernolide (**2**), and hydroxyvernolide (**3**) (Ohigashi et al., 1991a; Jisaka, et al., 1993a). These lactones are known to show cytotoxic activities (Kupchan et al., 1969; Jisaka et al., 1993a). Another class from an *n*-butanol-soluble part of the extract was a series of new stigmastane-type steroid glucosides, named

vernonioside A_1 (**5**), A_2 (**6**), A_3 (**7**), and A_4 (**8**) (Ohigashi et al., 1991b; Jisaka et al., 1992b; Jisaka et al., 1993b). Additionally, related nonbitter glucosides, vernonioside B_1 (**9**), B_2 (**10**), and B_3 (**11**), together with vernoniol A_4 (**8a**), the primary aglycone of vernonioside A_4, were isolated (Ohigashi et al., 1991b; Jisaka et al., 1992b; Jisaka et al., 1993b).

Based on these sick chimpanzees' symptoms—lack of appetite, malaise, diarrhea, constipation, and unusually dark-colored urine—along with the ongoing investigation of parasitic infection in Mahale chimpanzees, it has been hypothesized that the most probable use of this species by chimpanzees is for the relief of parasite-related diseases (Huffman et al., 1990; Huffman and Wrangham, 1993; Huffman et al., *in prep*). Hence, effects of the bitter and related compounds on *Schistosoma japonicum, Plasmodium falciparum, Entamoeba histolytica*, and *Leishmania infantum* were tested for their general anthelminthic activity. *Schistosoma*, *Plasmodium*, and *Entamoeba* are ecologically pertinent genera to test because they are known to be transmissible to chimpanzees (Brack, 1987). All three genera are known to infect humans living in the study area and yellow baboons that share the study group's home range have been confirmed to be infected with *Schistosoma mansoni* and *Entamoeba histolytica* (Huffman, unpublished data).

Previously, in vitro antischistosomal activities of the four sesquiterpene lactones (**1–4**) and some of the steroid glucosides (**5–7**, **9**), together with some aglycones (**7a**, **9a**, **9b**), have briefly been reported (Jisaka et al., 1992a). On the basis of the toxicity tests on mice, along with quantitative analyses of the major constituents in each class of compounds, vernodalin (**1**) and vernonioside B_1 (**9**), the steroid-related constituents were suggested to be more significant for chimpanzees' control of parasite related disease (Jisaka et al., 1992a).

In subsequent studies, further antiparasitic activities have been examined. This report describes the antischistosomal, plasmodicidal, amebicidal, and leishmanicidal activities of the isolated and some derivatized constituents. The significance of the ingestion by chimpanzees also is discussed from the viewpoint of the control of parasite-related disease.

METHODS AND MATERIALS

General Remarks. The coworkers of this large research group conducted the following activities in this study. Chemical analyses were conducted by H.O., D.I., and K.K. Field surveys and observation of chimpanzee behavior and plant collection were performed by M.A.H. In vitro and in vivo antischistosomal tests were conducted by M.K. and H.S. G.C.K., D.C.W., D.A., C.W.W., and J.D.P. carried out in vitro plasmodicidal and amoebicidal tests, and P.T.-D., F.D., R.E., and G.B. tested for leishmanicidal activity.

Test Chemicals. Vernodalin (**1**), vernolide (**2**), hydroxyvernolide (**3**), vernonioside A_1–A_4 (**5–8**), and vernonioside B_1 (**9**) were isolated from the whole plant parts of *V. amygdalina* as previously described (Ohigashi et al., 1991b; Jisaka et al., 1992b, 1993b). Vernodalol (**4**) was obtained as an artifact of vernodalin by extraction of the plant with MeOH (Ohigashi et al., 1991a; Jisaka et al., 1993a). The primary and secondary aglycones of the vernoniosides were prepared by enzymatic or acid hydrolysis of the respective glucosides also as previously reported (Jisaka et al., 1992b). 3-Oxo derivatives (**9c** and **9d**) of the B_1 aglycones (**9a** and **9b**) were both obtained by Jones oxidation of **9a** and **9b**, respectively.

In Vitro Antischistosomal Activity. The in vitro antischistosomal activity test using an adult pair of schistosomes of *Schistosoma japonicum* in RPMI 1640 supplemented with 10% fetal calf serum (1 ml) was conducted by the method reported previously by Kawanaka (1983) and Jisaka et al. (1992a). After incubation for 24 hr, inhibition of both the movement and egg-laying capability of the schistosomes were observed in triplicate experiments. The inhibitory activity of the schistosome movement was evaluated by three ranks (++, +, and −) as shown in Table 1 below. The ++ and + activities were found to be irreversible, when schistosomes treated with test compound for 24 hr were reincubated in a normal medium for another 48 hr. The inhibitory activity of the egg-laying capability was estimated by the average number of eggs laid as compared with that of control experiments.

In Vivo Antischistosomal Activity of Vernodalin. Prior to the in vivo test, the toxicity of vernodalin was examined by oral administration and abdominal, subcutaneous and muscular injection of it into NIH mice. In the in vivo test, vernodalin (2.5 mg) in DMSO (320 μl) was orally administered to the cercaria-infected mice (5 mice) once or twice with an interval of one day (Jisaka et al., 1992a). After 24 hr, schistosomes were collected from the portal vein and the number of mature schistosomes and the number of pairing schistosomes were counted and compared with those of the control experiments done with DMSO (320 μl), praziquantel (2.5 mg), and without any treatment. The egg-laying capability of the collected schistosomes also was measured by further cultivation of the pairing schistosomes for 48 hr.

In Vitro Plasmodicidal and Amebicidal Activities. The in vitro plasmodicidal and amebicidal activities (IC_{50}) using *Plasmodium falciparum* (multidrug-resistant strain K1) and *Entamoeba histolytica* (NIH 200), respectively, were conducted by the methods previously reported by Wright et al. (1991). The plasmodicidal activity was expressed by the IC_{50}. In the case of amebicidal activity, the concentration that clearly exhibited more than 50% inhibition of ameba growth was measured by visual inspection. For these tests DMSO was used as a solubilizing solvent for the test compounds.

In Vitro Leishmanicidal Activity. The in vitro leishmanicidal activity was

evaluated on *Leishmania infantum* isolated from the ganglia of dogs in Marseille. The promastigotes forms were cultivated in media RPMI 1640 supplemented with 15% of fetal calf serum. The leishmanicidal activity, expressed by minimum inhibitory concentration (MIC), was carried out according to the method previously described by Delmas et al. (1993), with DMSO as a solubilizing solvent for the test compounds.

Quantitative Analyses of Sesquiterpene Lactones, Steroid Glucosides, and Free Steroids by HPLC. The plant materials used for quantitative analyses were collected at Mahale in 1991. For detailed analyses of the levels of vernodalin and vernonioside B_1 by plant part, the leaf, bark, and pith parts from both the young and old aerial parts of one plant individual collected on October 28 (sample I) were separately prepared. The root of this sample was also collected. To investigate seasonal and individual variations of these compound levels, the young leaves and stem (pith with bark) were separately collected from three fixed individuals (plants A, B, and C) on October 27 (sample II) and December 22 (sample III): the beginning and middle of the rainy season. Each part (4 g) was divided in half, and one half (2 g) was extracted with MeOH (20 ml) for analyses of vernonioside B_1 and a free steroid, vernoniol B_1. The other half (2 g each) was extracted with acetone (20 ml) for analysis of vernodalin, because vernodalin was shown to be easily converted into vernodalol on treatment with MeOH. After two weeks, both extract solutions were removed and stocked as primary extracts. The remaining plant parts were further extracted twice in the same manner to obtain secondary and tertiary extracts. The primary–tertiary extracts were combined and concentrated. Next, the acetone extract was dissolved in 10 ml of 70% acetonitrile in water, and 4 ml of this solution was poured onto Cosmosil 140C_{18}-OPN gel (Nacalai Tesque, approximately 1 g). After the excess solvent was removed in vacuo, the gel was transferred into a syringe (7 cm × 0.8 cm ID) equipped with a Sep-pak C_{18} cartridge (Waters Chromatography Division) at the outlet side. The components absorbed on the Cosmosil gel were successively eluted with 10 ml of water, 10 ml of 90% acetonitrile in water, and 10 ml of acetonitrile under syringe pressure. The sesquiterpene lactone (vernodalin in this case) was found in the 90% acetonitrile fraction. This fraction was concentrated and redissolved with 1 ml of acetonitrile, filtered through an H-13-5 filter (TOSOH), and filled up to 2 ml with acetonitrile. Five microliters of this solution was injected into an HPLC column (YMC AQ-301, ODS, 4.6 × 100 mm) eluted with 25% acetonitrile in water at a flow rate of 1 ml/min. Vernodalin was detected at t_R = 12 min with a UV detector at 220 nm. In the case of analyses of the steroid-related constituents, MeOH was used in place of acetonitrile throughout sample preparation. The final fraction, eluted with 90% MeOH in water obtained by filtration with the H-13-5 filter, contained steroid glucosides and free steroids. The HPLC analyses on the same column as that of vernodalin were conducted with 33% acetonitrile

in water for the glucosides (vernonioside B_1) and with 50% acetonitrile in water for the free steroids (vernoniol B_1) at a flow rate of 1 ml/min. Vernonioside B_1 detected with a UV detector at 254 nm appeared at t_R = 18 min, and vernoniol B_1 analyzed by an HPLC (Waters U6K) equipped with a photodiode array detector appeared at t_R = 12 min. It was necessary for the solutions containing vernoniosides and their aglycones to be stored in the dark, because of their easy photochemical degradation.

Preparation of a Crude Free Steroid Fraction and Glucoside Fraction and Isolation of Vernoniol B_1 from Crude Free Steroid Fraction. A MeOH extract of *V. amygdalina* (3 kg) was partitioned with *n*-hexane–MeOH–water (5:9:1, 1 liter). The lower layer was concentrated and first partitioned between EtOAc and water (1:1, 500 ml). The lower layer was further partitioned between *n*-BuOH and water (1:1, 1 liter). The EtOAc soluble part and *n*-BuOH soluble part were indicated to contain several free steroids and the glucosides, respectively, by TLC and HPLC with a photodiode array detector. The EtOAc soluble part (62 g) was separated on Wako gel C-100 (460 g) eluted stepwise with toluene plus increasing amounts of EtOAc to give a free steroid-containing fraction eluted with 20% EtOAc in toluene. This fraction (7.9 g) was separated on YMC-Gel (ODS-A 60-400/230, 150 g) eluted stepwise with water plus increasing amounts of acetonitrile. The 55% acetonitrile fraction (1.9 g) was further chromatographed on Kieselgel 60H with chloroform–MeOH (99:1) to give a fraction (640 mg) containing vernoniol B_1. Recrystallization from toluene–chloroform gave vernoniol B_1 (**9a,** 480 mg), mp 205–210°C, which was identified as the primary aglycone of vernonioside B_1.

RESULTS AND DISCUSSION

Structures of the sesquiterpene lactones, vernodalin (**1**), vernolide (**2**), hydroxyvernolide (**3**) and vernodalol (**4**), and steroid glucosides, vernoniosides As and Bs (**5–11**), including their derivatives (**7a, 8a, 9a, 9c**), are shown in Figure 1.

Table 1 shows in vitro antischistosomal activities of the sesquiterpene lactones (**1–4**), steroid glucosides (**5–9**), and some of their aglycones (**7a, 8a, 9a, 9b**), including the 3-oxo derivatives (**9c, 9d**). All of the sesquiterpene lactones completely inhibited both the movement and egg-laying of schistosomes at a concentration of 200 μg/ml. At 20 μg/ml, vernodalin (**1**) and vernolide (**2**) inhibited both functions. Vernodalol also inhibited egg-laying capability at 20 μg/ml. All sesquiterpene lactones, however, showed no remarkable antischistosomal activities at 2 μg/ml, at which the known schistosomacides, praziquantel and niridazol, showed significant activities. Vernodalin was indicated to be the most active constituent in the sesquiterpene lactone class of compounds. Among

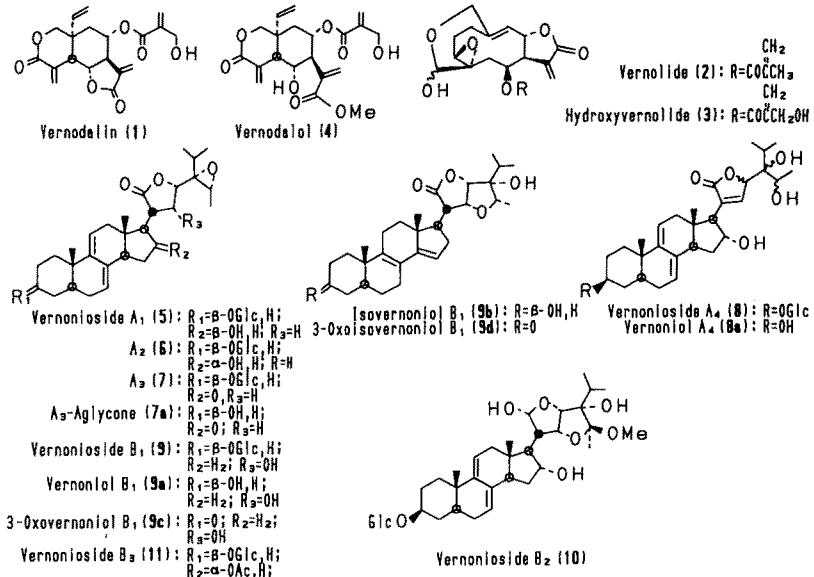

FIG. 1. Structures of the sesquiterpene lactones and the steroid-related compounds.

the steroid glucosides, on the other hand, only vernonioside B_1 at 200 μg/ml showed significant inhibitory activity against both the movement and egg-laying of the adult. The schistosome movement inhibition was, however, not observed at 20 μg/ml, while the inhibitory effect on egg-laying still remains active at 2 μg/ml. Vernonioside A_4 seems to be slightly active against egg-laying of the schistosome. Interestingly, the antischistosomal activity of vernonioside B_1 was enhanced when the glucose moiety was removed as observed in its primary aglycone, vernoniol B_1 (**9a**) and a secondary aglycone named isovernoniol B_1 (**9b**), both of which showed inhibitory effects on schistosome movement and egg-laying at 2 μg/ml. 3-Oxo derivatives (**9c** and **9d**) of the aglycones exhibited activity almost equal to those of their aglycones in egg-laying inhibition, although the effect on schistosome movement seemed to be slightly reduced. Such activity change was also detected in the conversion of vernonioside A_4 into the primary aglycone vernoniol A_4, whose natural occurrence has been confirmed (Jisaka et al., 1993b).

Table 2 shows plasmodicidal, amebicidal, and leishmanicidal activities of the sesquiterpene lactones and steroid-related compounds. The sesquiterpene lactones showed significant plasmodicidal activities, although the IC_{50} values were more than 20 times higher than that of the common antimalarial agent chloroquine. On the other hand, the activities of vernoniosides were far weaker

TABLE 1. *In Vitro* ANTISCHISTOSOMAL ACTIVITIES OF SESQUITERPENE LACTONES AND STEROID-RELATED CONSTITUENTS

	200 μg/ml		20 μg/ml		2 μg/ml	
Compound	IM[a]	EL[b]	IM	EL	IM	EL
Sesquiterpene lactones						
Vernodalin	++	2 ± 2	++	0	−	297 ± 194
Vernolide	++	0	+	24 ± 17	−	69 ± 6
Hydroxyvernolide	++	34 ± 47	−	154 ± 163	−	197 ± 116
Vernodalol	++	1 ± 1	−	25 ± 33	−	617 ± 24
Steroid related constituents						
Vernonioside B_1	+	0	−	0	−	20 ± 28
Vernoniol B_1	NT[c]	NT	+	0	+	33 ± 47
3-Oxovernoniol B_1	NT	NT	+	0	+	79 ± 108
Isovernoniol B_1	NT	NT	+	0	−	2 ± 2
3-Oxoisovernoniol B_1	NT	NT	−	0	−	122 ± 147
Vernonioside A_1	−	22 ± 17	NT	NT	NT	NT
Vernonioside A_2	−	350 ± 304	NT	NT	NT	NT
Vernonioside A_3	−	341 ± 236	NT	NT	NT	NT
A_3-Aglycone	NT	NT	−	106 ± 92	−	184 ± 24
Vernonioside A_4	NT	NT	+	61 ± 74	−	348 ± 264
Vernoniol A_4	NT	NT	+	10 ± 14	−	117 ± 43
	IM	EL				
Praziquantel (2 μg/ml)	+	0				
Niridazol (2 μg/ml)	+	18 ± 2				
Control (DMSO)[d]	−	662 ± 193				

[a] IM: inhibition of schistosome movement evaluated by ++ (complete inhibition), + (incomplete inhibition) and − (no inhibition).
[b] EL: the number of eggs laid (mean ± SD).
[c] NT: not tested.
[d] Data only with DMSO (20 μl) in quadruplicate experiments.

than those of the sesquiterepene lactones. However, a general trend for the enhancement of activity upon elimination of the sugar moiety from the respective glucoside was again detected in both plasmodicidal and amebicidal activities as indicated in parenthesis. In particular, the plasmodicidal activity of vernoniol A_4, the naturally occurring aglycone of vernonioside A_4, was significantly increased. Interestingly, vernodalin showed extremely high leishmanicidal activity. Its MIC was 10 times lower than that of pentamidine, a common antileishmanial agent.

The above analyses in vitro show vernodalin to be the most active, and thus most significantly antiparasitic, constituent tested thus far. Based on this,

TABLE 2. *In Vitro* PLASMODICIDAL, AMEBICIDAL, AND LEISHMANICIDAL ACTIVITIES OF SESQUITERPENE LACTONES AND STEROID-RELATED CONSTITUENTS[a]

Compound	PM[b] IC_{50} (μg/ml)		AM[c] IC_{50} (μg/ml)	LM[d] MIC (μg/ml)
Vernodalin	4.0		—	0.5
Vernolide	8.4		–	< 10
Hydroxyvernolide	11.4		–	–[f]
Vernodalol	4.2		–	–
Vernonioside A_1	139.7	(62.6)	– (< 12.5)	NT[g]
A_2	94.1	(248.0)	– (< 12.5)	NT
A_3	245.1	(55.1)	– (–)	NT
A_4	81.8	(15.3)[h]	– (< 12.5)[h]	NT
B_1	46.1	(45.0)[i]	– (< 12.5)[i]	NT
3-Oxovernoniol B_1	50.7		< 12.5	NT
Isovernoniol B_1	156.4		< 12.5	NT
Chloroquine diphosphate	0.2		NT	NT
Pentamidine	NT		NT	5

[a] Because of sample availability, each datum was obtained by single experiment.
[b] PM: plasmodicidal activity; the value in parenthesis: IC_{50} of the primary aglycone.
[c] AM: amebicidal activity; the value in parenthesis: IC_{50} of the primary aglycone.
[d] LM: leishmanicidal activity.
[e] – in the AM activity: inactive at less than 50 μg/ml.
[f] – in the LM activity: inactive at less than 50 μg/ml.
[g] NT: not tested.
[h] IC_{50} of vernoniol A_4 (**8a**).
[i] IC_{50} of vernoniol B_1 (**9a**).

we then tested the in vivo antischistosomal activity of vernodalin. It was, however, lethal to the cercaria-infected mouse when orally administered at 5 mg per mouse, and injected abdominally at 2 mg, or subcutaneously or intramuscularly at 5 mg, per mouse. Upon oral administration of 2.5 mg of vernodalin to cercaria-infected mice with an average body weight of 40 g, no effects were observed on both the number of recovered schistosomes and the egg-laying capability of the schistosomes recovered. On the other hand, praziquantel at a dose of 2.5 mg per mouse was effective (data not shown). Because of its high toxicity to the host, the use of vernodalin as well as other sesquiterpene lactones might be unfavorable for control of parasite-related disease in chimpanzees.

Based on the long-term observation of their feeding behavior at Mahale, the use of the pith of shoots seems to be the common part used by chimpanzees at Mahale (Nishida and Uehara, 1983). Therefore, quantitative analyses of vernodalin (**1**) and vernonioside B_1 (**2**), each of which is the major constituent of

the sesquiterpene lactones and steroid glucosides, respectively, were conducted by HPLC. The leaves, bark, and pith from the young and old aerial parts, as well as the root were collected on October 28 (sample I), and their extracts, after being partially purified, were submitted to analysis. As shown in Table 3, the level of vernodalin was highest in the leaves and next highest in bark. No significant occurrence of vernodalin was detected in the pith and the root. On the other hand, the level of vernonioside B_1 was comparably consistent in the parts except for the pith of the old aerial part. Seasonal and individual variation in the levels of vernodalin and vernonioside B_1 in the young leaves and stem (pith and bark) were also examined for the samples collected from three plant individuals fixedly marked as plant A, B, and C on October 27 (sample II) and December 22 (sample III). No significant variation in the relative levels of either constituent was found between the three individuals (Huffman et al., 1994). The levels of vernodalin in the leaves and stem of sample III were both lower than those of sample II, as shown in Table 3. Conversely, vernonioside B_1 in sample III increased by three fold in the leaves and 1.7-fold in the stem as compared with that in sample II. At Mahale, the rainy season starts in mid-October and

TABLE 3. LEVELS OF VERNODALIN AND VERNONIOSIDE B_1 BY PLANT PART[a]

Sample/Compound	Young aerial part			Old aerial part			Root	
Sample I on Oct. 28/	Lv[b]	Bk[c]	Pth[d]	Lv	Bk	Pth	Bk	Pth
Vernodalin	2.18	0.32	0.03	2.28	0.02	0	0	0
Vernonioside B_1	0.61	1.64	0.75	1.26	1.28	0.08	0.26	0
Sample II[e] on Oct. 27/	Lv		St[f]		Lv	St	Bk	Pth
Vernodalin	2.17 ± 0.47		0.28 ± 0.20		NM[g]	NM	NM	NM
Vernonioside B_1	0.55 ± 0.07		0.50 ± 0.12		NM	NM	NM	NM
Sample III[e] on Dec. 22/	Lv		St		Lv	St	Bk	Pth
Vernodalin	1.66 ± 0.27		0.13 ± 0.07		NM	NM	NM	NM
Vernonioside B_1	1.83 ± 0.68		0.88 ± 0.38		NM	NM	NM	NM

[a] The quantity (mg) per each fresh part (1 g).
[b] Lv: leaves.
[c] Bk: bark.
[d] Pth: pith.
[e] The mean value ± standard deviation in the data of three different plant individuals, A, B, and C.
[f] A total amount in pith plus bark.
[g] NM: not measured.

is well under way by December. The chimpanzees were reported to use *V. amygdalina* more often in the rainy season than in the dry season (Huffman et al., 1990). Also in this season, the number of chimpanzees with parasitic infections tends to increase (Huffman et al., 1990, 1992; Kawabata and Nishida, 1991). Hence, this increasing level of vernonioside B_1 in the stem part may be significant for the control of parasitic infections in chimpanzees.

HPLC analysis on sample III with a photodiode array detector also suggested the occurrence of several free steroids, which appeared as peaks carrying UV absorption maxima at 236, 242, and 251 nm characteristic for the 7,9(11)-diene moiety in the steroid ring system. The main peak was confirmed to be due to vernoniol B_1 (**9b**), the primary aglycone of vernonioside B_1, by several spectroscopic analyses of the purified sample by chromatography. Vernoniol B_1 was contained at levels of 0.14 mg/g fresh young leaves and 0.18 mg/g fresh young stem, respectively. In contrast to vernonioside B_1, vernoniol B_1 was more abundant in the stem than in the leaves. As described before, antiparasitic activities of vernoniol B_1 were higher than those of its glucoside, vernonioside B_1. Furthermore, antischistosomal activity of the crude free steroid fraction was higher than that of the crude glucoside fraction (data not shown). Thus, vernoniol B_1 is also considered to be significant for the control of parasite-related diseases of chimpanzees.

Huffman again observed in 1991 that a sick chimpanzee used this species in the same manner as the case in 1987. From the feces collected in the field, one intestinal nematode species, *Ternidens* sp. (Strongyloidea), and a symbiotic protozoon, *Troglodytella abrassarti*, were identified (Huffman et al., 1994). Based on the analysis of these fecal samples collected 1 hr and 23 hr after ingestion of the pith, the number of eggs of *Ternidens* sp. was indicated to be unusually reduced (Huffman et al., 1994).

At present, it has not been determined that chimpanzees that ingest *V. amygdalina* are infected with schistosomes, plasmodia, amebae, or leishmania, or whether the sesquiterpene lactones and/or steroid-related constituents are active against *Ternidens* sp. Effects of the constituents from *V. amygdalina* on several intestinal parasites are now being tested. However, on the basis of the above analyses, it may be concluded that chimpanzees avoid ingesting the leaves containing the abundantly toxic sesquiterpene lactones including vernodalin, and also they may selectively ingest the pith, which contains steroid-related compounds to control parasite-related diseases.

Rodriguez et al. (1985) found a biocide, thiaruburine A from *Aspilia mossambicensis*, which had been suggested to be another medicinal plant used by chimpanzee (Wrangham and Nishida, 1983). Page et al. (1992) recently isolated kaurenoic acid and grandiflorenic acid from *A. mossambicensis*. Both acids possess potent uterotonic activity together with antibacterial and antihepatotoxic activities, suggesting further possible medicinal value for chimpanzees. Thus,

wild chimpanzees may use a wide variety of plants for their medicinal effects. For a better understanding of the chemical–ecological interaction between chimpanzees and plants, more extensive studies based upon collaboration between researchers of several areas such as ethology, phytochemistry, pharmacognosy, pharmacology, and parasitology, are needed.

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PRODUCTION OF CARDENOLIDES VERSUS SEQUESTRATION OF PYRROLIZIDINE ALKALOIDS IN LARVAE OF *Oreina* SPECIES (Coleoptera, Chrysomelidae)

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Abstract—Adult leaf beetles of the genus *Oreina* are known to be defended either by autogenously produced cardenolides or by pyrrolizidine alkaloids (PAs) sequestered from the food plant, or both. In this paper we analyze larvae of different *Oreina* species and show that the larvae contain the same defensive toxins as the adults in quantities similar to those released in the adults' secretion. Both classes of toxins are found in the body and hemolymph of the larvae, despite their different origins and later distribution in the adults. Larvae of sequestering species differed in their PA patterns, even though they fed on the same food plants. The concentration in first-instar larvae of a PA-sequestering species was similar to that in fourth-instar larvae. In all stages examined, the amount of PAs per larva did not greatly exceed the estimated uptake of one day. Eggs of two oviparous species contained large concentrations of the adult's toxins, while neonates of a sequestering larviparous species had no PAs.

Key Words—*Oreina* spp., Coleoptera, Chrysomelidae, larval defense, cardenolides, pyrrolizidine alkaloids, sequestration.

INTRODUCTION

Leaf beetles show a great diversity of chemical defenses (Pasteels, 1993). In the adults, the toxic or deterrent substances are often associated with conspicuous aposematic coloration. The alpine genus *Oreina* (Chevrolat) (Coleoptera, Chrysomelidae, Chrysomelinae), which combines brilliant metallic coloration with toxic secretions (Pasteels et al., 1989), is a good example of this. The secretion

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of the adult *Oreina* contains either autogenously produced cardenolides (e.g., in *O. bifrons*, *O. gloriosa*, *O. luctuosa*, *O. speciosa*, and *O variabilis*) or pyrrolizidine alkaloid N-oxides (PAs) sequestered from the host plant (e.g., in *O. cacaliae*); both sorts of compounds occur together in some species (e.g., *O. elongata*, *O. intricata*, and *O. speciosissima*). Sequestration of PAs occurs only in species feeding on Senecioneae (Asteraceae), while most species of the genus feed on Apiaceae or Asteraceae devoid of PAs and synthesize cardenolides (Pasteels and Rowell-Rahier, 1991; Rowell-Rahier et al., 1991; Pasteels et al., in preparation). Thus, adult *Oreina* are apparently well defended against predators (summarized in Table 1).

However, the juvenile stages appear to be more vulnerable to predation. The larvae of *Oreina* do not have any mechanical protection and do not possess defensive glands, in contrast to the larvae of other tribes of the Chrysomelidae (Pasteels et al., 1984; Pasteels and Rowell-Rahier, 1991). The lack of defensive glands, however, does not exclude the possibility that the larvae store toxins in their body. In *O. gloriosa* the production of cardenolides starts in the larval instars (Eggenberger and Rowell-Rahier, 1993b). In some species of the related genus *Chrysolina* (Motschulsky), the larvae also produce cardenolides (Daloze and Pasteels, 1979, Hilker et al., 1992), yet in *Chrysolina* (*Dlochrysa*) *fastuosa* the adults do but the larvae do not (Pasteels, personal communication). The PAs that are sequestered by several *Oreina* species are also present in the larval food, since the larvae feed on the same plants as the adults. Whether the larvae are able to store PAs or not has not been investigated previously.

The site of storage of toxins in the larvae poses an interesting problem. In adult *Oreina*, cardenolides are found exclusively in glands producing defensive secretions. The larvae, however, do not possess these glands and the carden-

TABLE 1. DEFENSE CHARACTERISTICS OF ADULTS OF *Oreina* SPECIES

Species	Food plants	Mode of defense	Site of storage
O. bifrons *O. gloriosa* *O. luctuosa* *O. speciosa* *O. variabilis*	Apiaceae or Asteraceae without PA[a]	production of cardenolides	glands only
O. cacaliae	Asteraceae mostly with PA (Senecioneae)	sequestration of PA	body and glands
O. elongata *O. intricata* *O. speciosissima*	Asteraceae with and without PA	sequestration of PA + production of cardenolides	

[a]PA = pyrrolizidine alkaloids.

secretions. The larvae, however, do not possess these glands and the cardenolides must therefore be located elsewhere. In adults of the PA-sequestering species, on the other hand, the defensive compounds are not restricted to the glands but are also found in other parts of the body (Pasteels et al., 1992).

In this paper, we analyze the defensive toxin content of larvae of several *Oreina* species. Each species is tested for the presence of the compounds also found in adults, and the toxin concentration of the body tissue, the gut, and the hemolymph is assessed separately. We compare the content of species whose adults produce cardenolides de novo and species whose adults can sequester PAs from the food plant. Most of the species investigated here are larviparous, but two are oviparous. In the latter two, a transfer of defensive toxins from the female to the eggs should be beneficial for the offspring (Orians and Janzen, 1974). We therefore compare the toxin content of the eggs of the two oviparous species with the neonate of a larviparous species.

METHODS AND MATERIALS

Beetles and Food Plants. Our nomenclature follows the revision of the genus *Oreina* by Bontems (1978, 1981, 1984). Adult beetles were collected in June and July 1991 and 1992 at the following field sites: *Oreina bifrons* near Brülisau (Appenzell, Switzerland, 900 m); *O. cacaliae*, *O. speciosissima*, and *O. variabilis* near Zastler (southern Black Forest, Germany, 900 m); *O. elongata* at the Col du Lautaret (French Alps near Briançon, 1900 m) and at the Mattmark dam (Valais, Switzerland, 2400 m); *O. gloriosa* near Saas Grund (Valais, Switzerland, 1800 m); *O. intricata* and *O. speciosa* near Tschiertschen (Graubünden, Switzerland, 1800 m); and *O. luctuosa* near Hasslach (northern Bavaria, Germany, 500 m). Two populations of *O. elongata* were examined, because they live on different host plants in the field, one containing PA (*Adenostyles alliariae*, Col du Lautaret population) and one devoid of PA (*Cirsium spinosissimum*, Mattmark population). In the laboratory, the offspring of the beetles were raised in groups of approximately 20 individuals at 17°C on the food plants detailed below. Except for neonate and first- and third-instar larvae of *O. cacaliae*, all larvae were fed until the third day after the third (and last) molt, then starved for one day to empty the gut, killed by freezing, and stored at −20°C.

For cardenolide analysis, larvae of species that do not feed on PA-containing plants were raised on their most common field host: *O. bifrons*, *O. speciosa*, and *O. variabilis* on *Chaerophyllum hirsutum*; *O. gloriosa* on *Peucedanum ostruthium*; and *O. luctuosa* on *Centaurea nemorensis*. Larvae normally feeding on PA-containing plants were raised, when possible, on a host with no or low PA content: *O. elongata* on *Cirsium spinosissimum* (no PAs, field host of the Mattmark population), *O. cacaliae* on *Petasites albus* (no PAs), *O. speciosis-*

sima on *Adenostyles alliariae* (with PAs), and *O. intricata* on *Senecio fuchsii* (with PAs).

For PA analysis larvae were raised on *Adenostyles alliariae* to allow a comparison of PA concentrations. Additionally, larvae of *O. cacaliae* and *O. intricata* were raised on *Senecio fuchsii*, because both plants are common hosts in the field but are characterized by different PAs (Rowell-Rahier et al., 1991).

Plants were collected in the field at least once a week (*S. fuchsii*, *A. alliariae*, *P. albus*, *C. hirsutum*, and *C. nemorensis* at the Zastler field site; *C. spinosissimum* and *P. ostruthium* at the Mattmark field site) and kept in the refrigerator until used.

Dissection. Fourth-instar larvae were cut ventrally and the hemolymph collected in capillary tubes and stored in ethanol. The gut was removed and the body washed thoroughly in distilled water to remove remaining gut content and hemolymph. In the samples dissected for analysis of PAs, larvae that had not been starved were used and the guts collected separately. Additionally, exuviae of fourth-instar larvae of *O. cacaliae* were collected. In the samples dissected for cardenolide analysis, the gut and its content were not analyzed separately, as the uptake of cardenolides from the host plant can be excluded (Pasteels and Daloze, 1977; Van Oycke et al., 1987; Pasteels et al., 1992).

Cardenolide Extraction. As the cardenolides in the adult secretion comprise a large polarity range, including rather polar cardenolides, the usual extraction and purification methods based on chloroform extraction (e.g., Duffey and Scudder, 1972, Brower et al., 1982) did not appear appropriate.

Pooled samples of about 20 larvae were lyophilized, weighed, and homogenized in 5 ml methanol. Following centrifugation, the supernatant was evaporated to dryness and redissolved in 400 μl methanol, then 2 ml diethylether was added. Purification was effected using a silica gel column (glass pipet 0.85 cm diameter, 0.5 g silica gel 60 Macherey Nagel 0.04–0.063 mm). The extract was added to the column and eluted first with 15 ml diethylether and secondly with 30 ml diethylether–methanol (2:3). The latter fraction was dried and redissolved in 1 ml methanol for spectrophotometry.

We tested this purification method using the highly polar ouabain and the moderately polar digitoxin as standards that correspond to the polarity range of the cardenolides in the beetles' secretions. One hundred percent of these standards appeared in the diethylether–methanol fraction.

Spectrophotometry of Cardenolides. We modified the spectrophotometric method of Brower et al. (1982), using 3,5-dinitrobenzoic acid (Rowson, 1952) instead of 2,2′,4,4′-tetranitrodiphenyl. The 1-ml sample was divided between two cuvettes, 500 μl of 2% 3,5-dinitrobenzoic acid in methanol was added to the sample cuvette but not to the reference cuvette, and methanol was added to both to give a total volume of 1.7 ml. Then, 300 μl of 3% KOH in methanol was added to both cuvettes, which starts the reaction in the sample cuvette. The

difference in absorption at 535 nm between the two cuvettes was measured after exactly 10 mins, when the absorption is at its peak. With every set of measurements, a series of digitoxin standards (0 to 200 μg) was run. The concentrations of cardenolides in the samples were calculated in digitoxin equivalents, based on a regression equation derived from the standard values. The absorption increases linearly with increasing concentration within the range of 1–500 μg digitoxin. The calculated cardenolide content of twin samples derived from one original sample, that were extracted and measured in parallel, diverged by no more than 10%. Repeated extractions of identical samples gave a precision of 8.21 $\pm$ 2.94 μg cardenolides per larva in *O. variabilis* ($N = 6$, SD given) and of 18.11 $\pm$ 3.82 μg in *O. luctuosa* ($N = 4$). All twofold replications diverged by less than 10%, with one exception mentioned below.

PA Extraction. We followed the extraction and cleaning procedure described by Mattocks (1986). Pooled samples of larvae (about 20 individuals/sample), eggs, or body parts were lyophilized or cut and oven dried at 60°C, crushed, and weighed. The powder was then extracted with sulfuric acid and zinc dust, which also reduces PA N-oxides to tertiary PAs. After filtering and washing with petroleum ether, the solution was brought to pH 10 with ammonia and the PAs extracted with dichloromethane and evaporated to dryness. The precipitate was redissolved in 300 μl dichloromethane for TLC analysis.

Hemolymph samples were not submitted to the whole extraction process, because they have less interfering substances than the whole larvae and PAs are already in solution. They were centrifuged and the supernatant evaporated and redissolved in 300 μl ethanol for TLC analysis. No reduction of PA N-oxides was made in this case. The color reaction does not discriminate between tertiary PAs and N-oxides.

TLC Analysis. Samples were spotted on silica gel 60 plates (Merck) with a Camag Linomat III TLC Applicator. On each plate, samples of 2, 4, 8, and 16 μg seneciphylline (Roth AG) or monocrotaline (Sigma) were included as standards. The plates were developed and spotted according to the procedure described by Mattocks (1967, 1986). Spot intensity was measured with a Shimadzu CS-930 TLC Scanner. The minimum quantity detectable is 2 μg. For the precision of the method, see Speiser and Rowell-Rahier (1991). All PA concentrations are given in micrograms seneciphylline equivalents, which is the main PA of *Adenostyles alliariae* (Rowell-Rahier et al., 1991). When monocrotaline was used as a standard, seneciphylline equivalents were calculated later using a direct comparison of the absorption of both alkaloids.

RESULTS

Content of Cardenolides. Cardenolides were detected in the larvae of all species in which the adults also produce these compounds (Table 2). These include three species, *O. intricata*, *O. elongata*, and *O. speciosissima*, in which

TABLE 2. CARDENOLIDE CONTENT OF FOURTH INSTAR LARVAE[a]

	Species[b]	N	Cardenolide (μg/g dry wt)	Cardenolide (μg/larva)
I	*O. bifrons*	1	254	4.4
	O. gloriosa	2	565	9.9
	O. luctuosa	4	1840	18.1
	O. speciosa	2	389	5.1
	O. variabilis	6	559	8.2
II	*O. elongata* CL[c]	1	414	3.2
	O. elongata MM[d]	1	569	3.3
	O. intricata	1	1200	12.6
	O. speciosissima	2	621	5.8
III	*O. cacaliae*	1	51	0.6

[a] N = number of samples analysed; cardenolide = cardenolide content in digitoxin equivalents.
[b] I: adults produce only cardenolides, II: adults have cardenolides and PAs, III: adults have only PAs.
[c] CL = Col du Lautaret population.
[d] MM = Mattmark population.

the adults have a mixed defensive strategy and produce cardenolides as well as sequester PAs. The cardenolide concentrations of the different species varied by a factor of seven, with two exclusive cardenolide producers, *O. luctuosa* and *O. bifrons*, at both extremes. *O. cacaliae* larvae had only a low concentration of cardenolides. In this species, the adults do not possess cardenolides. The biological significance of this finding remains unclear. Possibly, the detected concentration lies within the background noise of our method.

The analysis of body and hemolymph of fourth-instar larvae of *O. gloriosa* showed that cardenolides were present in the body tissue at a concentration of 997 μg/g dry weight and in the hemolymph at a concentration of 300 μg/ml. Compared to the concentration of 565 μg/g dry weight found in extracts of whole larvae (Table 2), the concentration in the body alone is higher by a third, which indicates that most of the cardenolides are present in tissue consisting of integument, fat body, and muscles.

Sequestration of PAs and PA Pattern in Larvae. In all species sequestering PAs as adults, the larvae contained large amounts (Table 3). However, the concentration found in the larvae varied by more than a factor of 10 between species, or even between larvae of a single species reared on different plants. On the other hand, the two populations of *O. elongata* reared on *Adenostyles alliariae* contained similar concentrations of PAs, although one population (Col du Lautaret) lives on *A. alliariae* in the field while the other one (Mattmark)

TABLE 3. PA CONCENTRATIONS IN FOURTH-INSTAR LARVAE

Species[a]	Food plant	PA (μg/g dry wt)	PA (μg/larva)	Spot[b]
O. cacaliae	*A. alliariae*	4200	61	b
O. cacaliae	*S. fuchsii*	500	5	b
O. elongata CL	*A. alliariae*	1200	10	d
O. elongata MM	*A. alliariae*	1400	8	d
O. intricata	*A. alliariae*	1600	26	a, b
O. intricata	*S. fuchsii*	7300	81	a, b, c, d
O. speciosissima	*A. alliariae*	2400	18	d

[a] MM = Mattmark population, CL = Col du Lautaret population.
[b] Spot refers to the position on the plate: a, apolar spots, traveling shortly behind the solvent front (R_f = 0.69–0.86); b, spot at same height as seneciphylline standard (R_f = 0.45–0.58); c, more polar spot, halfway between seneciphylline and the baseline (R_f = 0.35); d, polar spots in the range of seneciphylline-N-oxide (R_f = 0.14–0.26).

lives on *Cirsium spinosissimum* and does not encounter *A. alliariae*. Similarly, *O. speciosissima* larvae, which in the field mostly feed on *Petasites albus*, a plant without PAs, contained concentrations similar to those found in the well-adapted PA plant feeders *O. cacaliae* and *O. intricata*, when fed on *A. alliariae*. In these latter species, the host plant species seems to influence the PA content of the larvae in a species-specific way: *O. cacaliae* larvae fed on *A. alliariae* had about 10 times higher concentrations of PAs than larvae raised on *Senecio fuchsii*. In *O. intricata*, on the contrary, larvae fed on *S. fuchsii* had four times higher concentrations of PAs than larvae fed on *A. alliariae*. The PA pattern in the two species also differed: extracts of *O. cacaliae* larvae from both plants showed only one spot at the height of the seneciphylline standard. This is the main PA component of *A. alliariae* (Rowell-Rahier et al., 1991) and the only one visible in our TLC of extracts of this plant. *O. intricata* larvae, on the other hand, showed additional components (one less polar spot in larvae from both plants, and a further more polar spot only in larvae from *S. fuchsii*). Seneciphylline was not found in *S. fuchsii* (Rowell-Rahier et al., 1991), and TLC of extracts of this plant gave three spots, all more polar than seneciphylline. Direct comparison of an extract of *O. intricata* larvae and an extract of *S. fuchsii* showed that the most polar spot of the larvae and the least polar spot of the plant extract are at the same height.

The dissection of *O. cacaliae* larvae raised on *Adenostyles alliariae* showed that PAs are found both in the body (at a concentration of 5000 μg PA/g dry weight) and in the hemolymph (940 μg PA/ml). No PAs could be detected in the gut of larvae raised on *A. alliariae*, although the sample of guts from 20 larvae was analyzed and the detection threshold of the method lies at 2 μg PA. Small concentrations, 190 μg PA/g dry weight, were detected in exuviae of

fourth-instar larvae. However, this accounted for only 0.3 μg PA per larva. The concentration in the hemolymph of larvae raised on *S. fuchsii* was similar and amounted to 1000 μg PA/ml. Hemolymph samples of *O. elongata* larvae, on the other hand, differed widely in concentration: in larvae of the Mattmark population we found 700 μg PA/ml, while larvae of the Col du Lautaret population had 1900 μg PA/ml, despite the similar content in extracts of whole larvae of the two populations.

Toxin Content of Eggs and Young Larvae. Eggs of the two oviparous species, *O. elongata* and *O. luctuosa*, contained high concentrations of cardenolides and of PAs in *O. elongata* fed on *A. alliariae* (Table 4). For *O. luctuosa* and *O. elongata* (Mattmark) the cardenolide concentrations in microgram per gram dry weight are in the upper range of the concentrations found in the fourth-instar larvae. For *O. elongata* (Col du Lautaret) the two samples analyzed differed too much to pool them—the cause of this difference is unclear. Nevertheless, both values are smaller than that of the *O. elongata* (Mattmark) population, which has to rely exclusively on cardenolides, as its food plant (*Cirsium spinosissimum*) is devoid of PAs. The eggs of the Col du Lautaret population, on the other hand, contained large amounts of PAs besides the cardenolides.

In the larviparous *O. cacaliae*, we analyzed different life stages (Table 5). No PAs could be detected in the neonate just after birth and before they started feeding (sample of 88 neonate compared to 33 1-day-old larvae). Yet, after one day of feeding on *A. alliariae*, the larvae contained concentrations similar to those of fourth-instar larvae (see Table 3). The mean concentration in *A. alliariae* leaves from the Zastler field site at the end of May is 1130 μg PA/g fresh weight (Speiser, unpublished). The amount detected in the 1-day-old larvae corresponds to a consumed leaf area of 2.9 mm^2, which fits well with the observed feeding capacity of young larvae. The larvae used in this analysis had not been starved, so that a contribution of the gut content to the measured PAs can not be excluded; in fourth-instar larvae, however, no PAs were found in the gut. The PA concentrations found in *O. cacaliae* larvae were highest in the

TABLE 4. CONTENT OF DEFENSIVE TOXINS OF EGGS OF TWO OVIPAROUS SPECIES

Species[a]	Egg weight (mg fresh wt)	Cardenolide		PA	
		μg/g dry wt	μg/egg	μg/g dry wt	μg/egg
O. elongata CL	0.77	500/1400	0.2/0.5	16700	4.5
O. elongata MM	0.70	1700	0.5		
O. luctuosa	1.27	1800	1.0		

[a] MM = Mattmark population, the females had been feeding on *C. spinosissimum*; CL = Col du Lautaret population, the females had been feeding on *A. alliariae*.

TABLE 5. CONTENT IN PA OF DIFFERENT LIFE STAGES OF *O. cacaliae* RAISED ON *Adenostyles alliariae*

Age	PA	
	μg/g dry wt	μg/larva
Neonate	ND	ND[a]
1 day old	4100	0.7
Third instar	6100	20.2

[a]ND = not detectable.

third instar. Expressed as consumed leaf area, the amount per larva would correspond to 76 mm^2 in the third instar and to 230 mm^2 in the fourth instar. As in the case of the 1-day-old larvae, these values roughly agree with the estimated daily consumption.

DISCUSSION

In all species examined, the larvae contain the same toxins as those found in the adult's defensive secretions. The quantities of cardenolides detected in *O. gloriosa* larvae are in agreement with those detected by HPLC (Eggenberger and Rowell-Rahier, 1993b). No differences in larval cardenolide content exist between solely cardenolide producing species and the species in which the adults can also sequester PAs. In *O. cacaliae*, a species in which the adults sequester only PAs, the concentration of cardenolides detected in the fourth-instar larvae is most likely too small to be considered a reliable result. In the species having cardenolides as adults, the amounts present in the last-instar larvae are roughly similar to those released in one adult secretion. Secretions of *O. speciosissima* contain between 2.5 and 6 μg cardenolides (Rowell-Rahier et al., 1991). Adults of *O. gloriosa* release, depending on age and sex, between 4 and 23 μg cardenolides in their pronotal secretions (Eggenberger and Rowell-Rahier, 1993a), and in *O. elongata* 1 μg only (*O. elongata* Mattmark populations; Rowell-Rahier et al., unpublished data). However, in these two species only the secretions released by the glands on the pronotum were quantified. This makes up approximately 50% of the total quantity released on both pronotum and elytra (in *O. gloriosa*, Eggenberger, unpublished). In *O. luctuosa*, in which the highest concentrations and amounts per larvae were detected, the amount of cardenolides in the adult secretion is so far unknown.

In contrast to the species producing cardenolides, the amount of PAs released in the secretion of the sequestering species is only part of the quantity

present in the beetles, as most of the PAs are stored in the body (Rowell-Rahier et al., 1991; Pasteels et al., 1992). Adult *O. cacaliae* collected on *Adenostyles alliariae* released on average 3.4 μg PA/secretion and had additionally 16.6 μg stored in the body. The amount detected in the corresponding larvae was three times higher than the total in the adults. On the other hand, *O. cacaliae* larvae raised on *Senecio fuchsii* and *O. speciosissima* larvae fed on *A. alliariae* contained more PAs than the adult secretion, but less than the adult's body. While adults of *O. cacaliae* and of *O. speciosissima* fed on *A. alliariae* sequestered PAs equally well in the secretion (Rowell-Rahier et al., 1991; Ehmke et al., 1991), the concentrations found in the larvae of *O. speciosissima* fed on *A. alliariae* were only half those detected in *O. cacaliae* on the same plant. This might reflect a difference in intake, since *O. speciosissima* larvae feed and grow less well on *A. alliariae* than *O. cacaliae* larvae and prefer the non-PA plant *P. albus* in the field (Rowell-Rahier et al., 1991, and unpublished observations).

In the adults of *O. cacaliae* and *O. speciosissima*, the concentration of PA in the secretion is dependent on the food plant; only certain PAs—namely the PAs present in *A. alliariae*—can reach the secretory glands, but storage in the body is less specific (Rowell-Rahier et al., 1991). Dependence on the food plant was also observed in the larvae: *O. cacaliae* larvae raised on *A. alliariae* had nearly 10 times the concentration of PAs of larvae fed on *S. fuchsii*. No difference detectable by TLC was seen in the PA pattern of larvae fed on the two plants. *O. intricata* larvae, on the other hand, had about five times higher concentrations on *S. fuchsii* than on *A. alliariae*. The difference in concentration between *O. cacaliae* and *O. intricata* larvae raised on *S. fuchsii* or on *A. alliariae* could be caused by differences in their ability to handle the different PAs typical of these plants. Selective sequestration of plant compounds not detected by TLC of plant extracts, or metabolic transformation of plant PAs, could explain the differences in the PA pattern between plant and larval extracts, which were also observed in *O. elongata* and *O. speciosissima*. In adult *O. cacaliae* no evidence for the metabolism of plant PAs was observed. The PAs found in the body of the beetles mirror the pattern present in the food plant. However, in *O. speciosissima*, a PA not present in the plant was found in the secretion, and metabolism is possible (Rowell-Rahier et al., 1991). Metabolism of plant PAs is the rule rather than the exception in arctiid moths (L'Empereur et al., 1989; Ehmke et al., 1990; Hartmann et al., 1990; Trigo et al., 1993), while an aphid and a coccinellid feeding on the aphid exactly mirrored the PA pattern of the aphid food plant (Witte et al., 1990).

In *O. gloriosa* larvae large proportions of the total toxins were contained in the body (=integument, muscles, and fat body). This agrees with findings in the milkweed bug (Scudder et al., 1986) and in the monarch butterfly (Brower and Glazier, 1975; Brower et al., 1988), both cardenolide-sequestering species, where the integument is the major storage site. Likewise, in ithomiid butterflies

(Brown, 1984) and arctiid moths (Egelhaaf et al., 1990, Ehmke et al., 1990) most of the sequestered PAs are found in the integument. Our data on the distribution of the PAs between body hemolymph are not clearly interpretable. Hemolymph of *O. cacaliae* larvae reared on *A. alliariae* and on *S. fuchsii* had similar concentrations of PAs, although the total concentration in the larvae differed by a factor of 10. Concentration of PAs in the hemolymph of *O. elongata* larvae differed by a factor of three between two populations with otherwise similar total concentrations. Possibly the hemolymph is only involved in transportation of the PAs. Interestingly, in *O. cacaliae* larvae reared on *A. alliariae*, no PAs could be detected in the gut. The uptake of PAs could occur early in the digestion and might be followed by rapid degradation or immobilization of remaining alkaloid in the gut. In adult *O. cacaliae*, radioactively labeled senecionine-N-oxide was found in the feces; however, a large proportion could neither be recovered from the insects nor from the feces (Ehmke et al., 1991) but was apparently transformed into a methanol-insoluble form.

The eggs of *O. elongata* and *O. luctuosa* contained cardenolides, as in the eggs of cardenolide-producing species of the genus *Chrysolina* (Daloze and Pasteels, 1979; Hilker et al., 1992). In the adults of the larviparous species *O. gloriosa*, on the other hand, no cardenolides could be found in the body, but were restricted to the defensive glands (Pasteels et al., 1992). However, the cardenolides are most likely stored in the eggs by maternal transfer. This should be the case in the oviparous *O. elongata* and *O. luctuosa*, whereas in the larviparous *O. gloriosa* this transfer is absent (otherwise cardenolides should be found in the body, which includes the reproductive organ). Similarly, PAs occur in the eggs of *O. elongata* of the population that had been feeding on *A. alliariae* in the field, yet no PAs were found in neonate larvae (before feeding) of the larviparous *O. cacaliae*. In this species, maternal transfer of toxins to the offspring might not be important, since the larvae feed on PA-rich plants immediately after birth and after one day contained concentrations similar to those of fourth-instar larvae. The difference in toxin transfer between ovi- and larviparous species could be explained if the toxins found in the eggs were located in the outer shell, which is reduced to the endochorion in the larviparous species (Bontems, 1989).

The concentrations of both PAs and cardenolides in *Oreina* larvae are far lower than those found in other insects in which a defensive function of the toxins could be shown [e.g., up to 20% dry weight of PAs in Ithomiinae (Brown, 1984); 0.9–3.4% dry weight of cardenolides in the monarch butterfly (Malcolm et al., 1989)]. Nevertheless, the PA and cardenolide quantities present in the larvae are equal to or higher than those in the adult secretion and higher than the amount of PAs stored in the body of *O. cacaliae*. Feeding experiments with adult cardenolide-producing *O. gloriosa* and PA-sequestering *O. cacaliae* and red-winged blackbirds (*Agelaius phoeniceus*) showed that the birds were deterred

from feeding if the beetle's secretion had not been removed. Moreover, adults of *O. cacaliae* from which the secretion had been removed still proved to be deterrent due to the PAs stored in the body (Rowell-Rahier et al., in preparation). The predator spectrum of the larvae certainly differs from that of the adults, and small invertebrate predators such as spiders and ants should be more important predators on the larvae than on the adults. As shown for *O. gloriosa* larvae (Eggenberger and Rowell-Rahier, 1993b), the pattern of cardenolides differs between larvae and adults, and it remains unclear how this affects the larval defense. However, at least in some species, the larvae are aposematic, either uniformly black (*O. luctuosa*, *O. elongata*) or bicolored with head and/or pronotum contrasting with the abdomen (yellow and black in last-instar larvae of *O. cacaliae*, *O. gloriosa*, *O. speciosissima*, and *O. variabilis*, black and white in *O. intricata*). The possible warning function of the coloration in these species and the potential crypsis of other species with sandy colored larvae (*O. speciosa*, *O. bifrons*), await a better understanding but correlate with high and low levels of defensive toxins.

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Trans-CERALURE ISOMERS: DIFFERENCES IN ATTRACTION FOR MEDITERRANEAN FRUIT FLY, *Ceratitis capitata* (WIED.) (DIPTERA: TEPHRITIDAE)

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Abstract—Differences in attractiveness of four individual *trans* isomers of ceralure (CRL) [ethyl 4- (and 5-) iodo-*trans*-2-methylcyclohexane-1-carboxylate] for male Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann), were investigated. One of the isomers, CRL-B1 (ethyl *cis*-5-iodo-*trans*-2-methylcyclohexane-1-carboxylate) was significantly superior to the three other *trans*-CRL isomers, CRL, trimedlure (TML) [1,1-dimethylethyl 4- (and 5-) chloro-*trans*-2-methylcyclohexane-1-carboxylate], and TML-C (1,1-dimethylethyl *cis*-4-chloro-*trans*-2-methylcyclohexane-1-carboxylate) on an equal weight basis.

Key Words—Insecta, Diptera, Tephritidae, *Ceratitis capitata*, attractant, ceralure, trimedlure, lure, medfly.

INTRODUCTION

Ceralure (CRL), ethyl 4- (and 5-) iodo-*trans*-2-methylcyclohexane-1-carboxylate (McGovern and Cunningham, 1987) is a mixture of iodo isomers analogous to the group of chloro isomeric compounds known collectively as trimedlure (TML) [1,1-dimethylethyl 4- (and 5-) chloro-*trans*-2-methylcyclohexane-1-carboxylate] (McGovern and Beroza, 1966), both of which are attractants of the male Mediterranean fruit fly (medfly), *Ceratitis capitata* (Wiedemann). The

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medfly is a worldwide pest of 253 varieties of fruits, nuts, and vegetables (Hagen et al., 1981; Jackson and Lee, 1985). It is found predominantly in Hawaii and Central America, but because it periodically invades the mainland of the United States, there is a great need for monitoring and controlling this pest (McGovern and Cunningham, 1988).

CRL was discovered by McGovern and Cunningham (1987; DeMilo et al., 1994a) while investigating various halogen and ester analogs of TML to find a better attractant for the medfly than the standard TML. Replacement of the chlorine of TML with fluorine, bromine, or iodine gave substances that showed equivalent initial attractiveness to TML, except for the weakly attractive iodo analog; only bromo TML (Warthen and McGovern, 1989) appeared to be persistent, but less attractive than TML. It was not until various ester analogs of the halogen analogs of TML were investigated, that McGovern and Cunningham (1987) and DeMilo et al. (1994a) discovered that the ethyl ester of iodo TML was the most attractive and persistent of the tested samples (McGovern and Cunningham, 1988).

As with TML, the synthesis of CRL results in a preponderance (90–95%) of four *trans* isomers, as with TML (McGovern et al., 1986), where the *trans* designation refers to the diequatorial relationship between the vicinal 1-carboxylic ester group and the 2-methyl group (Figure 1). The four *trans*-CRL isomers are arbitrarily designated A, B1, B2, and C (McGovern and Cunningham, 1987; Warthen and McGovern, 1990; DeMilo et al., 1994a,b) as with TML (McGovern and Beroza, 1966), with the iodine atom in the 4- or 5-position being either equatorial or axial (DeMilo et al., 1994c). The remaining 5–10% of CRL consists of four *cis* isomers (Figure 1) (Warthen and McGovern, 1990) that would follow the convention of V, W, X, and Y that was used in naming the *cis*-TML

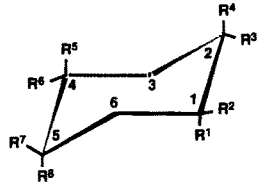

FIG. 1. *trans*- (A, B1, B2, C) and *cis*- (V, W, X, Y) CRL isomers. CRL-A: $R^2 = CO_2Et$, $R^3 = CH_3$, $R^8 = I$, $R^1 = R^4 = R^5 = R^6 = R^7 = H$; CRL-B1: $R^2 = CO_2Et$, $R^3 = CH_3$, $R^7 = I$, $R^1 = R^4 = R^5 = R^6 = R^8 = H$; CRL-B2: $R^2 = CO_2Et$, $R^3 = CH_3$, $R^6 = I$, $R^1 = R^4 = R^5 = R^7 = R^8 = H$; CRL-C: $R^2 = CO_2Et$, $R^3 = CH_3$, $R^5 = I$, $R^1 = R^4 = R^6 = R^7 = R^8 = H$; CRL-V: $R^1 = CO_2Et$, $R^3 = CH_3$, $R^7 = I$, $R^2 = R^4 = R^5 = R^6 = R^8 = H$; CRL-W: $R^1 = CO_2Et$, $R^3 = CH_3$, $R^5 = I$, $R^2 = R^4 = R^6 = R^7 = R^8 = H$; CRL-X: $R^1 = CO_2Et$, $R^3 = CH_3$, $R^6 = I$, $R^2 = R^4 = R^5 = R^7 = R^8 = H$; and CRL-Y: $R^2 = CO_2Et$, $R^4 = CH_3$, $R^7 = I$, $R^1 = R^3 = R^5 = R^6 = R^8 = H$.

isomers (McGovern et al., 1986; Leonhardt et al., 1982). These *cis* isomers have an axial–equatorial relationship between the vicinal 1-carboxylic ester group and the 2-methyl group, as with TML (McGovern et al., 1986; Warthen et al., 1987).

The medfly attractiveness of TML-like compounds is dependent upon a specific trisubstitution on a cyclohexane ring; the location and conformation of the halogen on the ring plays a big role in the differences in attractiveness of the various isomers (McGovern and Cunningham, 1987; DeMilo et al., 1994a). The semipreparative high-performance liquid chromatographic (HPLC) separation of the four *trans*-TML isomers (Sonnet et al., 1984) and the analytical HPLC separation of the *cis*-TML isomers (Warthen and McGovern, 1986) preceded the subsequent semipreparative HPLC separation of all eight TML isomers (Warthen and McGovern, 1988). The latter separation made possible a field study on the relative attractiveness of all eight TML isomers; the order of attractiveness was C > A > Y > B1 > V > X > W > B2 (McGovern et al., 1990). More specifically, the two most attractive *trans* enantiomers are (1*S*,2*S*,4*R*)-TML-C and (1*R*,2*R*,5*S*)-TML-A, the former being more attractive (Sonnet et al., 1984; Doolittle et al., 1991); the individual *cis* enantiomers have not yet been synthesized for biological evaluation to complete the study on relative attractiveness of enantiomers. The quantitative structure–activity relationship of the eight-isomer (racemic) TML series also was studied via computer molecular modeling (Warthen et al., 1993). The study revealed that medfly catch correlates well with the ratio of TML molecular volume to surface area with modulation of the molecular surface area by a torsion angle and an average of two interatomic distances.

This paper evaluates the differences in attractiveness of the four *trans*-CRL isomers and compares them with TML-C, standard TML, and CRL via two field tests.

METHODS AND MATERIALS

Chemicals. The TML, the attractant standard, came from a commercial lot (United Oil Products, Chemical Division, East Rutherford, New Jersey). CRL was synthesized (McGovern and Cunningham, 1988) for field use; the sample was stored over a copper coil to prevent discoloration. Commercial CRL AG was synthesized by AgriSense, Fresno, California. TML-C (98.7%+ pure) was isolated and purified from a TML mixture by HPLC (Warthen and McGovern, 1988). CRL-B1 and -B2 were isolated from a CRL mixture by HPLC. The *t*-butyl esters of CRL-A and -C were isolated via HPLC from a *t*-butyl ester mixture of CRL; they were then hydrolyzed and converted to the ethyl esters (CRL-A and -C). All of the CRL isomers were 95%+ purity (Warthen and McGovern, 1990; DeMilo et al., 1994b).

Bioassay. Field tests were conducted in Keaau, Hawaii. The differences in attractiveness of CRL isomers (A, B1, B2, and C), TML, TML-C, and CRL were determined in two field tests (Tables 1 and 2, Figures 2 and 3) of nine-day duration each during January 1990. Clear CRL samples over a copper coil were utilized. The liquid test samples were pipetted in 10-μl ($\approx$ 10 mg) amounts onto cotton dental-roll wicks (9.53 mm diameter $\times$ 12.7 mm length; Johnson & Johnson No. 2); the wicks were secured in standard Jackson traps (Harris et al., 1971). Since TML-C is a solid, it was dissolved in acetone and an appropriate aliquot containing 10 mg was pipetted onto the cotton wick. No adjustments were made for the different volatilities of the CRL isomers; this was less of an issue than with TML isomers since CRL does not precipitate crystalline isomers during cool conditions (McGovern and Cunningham, 1987; DeMilo et al., 1994a). Even with TML isomers that do precipitate crystalline isomers, volatility was not considered because the attractiveness of the *trans*-TML isomers is related more to their stereochemistry than to their volatilities (McGovern et al., 1966); TML-A and TML-B1 are liquids and TML-B2 and C (the most attractive TML isomer; McGovern et al., 1990) are solids. Volatilities of the *cis*-TML isomers have not been studied, but V is a liquid, Y is a semisolid, and W and X are solids (Warthen and McGovern, 1988); semisolid TML-Y (the most active *cis*-TML isomer) is more attractive than liquid TML-V (McGovern et al., 1990).

The traps were hung on trees in a large macadamia nut orchard about 8 m apart in a randomized complete block design (one replicate per block; eight blocks) in Keaau, Hawaii. Sterile laboratory-reared *C. capitata* were released throughout the test plot at regular intervals. *C. capitata*, caught on the removable sticky inserts in the traps, were counted one day after each fly release; new sticky inserts were placed in each trap at that time. A freshly baited TML wick was also introduced into the test at the beginning of each evaluation period to permit the assessment of potency with time.

Data were analyzed by analysis of variance on untransformed catch data and means were separated by the k ratio test of mean separation (Waller and Duncan, 1969) at the $P = 0.05$ level.

RESULTS AND DISCUSSION

Results from the first attractancy test for medflies with the four *trans*-CRL isomers (A, B1, B2, and C) are presented in Table 1 and Figure 2. TML-C, CRL, fresh TML standard, and aged TML were included for comparison with each of the CRL isomers. Examination of the overall accumulation of data for nine days from Table 1 shows that the medfly attractiveness of CRL-B1 was significantly different and greater than that of CRL-A, CRL-B2, CRL-C, the

TABLE 1. MEAN CATCH OF MALE MEDITERRANEAN FRUIT FLIES WITH *trans*-CERALURE ISOMERS, TEST 1 (KEAAU, HAWAII)

Lure[a]	Mean catch (± standard error) per trap after indicated days of exposure in the field[a]								
	0	1	2	3	6	7	8	9	10
TML fresh[c,d]	101.38 ± 12.57b	67.12 ± 16.20b	88.62 ± 17.08a	98.88 ± 11.46b	86.62 ± 12.54a	96.25 ± 11.87a	96.12 ± 9.24a	95.62 ± 6.75a	129.75 ± 11.95a
TML aged[c,d]	98.12 ± 14.80b	56.00 ± 6.71bc	56.12 ± 10.11b	8.12 ± 3.70d	0.0 ± 0.0c	0.0 ± 0.0c	0.0 ± 0.0d	0.0 ± 0.0c	0.12 ± 0.12c
TM-C[e]	73.88 ± 7.78c	42.75 ± 9.56c	55.75 ± 8.97b	60.12 ± 8.97c	45.25 ± 7.45b	50.12 ± 12.17b	35.00 ± 14.88c	14.62 ± 6.39c	11.12 ± 5.89c
CRL[d,f]	86.50 ± 8.74bc	75.50 ± 9.06b	93.62 ± 10.81a	97.88 ± 9.72b	78.12 ± 9.54a	92.50 ± 10.53a	71.38 ± 10.44b	61.38 ± 10.87b	56.75 ± 10.63b
CRL-A[g]	5.12 ± 2.17d	1.12 ± 0.48d	1.38 ± 0.84c	2.12 ± 0.89d	1.88 ± 1.01c	0.0 ± 0.0c	0.0 ± 0.0d	0.0 ± 0.0c	0.0 ± 0.0c
CRL-B1[g]	123.50 ± 9.30a	105.38 ± 6.13a	103.38 ± 9.27a	143.50 ± 10.35a	93.75 ± 11.41a	106.25 ± 11.65a	104.38 ± 8.70a	71.38 ± 9.36b	44.38 ± 22.18b
CRL-B2[h]	3.12 ± 1.24d	0.25 ± 0.16d	0.50 ± 0.50c	0.25 ± 0.16d	0.25 ± 0.25c	0.0 ± 0.0c	0.75 ± 0.53d	0.0 ± 0.0c	0.0 ± 0.0c
CRL-C[h]	0.62 ± 0.37d	0.12 ± 0.12d	0.0 ± 0.0c	0.12 ± 0.12d	0.12 ± 0.12c	0.0 ± 0.0c	0.25 ± 0.25d	0.0 ± 0.0c	0.38 ± 0.37c
F test	39.380	25.751	27.119	69.551	33.602	34.638	37.816	44.449	20.241
k	20.425	20.087	21.651	17.159	18.732	21.130	18.868	14.959	26.311

[a] Initial dosage 10 μl ≈ 10 mg/wick.

[b] Means followed by the same letter within a column are not significantly different [$P > 0.05$; Waller and Duncan (1969) k ratio test of mean separation]. Randomized complete block field plot design with eight replicates. ANOVA statistics on untransformed data for all days, degrees of freedom = 7, 49.

[c] Contains 27% A, 7% B1, 18% B2, 41% C isomers (Doolittle et al., 1991).

[d] TML and CRL are mixtures of 1,1-dimethylethyl 4- (and 5-) chloro-*trans*-2-methylcyclohexane-1-carboxylate and ethyl 4- (and 5-) iodo-*trans*-2-methylcyclohexane-1-carboxylate, respectively.

[e] 1,1-Dimethylethyl *cis*-4-chloro-*trans*-2-methylcyclohexane-1-carboxylate.

[f] w/Cu coil, contains 11.83% A, 25.76% B1, 39.50% B2, 18.01% C isomers (DeMilo et al., 1994b; personal communication).

[g] CRL-A and -B1 are ethyl *trans*- (and *cis*-5-)iodo-*trans*-2-methylcyclohexane-1-carboxylate, respectively.

[h] CRL-B2 and -C are ethyl *trans*- (and *cis*-4-)iodo-*trans*-2-methylcyclohexane-1-carboxylate, respectively.

TABLE 2. MEAN CATCH OF MALE MEDITERRANEAN FRUIT FLIES WITH *trans*-CERALURE ISOMERS, TEST 2 (KEAAU, HAWAII)

Lure[a]	Mean catch (± standard error) per trap after indicated days of exposure in the field[b]								
	0	1	2	3	4	7	8	9	10
TML fresh[c,d]	134.12 ± 10.58ab	104.00 ± 8.86b	88.75 ± 11.19b	101.25 ± 8.63b	135.75 ± 15.90b	138.50 ± 12.76a	133.75 ± 20.48a	160.38 ± 16.12a	182.75 ± 13.94a
TML aged[c,d]	110.25 ± 29.68bc	88.25 ± 20.55bc	64.62 ± 11.63c	9.88 ± 5.78c	0.12 ± 0.12d	0.0 ± 0.0c	0.0 ± 0.0c	0.25 ± 0.25d	0.0 ± 0.0d
TML-C[e]	96.88 ± 9.83cd	82.50 ± 18.01bc	81.38 ± 9.67bc	82.75 ± 10.63b	98.50 ± 6.88c	64.62 ± 13.11b	73.38 ± 13.81b	76.62 ± 11.20bc	82.75 ± 10.88b
CRL[d,f]	76.12 ± 12.53d	82.12 ± 13.30bc	72.38 ± 12.78bc	83.12 ± 12.28b	105.38 ± 9.21c	68.75 ± 11.93b	78.87 ± 14.95b	70.62 ± 16.69c	74.62 ± 16.19b
CRL-A[g]	12.75 ± 3.83e	0.0 ± 0.0d	1.38 ± 0.73d	3.88 ± 1.48c	3.50 ± 0.73d	0.88 ± 0.52c	0.0 ± 0.0c	0.62 ± 0.52d	0.0 ± 0.0d
CRL-B1[g]	165.25 ± 18.37a	138.62 ± 11.61a	151.75 ± 15.69a	149.12 ± 13.80a	161.88 ± 16.36a	121.88 ± 11.54a	141.50 ± 24.13a	102.38 ± 22.28b	27.00 ± 12.73c
CRL-B2[h]	15.88 ± 4.90e	0.0 ± 0.0d	0.38 ± 0.26d	1.62 ± 0.93c	0.38 ± 0.18d	0.0 ± 0.0c	0.0 ± 0.0c	0.38 ± 0.37d	0.0 ± 0.0d
CRL-C[h]	12.12 ± 5.64e	0.0 ± 0.0d	0.88 ± 0.74d	0.88 ± 0.40c	0.12 ± 0.12d	0.62 ± 0.26c	0.0 ± 0.0c	0.0 ± 0.0d	0.0 ± 0.0d
CRL AG[i]	77.88 ± 8.66cd	73.75 ± 13.53c	75.88 ± 10.94bc	90.88 ± 9.52b	96.62 ± 12.90c	61.62 ± 15.58b	62.75 ± 11.60b	51.25 ± 10.84c	26.50 ± 11.47c
F test	18.528	21.707	38.634	55.847	60.625	33.218	28.700	24.850	43.593
k	33.093	27.968	20.623	18.496	21.181	23.826	27.345	29.043	23.393

[a] Initial dosage 10 μl ≈ 10 mg/wick.

[b] Means followed by the same letter within a column are not significantly different [$P > 0.05$; Waller and Duncan (1969) k ratio test of mean separation]. Randomized complete block field plot design with eight replicates. ANOVA statistics on untransformed data for all days, degrees of freedom = 8, 56.

[c] Contains 27% A, 7% B1, 18% B2, 41% C isomers (Doolittle et al., 1991).

[d] TML and CRL are mixtures of 1,1-dimethylethyl 4- (and 5-) cloro-*trans*-2-methylcyclohexane-1-carboxylate and ethyl 4- (and 5)-iodo-*trans*-2-methylcyclohexane-1-carboxylate, respectively.

[e] 1,1-Dimethylethyl *cis*-4-chloro-*trans*-2-methylcyclohexane-1-carboxylate.

[f] w/Cu coil, contains 11.83% A, 25.76% B1, 39.50% B2, 18.01% C isomers (DeMilo et al., 1994b; personal communication).

[g] CRL-A and -B1 are ethyl *trans*- (and *cis*-5-)iodo-*trans*-2-methylcyclohexane-1-carboxylate, respectively.

[h] CRL-B2 and -C are ethyl *trans*- (and *cis*-4-)iodo-*trans*-2-methylcyclohexane-1-carboxylate, respectively.

[i] Contains 9.10% A, 31.11% B1, 51.04% B2, 8.80% C isomers (DeMilo, personal communication).

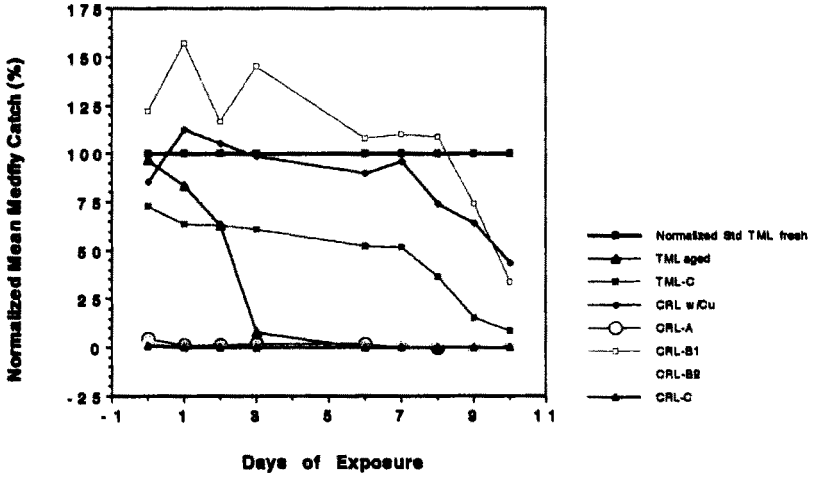

FIG. 2. Data from Table 1: means were normalized to the standard; fresh TML standard was replenished on the days of medfly catch determination.

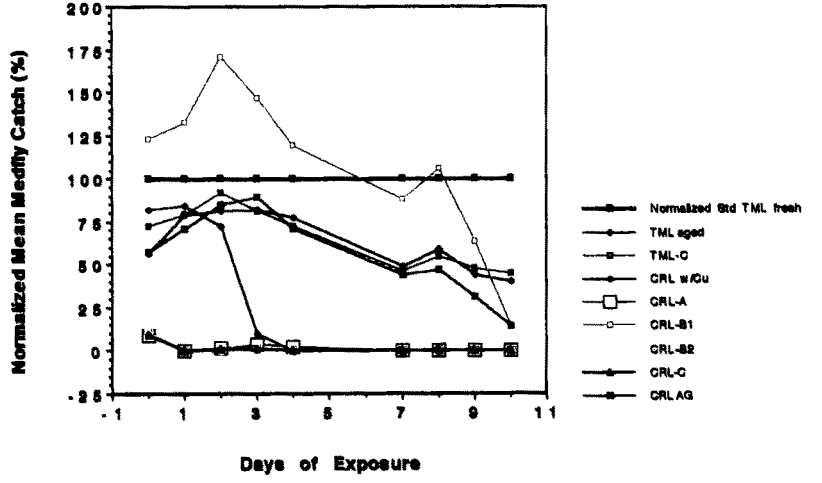

FIG. 3. Data from Table 2: means were normalized to the standard; fresh TML standard was replenished on the days of medfly catch determination.

CRL mixture, TML-C, and aged TML on an equal-weight basis in comparison to the fresh TML standard (Figure 2); however, it was not significantly different from that of the fresh TML standard even though this standard was replenished on the days of examination. In this first test, the medfly attractiveness of each of the CRL isomers A, B2, and C did not significantly differ from one another in comparison to the fresh TML standard; these isomers were essentially inactive.

The observation that CRL-B1 is the most medfly attractive *trans*-CRL isomer is in sharp contrast with the TML series of isomeric attractants; even though the most medfly attractive TML isomer (TML-C) and CRL-B1 are both *trans* isomers, TML-C has an axial 4-chloro and CRL-B1 has an equatorial 5-iodo and there is also the difference in their ester groups. Actually, the greatest difference is in the comparison (on an equal-weight basis) of the medfly attractiveness for TML-C and CRL-C, which differ in their *t*-butyl and ethyl esters, respectively but have the same configurations and conformations with different halogens. CRL-C is virtually unattractive to medflies.

A second test was conducted with the four *trans*-CRL isomers for confirmation of their differences in medfly attractiveness, with the addition of an AgriSense CRL sample. Results (Table 2 and Figure 3) followed the observations made in the first test (Table 1 and Figure 2); medfly attractiveness of the AgriSense CRL AG sample was not significantly different from the CRL w/Cu sample.

The overall accumulation of data for nine days in Tables 1 and 2 (Figures 2 and 3, respectively) revealed that CRL-B1 was significantly superior in medfly attractiveness to that of CRL and TML-C, with these latter two substances being significantly superior in medfly attractiveness to aged TML, all in comparison to the fresh TML standard. CRL-B1 was 1.6 ± 0.3 to $2.25 \pm 0.35 \times$ (Tables 2 and 1, respectively) as medfly attractive as TML-C. CRL was 2.8 ± 0.9 to $3.35 \pm 0.65 \times$ (Tables 2 and 1, respectively) as medfly attractive as TML aged; CRL Ag was 2.45 ± 0.75 times (Table 2) as medfly attractive as TML aged. It must be pointed out that the CRL samples have 22% fewer molecules than an equal weight of TML samples to which they are being compared since the molecular weight of CRL is 27% more than TML because of the heavy iodine atom, even with two less carbon atoms.

This 27% increase in molecular weight of CRL is accompanied by a decrease in volatility, improving the persistency of the lure mixture. This makes CRL a candidate for a male medfly annihilation formulation of attractant plus insecticide (McGovern and Cunningham, 1988; Wood, 1989; Anonymous, 1990).

CONCLUSIONS

The overall accumulation of data for nine days (Tables 1 and 2) shows that the medfly attractiveness of CRL-B1 was significantly different and superior to the other three *trans*-CRL isomers, TML-C, CRL, and aged TML on an equal-

weight basis (Figures 2 and 3). However, the attractiveness of each of the CRL isomers A, B2, and C did not significantly differ from one another; they were essentially not attractive. CRL-B1 was almost twice as attractive as TML-C. CRL was about three times as active as aged TML. The persistence of medfly attractiveness of CRL-B1 and CRL in comparison to TML-C and aged TML, respectively, was evident from the data even though the CRL samples have 22% fewer molecules than the TML samples on an equal-weight basis.

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FIELD TESTS OF SYNTHETIC *Manduca sexta* SEX PHEROMONE

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Abstract—In field experiments traps were baited with live females or with a two-, four-, or eight-component blend of the 16-carbon aldehydes previously identified as components of the sex pheromone emitted by female *Manduca sexta* moths. The blends were formulated on rubber septa. Traps baited with a blend of all eight aldehydes captured more *M. sexta* males than any other treatment. Septa loaded with 600 μg of the eight-component blend were attractive to males for about seven days in the field. Septa loaded with the eight-component blend and stored in a refrigerator at 4°C for a year released the conjugated diene and triene aldehydes at the same rate as freshly prepared septa and were equally attractive in the field.

Key Words—Aldehydes, formulation, 10,12-hexadecadienal, 10,12,14-hexadecatrienal, moth, *Manduca sexta*, Lepidoptera, Sphingidae, tobacco hornworm, trapping.

INTRODUCTION

In the United States the tobacco hornworm moth, *Manduca sexta* (L.) (Lepidoptera: Sphingidae) is not only a common pest of tobacco, but also its larvae eat the leaves of a wide range of other solanaceous plants including tomato, eggplant, jerusalem cherry, and potato (Madden and Chamberlin, 1945). *M. sexta* occurs throughout the greater part of the United States, the West Indies, Mexico, Central America, and parts of South America.

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The sex pheromone glands of *M. sexta* females produce a series of saturated and mono-, di-, and triunsaturated aldehydes with chain lengths of 16 and 18 carbons (Tumlinson et al., 1989). A blend of two of these compounds is required to stimulate *M. sexta* males to complete a characteristic behavioral sequence in a laboratory wind tunnel: upwind-oriented flight in the pheromone plume, approaching and touching the pheromone source, and bending their abdomens in apparent copulatory attempts. One of the two essential pheromone components is the previously identified (*E,Z*)-10,12-hexadecadienal (*E*10,*Z*12-16:Ald)[3] or bombykal (Starrat et al., 1979). The other is (*E,E,Z*)-10,12,14-hexadecatrienal (*E*10,*E*12,*Z*14-16:Ald). Further tests in the wind tunnel suggested, but did not clearly demonstrate, that other components of the gland rinse played a role in mating communication in this species. However, a blend of four of the compounds found in the sex pheromone gland, *E*10,*Z*12-16:Ald, (*E,E*)-10,12-hexadecadienal (*E*10,*E*12-16:Ald), *E*10,*E*12,*Z*14-16:Ald, and (*E,E,E*)-10,12,14-hexadecatrienal (*E*10,*E*12,*E*14-16:Ald), was less effective in the wind tunnel than either the two-component blend or a blend of all the compounds identified (Tumlinson et al., 1989).

Electrophysiological studies showed that *E*10,*Z*12-16:Ald selectively stimulates one of the two pheromone receptor cells in each trichoid sensillum on the antennae of *M. sexta* males. In most of these sensilla the second receptor responds to *E*10,*E*12,*Z*14-16:Ald, but in some sensilla the second receptor responds to the *EEE* isomer (Kaissling et al., 1989). Furthermore, Christensen et al. (1989) showed that all the 16-carbon aldehydes found in the pheromone gland rinses elicit some form of response in olfactory interneurons in males, but *E*10,*Z*12-16:Ald, *E*10,*E*12,*Z*14-16:Ald, and *E*10,*E*12,*E*14-16:Ald evoke the greatest responses.

The wind-tunnel and electrophysiological studies strongly suggest that all eight 16-carbon aldehydes found in pheromone gland rinses have a role in the communication system of this insect. The goal of this study was to determine if the pheromonal compounds that attracted *M. sexta* males in the wind tunnel would also lure them into traps in the field. We also wanted to determine if a blend of all eight 16-carbon aldehydes was more attractive in the field than the blend of the two essential components. One of the difficulties in working with this pheromone blend is the instability of the triene aldehydes. Therefore, we attempted to determine how long blends formulated on rubber septa could be stored in the laboratory and how long they would remain active in field tests.

[3] A standard shorthand notation for pheromone molecules of this type will be used in this paper. For example (Z)-9-hexadecenal is abbreviated Z9-16:Ald; the corresponding acetate is Z9-16:Ac; hexadecanyl acetate is abbreviated *S*-16:Ac.

METHODS AND MATERIALS

Chemicals. The saturated and monoene 16-carbon aldehydes used in this study were obtained from commercial sources and purified by HPLC on a $AgNo_3$-coated silica column (Heath et al., 1977). Both *E*10,*Z*12–16:Ald and *E*10,*E*12–16:Ald were synthesized in this laboratory by the general method of conjugated diene synthesis described by Zweifel and Backlund (1978) and applied to the synthesis of functionalized dienes by Doolittle and Solomon (1986). The conjugated triene aldehydes were synthesized as described by Doolittle et al. (1990). The dienals and trienals were purified by preparative HPLC (see later). All synthetic compounds were analyzed on both polar and nonpolar capillary gas chromatographic (GC) columns and determined to be greater than 99% pure. Solvents used in this study were: hexane, methylene chloride, and methanol (B&J GC,[2] Burdick and Jackson, Muskegon, Michigan); acetonitrile, (B&J UV); ethyl ether (anhydrous AR, Mallinckrodt, Paris, Kentucky); 2-propanol, (HPLC grade, Fisher); and water (B&J HPLC grade).

Formulations. Synthetic blends were formulated on two types of rubber septa. Septa from Thomas Scientific Co. (Philadelphia, Pennsylvania) (5 × 9 mm, catalog #1780J07) were Soxhlet extracted with methylene chloride for 24 hr and dried under vacuum prior to loading. Septa from Wheaton (Millville, New Jersey) (5 × 11 mm, catalog #224100-020) were not extracted prior to loading with pheromone blends. The retention indices in equivalent chain length units (ECLUs), relative to esters of saturated straight chain alcohols, were determined for all the 16-carbon aldehydes used in this study by gas chromatography on a liquid crystal column (see later) and the relative vapor pressures of these compounds were calculated from these data as described by Heath and Tumlinson (1986). All blends prepared for formulation on septa were analyzed by capillary GC on a 50-m CPS-1 capillary GC column to confirm the component ratios. Each septum was loaded with 88–175 μl (depending on dose) of a hexane solution of the blend pipetted into the well on the large end of the septum. Septa were aired for 24 hr at room temperature before use.

To determine the release rates of components of the various blends and to verify that the release ratios were similar to the calculated values, septa were aerated in a volatile collection apparatus. A septum was placed in a 1.26-cm-ID × 2.4-cm aeration chamber made from Swagelok (Crawford Fitting Co., Solon, Ohio) stainless steel fittings. The downstream end of the chamber consisted of a reducer with a $\frac{5}{8}$-in.-OD tube coupled to a $\frac{1}{4}$-in. ID threaded male fitting to which a filter trap was connected via a $\frac{1}{4}$-in.-OD stainless steel tube with a Teflon sleeve. The filter trap consisted of a $\frac{1}{4}$-in.-OD glass tube packed with a 5-mm-long bed (ca. 20–25 mg) of 80/100 mesh Super Q adsorbent. The upwind end of the $\frac{5}{8}$-in. tube was connected to a $\frac{5}{8}$- to $\frac{3}{8}$-in. reducing union

through which charcoal filtered air entered the aeration chamber. An airflow of 1 liter/min was maintained over the septum for 2–3 hr. Volatile compounds trapped on the Super Q filter were eluted with three 50-μl aliquots of hexane–ether (80:20).

Volatiles eluted from the filter traps were analyzed in two ways. When the volatiles were collected from the extracted Thomas septa, 10 ng of *S*-16:Ac in 10 μl of hexane was added to the filter rinse as an internal standard, the solution was concentrated by evaporation under a gentle stream of argon to about 2.5 μl, and the sample was analyzed by capillary GC on a 15-m CPS-1 capillary GC column. Volatiles collected from the Wheaton septa were analyzed by reverse-phase HPLC to quantitate the conjugated dienals and trienals. Hexyl cinnamate (20 ng in 20 μl of hexane) was added to the filter rinse, and the sample was concentrated as before to about 5 μl. Then 20 μl of acetonitrile was added and the total volume was injected onto the HPLC column.

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. Data from the GC analyses were collected, stored, and analyzed with a Perkin Elmer chromatographic data system. Helium was used as the carrier gas at a linear flow velocity of 19 cm/sec. Columns used for analyses were: a glass column, 17 m × 0.25 mm ID, drawn and coated in this laboratory as described by Heath et al. (1979, 1981) with a mixture of 60% cholesteryl *p*-chlorocinnamate (liquid crystal) and 40% OV-101, column temperature 145°C, isothermal, injections made in the split mode; OV-101, 47 m × 0.25 mm ID fused silica, 0.25-μm film thickness, initial temperature 60°C for 1 min, then temperature programmed at 10°C/min to 180°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, temperature programmed at 20°C/min to 180°C; CPS-1, 15 m × 0.25 mm ID, 0.25-μm film thickness, operated under same conditions as other CPS-1 column. Injections on the fused silica columns were made in the splitless mode with a 30-sec delay before injector purging. Injector and detector temperatures were 220°C and 260°C, respectively.

HPLC analyses were conducted on an Adsorbosphere (AllTech Associates, Deerfield, Illinois) 5-μm C18 reverse-phase, 250 mm × 4.6 mm ID stainless steel column, eluted with CH_3CN–H_2O (75:25) at a flow rate of 2 ml/min delivered by a Constametric IIG pump (Laboratory Data Control, Riviera Beach, Florida). Eluting peaks were monitored by a Kratos Spectroflow 757 UV detector (Kratos, Ramsey, New Jersey) at a wavelength of 267 nm for retention volumes at which the triene aldehydes elute, and then at 230 nm to detect the conjugated diene aldehydes (Tumlinson et al., 1989).

Preparative HPLC for the purification of conjugated trienals was conducted on a 250 × 22.5-mm-ID column packed with Adsorbosphere HSC18 (7 μm)

and eluted with MeOH/H_2O (87:13) at a flow rate of 18 ml/min delivered by a Kratos Spectroflow 400 pump monitored at a wavelength of 267 nm (Tumlinson et al., 1989). The dienals were purified on the same column with a mobile phase consisting of $CH_3CN/CH_3CHOHCH_3/H_2O$ (68:5:27) and elution was monitored at 230 nm.

Field Tests. Trapping experiments were conducted in tobacco fields located in northwest Alachua County, Florida, during the summers of 1990 and 1991. Screenwire cone traps (cone 75-50 trap) described by Hartstack et al. (1979) were used in all trials. The traps were spaced ca. 50 m apart in an east–west alignment, perpendicular to the prevailing wind.

Treatments were deployed in randomized complete blocks. When two blocks were set out on the same day, the blocks were located ca. 100 m apart. In all but one test, the bait septa and virgin tobacco hornworm females (three per trap, each 1–3 days old) were placed in the traps ca. 1 hr before sunset. The bait septa were shielded from direct sunlight by a small, white paper condiment cup. The *M. sexta* females were reared at the Crops Research Laboratory, USDA, ARS, Oxford, North Carolina, by the method of Baumhover et al. (1977) and shipped as pupae to Gainesville, Florida. They were sexed in the pupal stage (Baumhover, 1985) and the females were held under natural light in a greenhouse at ca. 30°C until they emerged. Females (unfed) used as bait in traps were replaced every second day.

Captures of tobacco hornworm males were recorded the following morning ca. 1 hr after sunrise. The bait septa and female moths then were removed from the traps and returned to the laboratory. The septa were held in a glass jar in the refrigerator at 0–4°C, and the females were held in the greenhouse until late in the afternoon when they were returned to the field. The treatments were randomized each time they were placed in the field.

The attractiveness of two-, four-, and 8-component pheromone blends to tobacco hornworm males were evaluated in test 1 (see Table 1 below for blends, component ratios, and load of total blend per septum). Female *M. sexta* moths were used as controls. Each treatment was replicated 12 times (1 or 2 replicates/night) between June 5 and July 18, 1990.

The response of tobacco hornworm males to different dosages of the eight-component pheromone blend was recorded in two different trials. In the first trial (test 2, August 8–12, 1990) dosages of 60, 200, 600, and 2000 μg/septum were evaluated. The second trial (test 3, August 28–September 4, 1990) compared septa loaded with 2, 6, 20, and 60 μg of total blend per septum. Dosages in each test were replicated a total of 8 times.

Test 4 was conducted August 21–27, 1990 to determine how long the eight-component blend would remain attractive to tobacco hornworm males when left outdoors continuously for periods ranging from one to eight days. Two septa were loaded with 600 μg of the eight-component blend each day over a period

of a week, Friday and Saturday excluded. After airing in a fume hood for 24 hr, the septa were placed in traps in the field at about sundown each day where they remained until the end of the test. Moth captures were recorded daily, except Saturday and Sunday, until the first septa had been in the field eight days. After each collection, the baits and control female baits were rotated one position.

Test 5, conducted August 20–28, 1991, was designed to determine the effects of long-term storage at 0–4°C on the attractiveness of septa loaded with 700 μg of the eight-component pheromone blend to tobacco hornworm males. Fresh septa loaded with 700 μg of the eight-component blend were used as the standard, and blank septa loaded with hexane only and female tobacco hornworm moths (3/trap) were used as controls. Each treatment was replicated six times.

For statistical analysis, moth captures were transformed to $\sqrt{x + 0.5}$ and subjected to analysis of variance. Differences between means were separated using Duncan's (1955) multiple range test (Scholtzhauer and Littel, 1987).

RESULTS AND DISCUSSION

The preparation of pheromone blends was based on the volatility of the individual components, as previously determined. The relative vapor pressures of the eight 16-carbon aldehydes found in the hexane rinses of the pheromone glands of *M. sexta* females (Tumlinson et al., 1989), calculated from their ECLUs on a liquid crystal capillary GC column (Heath and Tumlinson, 1986), are listed in Table 1. Three blends were prepared for formulation and field testing, based on the results of earlier wind tunnel studies (Tumlinson et al., 1989). These were: a blend of the two compounds found to be essential to elicit the full range of behaviors in the wind tunnel, a four-component blend of the conjugated dienals and trienals, and a blend of all eight 16-carbon aldehydes. The percentage of each component loaded onto septa in each blend was based on the composition of the hexane rinses of pheromone glands. The theoretical percentage of each component predicted to be released from rubber septa for each blend was calculated from the relative vapor pressures of each compound by using the method of Heath et al. (1986). The percent of each compound loaded onto septa and the predicted release ratios of the components in each blend are given in Table 1.

The first set of experiments compared the attractiveness of the three blends to *M. sexta* males in the field. However, it was also necessary to evaluate the volatile blends released from each formulation to ensure that the release rates and percentages of the components of each blend were comparable and consistent. Therefore, in each set of experiments two septa were loaded with each blend. Volatiles were collected from one septum while the other was placed in

TABLE 1. RELATIVE VAPOR PRESSURES, THEORETICAL PERCENT RELEASE, MEASURED PERCENT RELEASE, AND RELEASE RATE OF COMPONENTS OF SYNTHETIC PHEROMONE BLENDS FORMULATED ON RUBBER SEPTA

			2-component blend, 250 μg/septum[d]; N = 9				4-component blend, 350 μg/septum[d]; N = 10				8-component blend					
													700 μg/septum[d]; N = 12		350 μg/septum[e]; N = 6	
Compound	ECLU[a]	Po[b]	% Load	Theor[c] % Rel	Measured % Rel. (±SD)	Rel. rate, ng/hr (±SD)	% Load	Theor[c] % Rel	Measured % Rel. (±SD)	Rel. rate, ng/hr (±SD)	% Load	Theor[c] % Rel.	Measured % Rel. (±SD)	Rel. rate, ng/hr (±SD)	Measured % Rel. (±SD)	Rel. rate, ng/hr (±SD)
Z9-16:Ald	1394	0.0661									1.0	1.8	[f]	[f]	2.4 (±0.3)	0.6 (±0.2)
Z11-16:Ald	1405	0.0596									17.4	27.2	[f]	[f]	27.5 (±0.3)	6.4 (±1.0)
E11-16:Ald	1408	0.0576									8.8	13.3	[f]	[f]	12.1 (±0.7)	2.8 (±0.5)
S-16:Ald	1425	0.0484									20.4	25.9	[f]	[f]	28.8 (±0.8)	6.7 (±1.0)
E10,Z12-16:Ald	1467	0.0320	67.8	90.8	88.9 (±2.8)	12.5 (±2.4)	59.2	81.4	80.0 (±2.5)	15.9 (±2.7)	31.0	25.9[g]	78.9 (±2.8)	14.8 (±3.2)	24.4 (±0.8)	5.6 (±0.7)
E10,E12-16:Ald	1500	0.0229					9.7	9.5	10.5 (±2.0)	2.0 (±0.3)	5.1	3.0[g]	10.8 (±2.0)	2.1 (±0.8)	4.9 (±0.4)	1.1 (±0.1)
E10,E12,Z14-16:Ald	1622	0.0068	32.2	9.2	11.1 (±2.8)	1.6 (±0.5)	28.1	8.2	8.8 (±0.7)	1.7 (±0.2)	14.7	2.6[g]	9.1 (±1.1)	1.7 (±0.4)	[h]	[h]
E10,E12,E14-16:Ald	1629	0.0063					3.0	0.8	0.7 (±0.4)	0.3 (±0.5)	1.6	0.3[g]	1.1 (±0.6)	0.2 (±0.1)	[h]	[h]

[a] ECLU = Equivalent chain length unit based on retention time on liquid crystal GC column (see text).
[b] P_0 = Relative vapor pressure.
[c] Theoretical % release of each component of a blend calculated from relative vapor pressures (see text).
[d] Blends loaded on Wheaton septa. Collected volatiles analyzed by reverse phase HPLC with UV detection.
[e] Blends loaded on extracted A.H. Thomas septa. Collected volatiles analyzed by capillary GC on CPS-1 column.
[f] Not detectable by UV.
[g] If the theoretical % release were calculated for only those components of this blend measurable by UV detection these values would be changed as follows: 25.9 = 81.4, 3.0 = 9.4, 2.6 = 8.2, 0.3 = 0.9.
[h] Not measurable due to lability of compounds on GC.

a trap in the field on the evening of the same day. The next morning the septum in the field was returned to the laboratory for volatile collection, and on that evening the septum initially used for volatile collection was placed in a trap in the field. The septa were alternated in this manner for four days until volatiles had been collected twice from each septum for each blend.

For these experiments Wheaton septa were used, and the collected volatiles were analyzed by HPLC. Preliminary experiments indicated that *E*10,*Z*12–16:Ald was released at a different rate from septa loaded with different blends. Therefore the amount of each blend loaded on a septum was adjusted so that the release rate of *E*10,*Z*12–16:Ald was about the same in all cases. As indicated in Table 1, twice as much of the eight-component blend as the four-component blend was required to be loaded on a septum to yield equal release rates. However, the percentage of each component released was not affected by the amount loaded, but only by the percent loaded and the relative vapor pressure of the compounds (Table 1).

To determine the release rates and percentages of the components not detectable by UV, the eight-component blend was loaded on extracted Thomas septa and volatiles were collected for gas chromatographic analysis. Extracted septa were used to reduce the background impurities that would interfere with GC analyses. Previous studies (Heath et al., 1991) have shown that release rates of pheromonal compounds from Wheaton septa and extracted Thomas septa are nearly identical.

The results of the analyses of the volatiles collected from septa loaded with each blend (Table 1) indicate that the percentage of each component released was similar to the calculated value for each blend. The variability in percentages or rates of release for a given blend and load was relatively low, even for septa that had been used in the field for two nights.

In the field trapping tests (test 1), the eight-component blend attracted significantly more *M. sexta* males than any other lure (Figure 1). As we found in the wind tunnel (Tumlinson et al., 1989), the two-component blend appeared to be slightly more attractive than the four-component blend, although the difference was not significant. Since the volatile collection data indicated that the release rates from all septa were approximately equal, it appears that one or more of the compounds, *S*-16:Ald, *Z*9–16:Ald, *Z*11–16:Ald and *E*11–16:Ald, increased the attractiveness of the two compounds, *E*10,*Z*12–16:Ald and *E*10,*E*12,*Z*14–16:Ald, required for attraction. This is consistent with the results of the neurophysiological experiments reported by Kaissling et al. (1989) and Christensen et al. (1989) that indicate that all the 16-carbon aldehydes in the blend released by *M. sexta* females are detected by pheromone receptors on male antennae.

Experiments were also conducted to determine the effect of septum load or dose on the response of male *M. sexta* to the eight-component blend in traps in

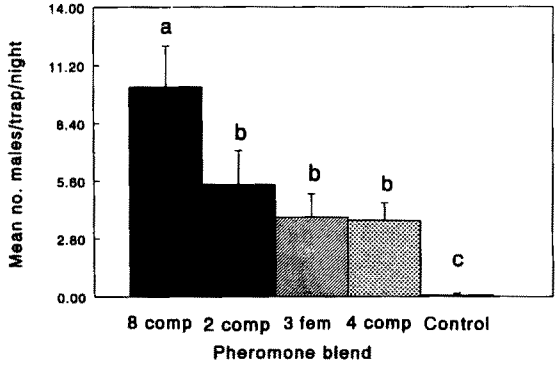

FIG. 1. Results of test 1. Capture of THW males in screenwire cone traps baited with different blends of 16-carbon aldehydes formulated on Wheaton rubber septa. $N = 12$ for all treatments. Error bars represent standard error of the mean. Bars not topped by the same letter differ significantly at the 5% probability level (Duncan's multiple range test). Composition of each blend is given in Table 1. The two-, four-, and eight-component blends were loaded at doses of 250, 350, and 700 μg/septum, respectively.

the field. The dose was varied from 2 to 2000 μg/septum (Figure 2) while the percentage of each component in the blend was held constant (Table 1). In test 2 septa loaded with 60–2000 μg were placed in traps in the field in the evening and then returned to the laboratory the next morning for volatile collection and analysis by HPLC to determine release rates. In the third test, in which septa were loaded with 2–60 μg of the blend, the release rates of volatiles from the septa were too low to be measured in the laboratory. While it is difficult to extrapolate release rates for doses below 60 μg, it is obvious that *M. sexta* males are attracted by very low doses of this blend because even the 6-μg dose captured significant numbers of males. Furthermore, it appears that it is not necessary to use doses higher than about 60 μg, since trap captures do not improve at the higher doses (Figure 2).

Because the conjugated diene and triene aldehydes are notoriously unstable, an experiment was conducted to determine how long the eight-component blend formulated on rubber septa would remain active in the field. In test 4, septa that had been aged in the field for different lengths of time were directly compared in two tests. The data in Figure 3 indicate that while activity appears to gradually decrease over a period of a week, this formulation attracted a significant number of males for at least seven days. Thus, it should be possible to formulate these compounds to remain active in field tests for at least a week.

Finally, an experiment was conducted to determine the amount of time rubber septa formulations of this pheromone could be stored without losing activity. Three sets of two septa, each loaded with 700 μg of the eight-com-

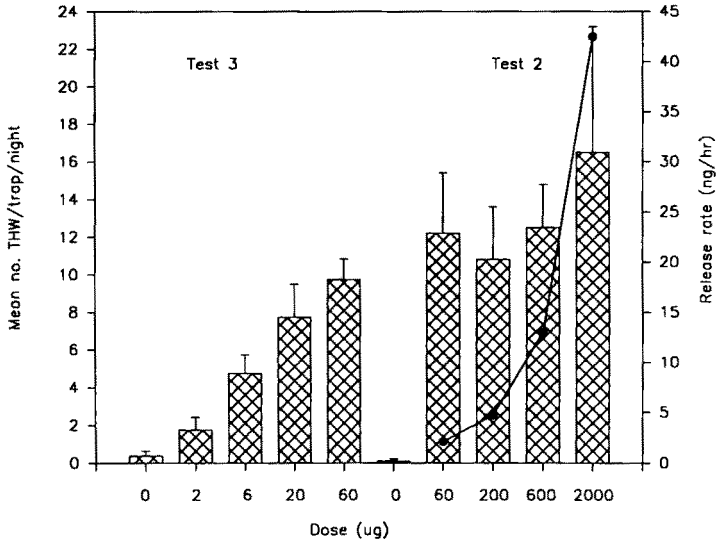

FIG. 2. Results of tests 2 and 3. Capture of THW males in screenwire cone traps baited with different doses of the eight-component blend of 16-carbon aldehydes loaded on rubber septa. See Table 1 for blend composition. $N = 18$ for all treatments. Error bars represent standard error of the mean. Release rates of $E10,Z12$-16:Ald from septa loaded with each dose (mean of two replicates) represented by black filled circles in test 2.

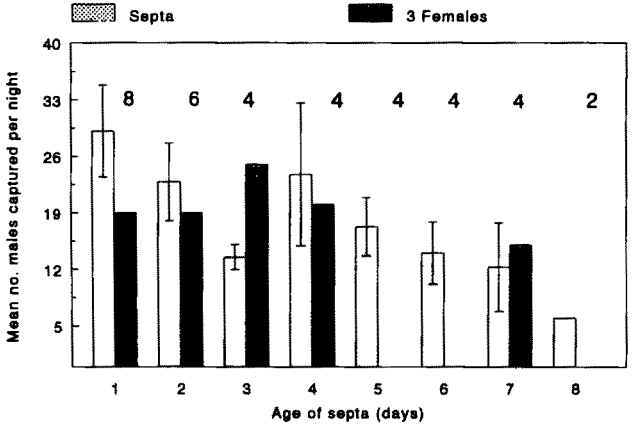

FIG. 3. Results of test 4. Capture of THW males in screenwire cone traps baited with three virgin females or with septa loaded with 600 μg of the eight-component blend (see Table 1 for composition) aged in the field for different lengths of time. Error bars represent standard error of the mean. Values above bars represent replicates for each age group.

ponent blend, which were used in field test 1 during June and July of 1990, were then stored in glass vials with Teflon-lined screw caps at 0–4°C in a refrigerator and removed at intervals as indicated in Figure 4 for volatile collection. Volatiles were collected and analyzed by HPLC as before from one septum in each set before field use and after one night in the field. Volatiles from the other septum in each set were analyzed after one night in the field and then again after the second night in the field. On subsequent days when volatiles were to be collected, the septa were removed from the refrigerator, allowed to warm to room temperature, and aired for 1 hr in a fume hood prior to volatile collection. The data in Figure 4 indicate that the release rate of each component measured in the volatiles remained very constant even after the septa had been stored for nine months. At the end of a year, the septa were tested in the field

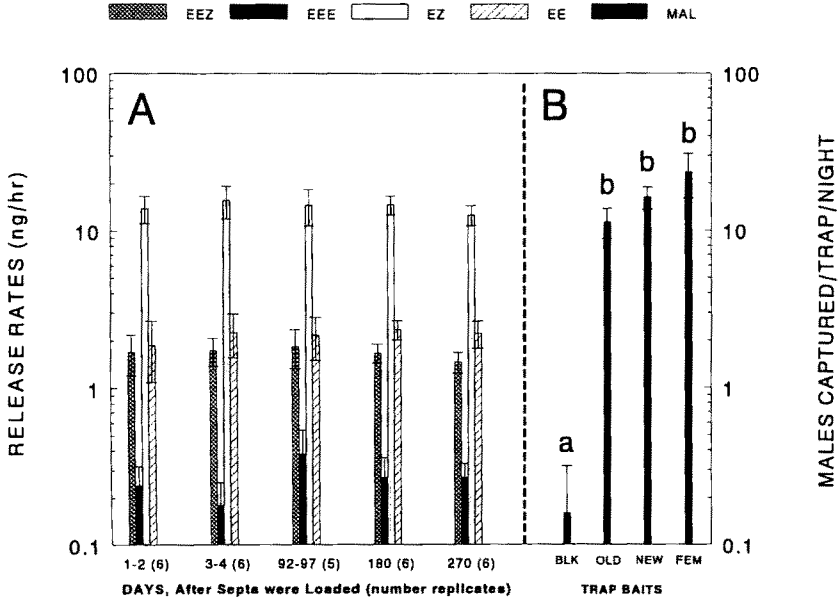

FIG. 4. (A) Release rates, measured in the laboratory, of the conjugated diene and triene aldehydes from septa loaded with 700 μg of the eight-component blend (see Table 1 for composition) and stored at 0–4°C. Error bars represent standard deviation from the mean. (B) Capture of male THW in screenwire cone traps in the field baited with same septa used in release rate studies (N = 6). BLK = septa loaded with hexane; OLD = septa that had been stored at 0–4°C for approximately one year; NEW = septa loaded with the eight-component blend just prior to the field test; FEM = live females; MAL = males captured per trap. Bars topped with different letters differ significantly at the 5% probability level (Duncan's multiple range test).

and their attractiveness was compared directly to septa loaded with the same blend just prior to the field test and to live females (Figure 4b). The results of this experiment suggest that when formulated on rubber septa the eight-component blend can be stored at 0–4 °C for at least a year without losing activity.

In summary, these studies show that the blend of 16-carbon aldehydes previously identified as the sex pheromone produced by *M. sexta* females on the basis of laboratory wind-tunnel assays are also active in the field. It appears that in addition to *E*10,*Z*12–16:Ald and *E*10,*E*12,*Z*14–16:Ald, one or more of the saturated and monounsaturated components play a role in sexual communication in this species. It is possible that all eight 16-carbon aldehydes are active. The results of this study also indicate that a rubber septum formulation of this aldehyde blend is stable enough to remain active in the field for seven days and that it is possible to store such a formulation at low temperature for at least a year. It is likely that formulations of this blend could be developed that would remain active in the field for a sufficient time to have practical utility in monitoring for this species.

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AN AUTOMATED SYSTEM FOR USE IN COLLECTING VOLATILE CHEMICALS RELEASED FROM PLANTS

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Abstract—A system is described for the collection of volatiles produced by plants that minimizes stress on the plant in an environment that is free from chemical impurities. Air entering a volatile collection chamber containing a plant is purified using a nonwoven fabric media infused with charcoal. A multitasking, computer-automated system is described that can simultaneously collect volatilized chemicals from plants as well as monitor and record environmental conditions associated with those collections. Collection of up to 16 samples can be made in varying sampling order, flow rates, and user-specified time periods, without disturbing the sampling environment. During the same time period, this system is capable of simultaneously monitoring up to eight environmental parameters using any type of sensor with electrical signal outputs. A multiport base assembly was designed to fit around the base of the plant permitting air samples to be collected at the bottom of the chamber. The chamber can pass ambient light so the plant may follow its natural photocycles. The entire system can be configured for continuous laboratory duty or portable field use by utilizing components that run on DC voltages. For the purpose of testing the system's performance, we determined the periodicity of the release of volatiles from red and yellow flowering four o'clock plants, *Mirabilis jalaba* (Nyctaginaceae). The major chemical released from four o'clocks was identified as ocimene. The onset of release occurred between 1400 and 1600 hr and increased with time with maximum amount of ocimene released during 1800–2000 hr, followed by a decrease in emission. No ocimene was detected after 2400 hr. Determination of the amount of ocimene released per flower was calculated for the 1800- to 2000-hr time period. Based on the number of open flowers during the 1800- to 2000-hr period, yellow four o'clock's released 80.9 (±7.3 SD) ng/hr/flower, while the red flowers released 51.9 (±7.0 SD) ng/hr/flower.

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Key Words—Automated volatile collection system, plant volatiles, whole plant sampling, four o'clock plants, *Mirabilis jalaba*, volatiles collection.

INTRODUCTION

Many plants, ranging from crop species to ornamentals, emit volatile compounds in varying quantities for a variety of reasons. Chemicals released range from gases such as ethylene, ethane, and oxygen, as a result of biochemical processes within the plant, to complex terpenoids that are used to lure insects for pollination (Wyatt, 1983). Chemicals released by flowering plants have been investigated, and the circadian and diurnal rhythms of floral fragrance emissions of plants have been documented by several researchers (see for example, Matile and Altenburger, 1988; Altenburger and Matile, 1988, 1990; Loughrin et al., 1990a,b, 1991). Recently we identified the chemicals and periodicity of release from flowers of the night-blooming jessamine, *Cestrum nocturnum* L., that are attractive to female cabbage looper moths, *Trichoplusia ni* (Hübner) (Heath et al., 1992). The release of these chemicals was shown to correspond to periods of feeding activity of female cabbage loopers. The chemicals released were collected and identified as benzaldehyde, benzyl acetate, and phenylacetaldehyde. Flight-tunnel bioassays of female cabbage loopers demonstrated that the moths exhibited upwind-oriented flight and contact with dispensers after releasing an artificial blend of these compounds.

In addition to developing better chemical lures for monitoring insects, there is interest in the volatiles released from plants in response to environmental changes or plant stresses such as lack of nutrients and water and stress due to disease (Kimmerer and Kozlowski, 1982; Biddington, 1986). Recent research has demonstrated that many plants release chemicals in response to larval feeding, and these volatiles have been shown to attract secondary insect predators (Turlings and Tumlinson, 1992; Tumlinson et al., 1993). In addition to the relationship of plant volatiles to insects, there also is interest in the volatiles emitted by plants as they relate to chemicals used in the flavor and perfume industry and other allied areas of research (Buttery, 1981).

Several indirect methods have been developed for elucidating potential volatile chemicals emitted from plants. Methods such as extraction with solvent or steam distillation used to identify "volatile" components from plants most often result in the identification of a large number of compounds that are not representative of the chemicals released by the plant (Tollsten and Bergstrom, 1988). Rearrangement and/or decomposition of many labile compounds often occur when many chemicals are subjected to heat or solvent. In addition to questions raised on the validity of compounds that are suggested to be "vola-

tilized," no quantitative data were obtained on the amount of the chemicals released.

Collection of chemicals from plants in a natural environment is difficult due to the fact that plants are sensitive to contact and movement such as wind [types of mechanically induced stress (MIS)]. Changes in growth rates and amounts of released volatile gases such as ethylene in response to these agitations has been documented (Biddington, 1986). Plant responses to stimuli become more pronounced with increased stress and this results in increased release of chemicals (Yu and Yang, 1980; Kimmerer and Kozlowski, 1982; Hyodo, 1991). Thus, it is not surprising that the cutting of flowers or sections of plants has been shown to alter the chemicals released from those plants (Heath et al., 1992). Because of this, it is important that the collection of plant volatiles is done in a manner that does not introduce stress on the plant.

Several systems have been designed to identify chemicals released from plants (Panasiuk, 1984; Loughrin et al., 1990b; Heath and Manukian, 1992). These systems were limited in the number of samples that could be collected during an experiment without having to go into the chamber to replace collection traps. Systems previously described typically were open at the bottom of the plant container and as such were subject to intrusion of unpurified air (Heath et al., 1992; Heath and Manukian, 1992). To prevent this from occurring, large volumes of pure air were required for positive purging of the test chamber, thereby allowing only a small percentage of the total released volatiles to be collected, which could potentially compromise accurate quantification of those chemical(s) released in trace amounts.

We describe here an automated system that can be used to collect chemical samples in any combination, sample volume, and time period in the plant's ambient surroundings with minimal stress placed on the plant. This system, using various electronic sensors, can simultaneously collect environmental data from the surroundings while the collection is occurring. To test the effectiveness of this system, we used ornamental red and yellow four o'clock flowering plants, *Mirabilis jalaba* (Nyctaginaceae), as a model to demonstrate the system's ability in collecting volatile chemicals.

METHODS AND MATERIALS

The entire system consists of three main parts, a guillotine collection assembly (GCA), an automated volatile collection system (AVCS), and an environmental data monitoring system (EDMS), each having several subcomponents. The GCAs are glass chambers into which the plants are inserted and from which volatiles are collected. The remaining two subsystems are used to control the overall experiment, make collections, and record all experimental parameters.

The AVCS consists of electronic hardware and pneumatics necessary for performing automated air sampling of volatiles from the GCAs. The EDMS consists of electronic sensors and hardware used to monitor environmental conditions affecting an experiment and store these data on a computer. Both the AVCS and EDMS can be used separately as independent modules or concurrently as one complete system. This is done through the MACDAS (Monitoring And Control, Data Acquisition System) for Windows control software developed specifically for this application at this laboratory (Manukian and Heath, 1993). The software contains separate modules for each system that can be multitasked in any combination on one computer using the Microsoft Windows 3.1 operating system.

Guillotine Collection Assembly (GCA). The GCA is shown in Figure 1. Air entering the system is purified by using five layers of 1.27-cm-thick carbon-infused polyester media containing 150% activated carbon (340 g fabric weight with 510 g carbon/sq yd, p/n ACF-NWPE-12-150P, Lewcott Corp., Millbury, Massachusetts) cut into 15.25-cm-diameter wafers. The use of this material to purify air in a manner that does not alter the ambient relative humidity has been described previously (Heath and Manukian, 1992). The five layers were compressed using an 11 metric ton Carver model 150-C hydraulic press (Menomonee Falls, Wisconsin) to a total load force of 1.1×10^5 newtons (24,000 lbf) applied to the filter media for 15 min. From the force of compression, the multiple layers are fused together, and the resulting disk is approximately 3 cm thick $\times$ 15.25 cm in diameter. Pressurized air (420 kPa), set at a constant flow at 5 liters/min by an adjustable flowmeter (Aalborg p/n P14/044-40ST, Monsey, New York), enters through a cap on top of the chamber and is diffused due to the back-pressure created by the compressed filters and a uniform air flow occurs in the test chamber.

The chamber used to house the plant is a 15.25-cm-OD $\times$ 40-cm-long $\times$ 0.4-cm-thick Pyrex glass tube with a 0.64-cm glass flange on the top end. The flanged end is used to seal the compressed carbon filters between the glass tube and air diffuser inlet cap using a 15.25-cm-ID phenolic coupler union (model S-6750-013, Southern Scientific Inc., Micanopy, Florida) (Figure 2). The glass test chamber with its attached filters is then placed on top of a multiport guillotine base using a 15.25-cm-ID $\times$ 7.62-cm-long piece of metal tube sleeve, which serves as a coupler to hold both sections together.

A multiport guillotine base (MGB) is used to close off the bottom of the test chamber around the stem of a plant. Two Teflon-coated blades, which come together to a tongue-and-groove joint with one half of a 2.54-cm hole cut into each blade, form a circular cutout around the stem of the plant resembling a guillotine (Figures 1 and 2). After the plant is inserted into the chamber, a piece of cotton is placed around the stem to prevent the plant stem from touching the MGB. Attached to the MGB above the closed blades, is a 15.25-cm-OD $\times$

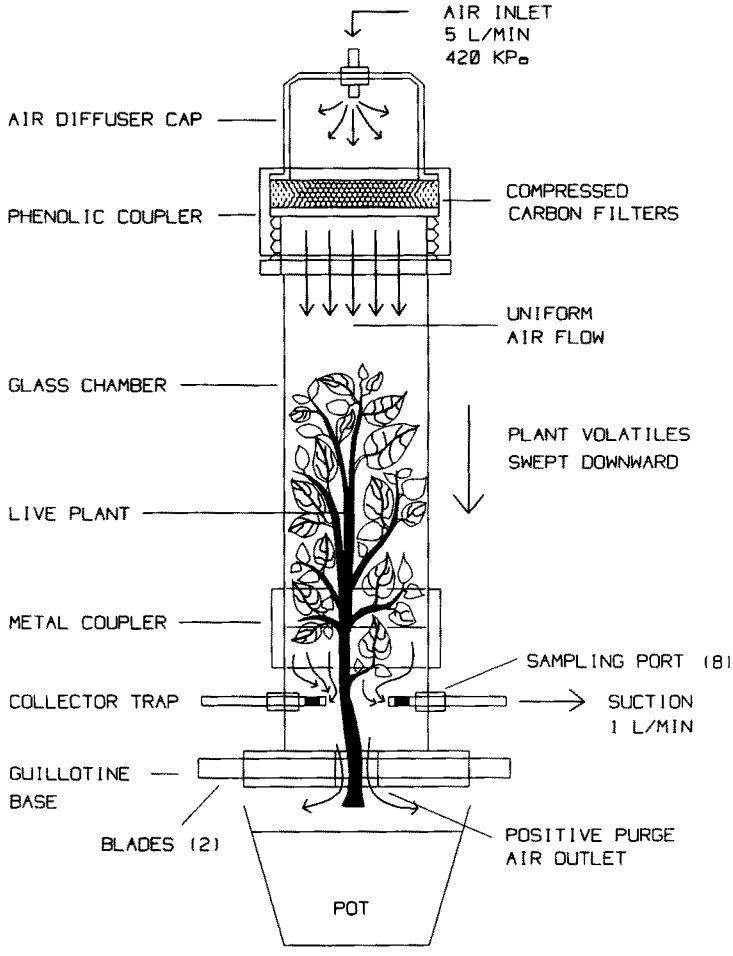

FIG. 1. 2-D side view/function diagram of the guillotine collection assembly (GCA) used for plant volatile sampling with the AVCS.

9-cm-long piece of 0.64-cm-thick clear acrylic Plexiglas tube with eight 0.64-cm Swagelok compression to M-NPT adapter fittings attached along the circumference of this tube at 45 degree angles from center, which act as filter ports for sampling. These bulkhead fittings serve as connectors for the volatile collector traps (VCT). The Plexiglas tube that contains the bulkhead connectors is lined with Teflon on the inside.

Monitoring And Control, Data Acquisition System (MACDAS). The auto-

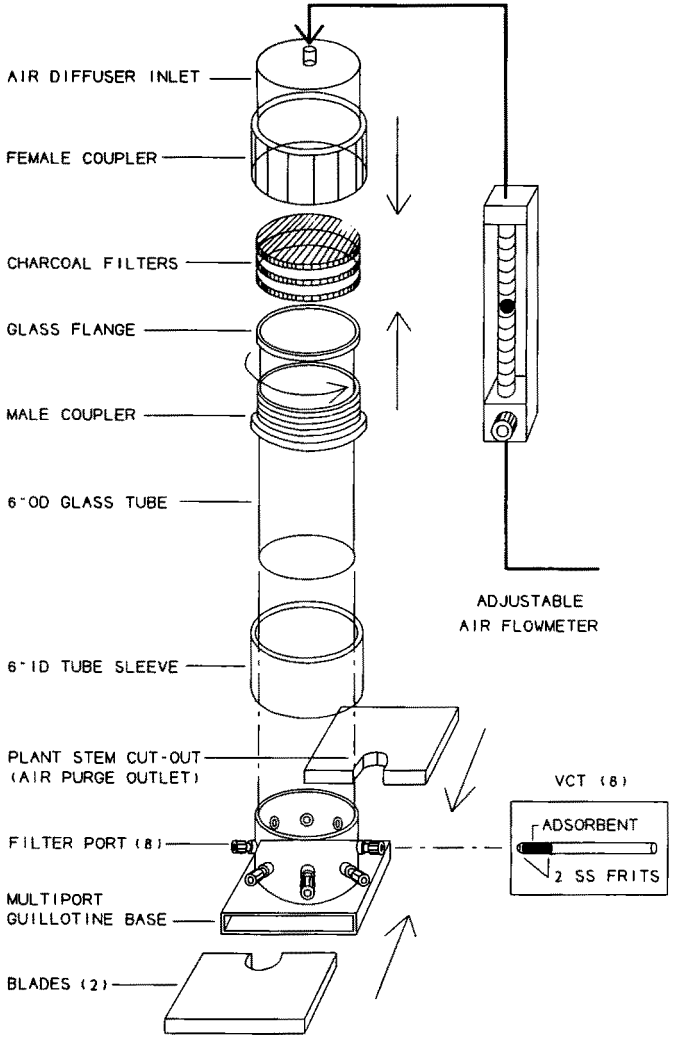

FIG. 2. 3-D exploded view of components of the guillotine collection assembly (GCA) used for plant volatile sampling with the AVCS.

mated volatile collection system (AVCS) and environmental data monitoring system (EDMS) used to set up, operate, and monitor all aspects of the system during an experiment has been described (Manukian and Heath, 1993). Briefly, a computer data system (CDS) consists of an Intel-based i80386DX-25Mhz CPU

computer capable of running Microsoft Windows 3.1 in the enhanced mode. Data acquisition is done using a ComputerBoards Inc., model CIO-DAS08 (Mansfield, Massachusetts) multifunction eight-channel analog and 32-channel digital I/O board. The digital section of the CIO-DAS08 is used for controlling the collection of air samples during an experiment, as well as controlling any additional external events by outputting control signals directed from the AVCS software module. This board contains 24 lines of bidirectional TTL digital I/O that are divided into three ports; A, B, and C, each containing eight channels, are configured as outputs used to control external solid state relays (SSRs), which act as electronic switches in turning on or off solenoid valves and equipment (Figure 3).

Two computer interface boards are used with the system to interface all external electrical hardware with the CIO-DAS08 data acquisition board (DAC) inside the computer. These boards are independent of each other and only share

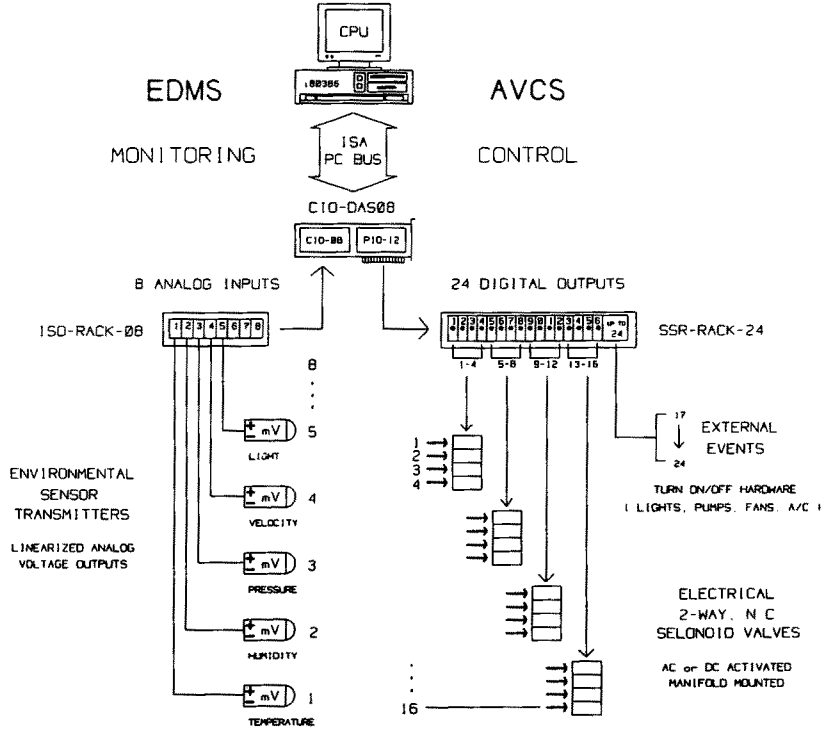

FIG. 3. AVCS and EDMS electrical components of the Monitoring And Control Data Acquisition System (MACDAS).

a common power source and electrical ground with the computer's main power supply. A ComputerBoards Inc., model SSR-RACK24 solid state relay rack equipped with 24 Grayhill Inc., type 70-OAC digital output AC modules (La Grange, Illinois) is used to turn the DAC boards TTL digital outputs directed from the AVCS module into control signals to turn on/off hardware and select samples for collection. A ComputerBoards Inc., model ISO-RACK08 universal analog isolation and interface board is used to isolate, amplify, filter, and condition all analog input sensory data from the ESTs into the EDMS module for display and storage.

To monitor environmental parameters associated with the experiment, we utilized an Omega Instruments model RH-411 digital thermohygrometer to record both temperature and relative humidity data (Stamford, Connecticut). For measuring light intensities during experiments, we used a three-decade digital light meter covering a range from 0 to 50,000 lux utilizing a selenium photovoltaic detector with a 300-nm bandwidth centered at 570 nm (Davis Instruments Inc., C/N# EH1191025, Baltimore, Maryland). Both of these instruments produced linearized mV analog outputs for input to the EDMS.

The selection of multiple samples for collection during an experiment is done through a sample switching manifold (SSM), which is controlled by the AVCS module in the MACDAS control software. This manifold consists of four separate banks of four, two-way, normally closed (NC), electrical (AC) solenoid valves, manifold-mounted with a common outlet (Versa Valves Inc., no. EZM-2180-4-0-243-120V60, Gulf Controls Corp., Tampa, Florida). Each bank is connected in parallel to a vacuum header, through a 0–1 liter/min adjustable flowmeter (Aalborg Instruments, p/n PO4/1-112-02C), which is in series with the outlet port of each bank of valves, making a total of 16 individual sampling lines that can be used in any combination. The parallel groups of four banks enable eight collections from up to two different chambers at the same time (Figure 4). The vacuum header is connected to a vacuum system, which consisted of a standard 115 liter (30-gallon) compressor tank with an oilless Teflon rotary vane vacuum pump (Gast model 1023-V126T-G272X, Gulf Controls Corp.).

Control of an experiment is achieved through the MACDAS for Windows software which was developed at this laboratory using the Microsoft Visual BASIC version 1.0 programming language along with the Microsoft Professional Toolkit for Visual BASIC and the DriverLINX/VB dynamic link library for accessing control functions of the DAC board through Windows (Scientific Software Tools, Inc., Malvern, Pennsylvania).

Volatile Collector Traps (VCT). The collector traps used were based on modification of traps previously described (Heath and Manukian, 1992). For this system the VCTs are made using a 12-cm-long × 0.4-cm-ID piece of glass tubing and contains 30 mg of 80/100 mesh Super-Q (Altech Assoc. Inc., cat.

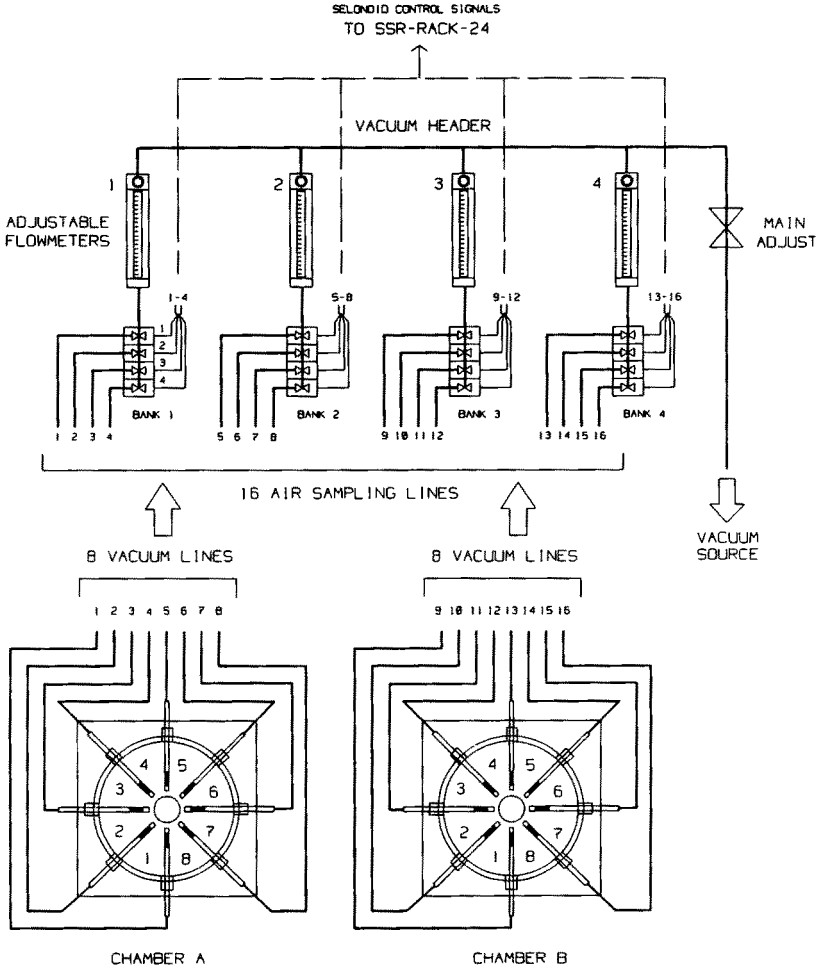

FIG. 4. Electrical and pneumatic schematic of the sample switching manifold (SSM) and vacuum line connections to multiport base of the GCA used for plant volatile sampling with the AVCS.

no. 2735, Deerfield, Illinois) as the adsorbent. Two 325-mesh stainless steel cloth frits are used to contain the adsorbent. The collector traps are connected directly to soft Tygon sampling lines from the SSM and are then inserted (adsorbent end first) through the filter ports in the guillotine base assembly (Figure 2, inset). Prior to use, the traps are cleaned by Soxhlet extraction using ultra-high purity methylene chloride for 24 hr. Volatiles collected on the traps are eluted

with 100 μl methylene chloride and then 20 ng of tridecan-1-ol acetate (*S*-C13 : Ac) is added as an internal standard for subsequent analyses and quantification in the laboratory.

Plants and Collection of Volatiles. Ornamental red and yellow four o'clock flowering plants *Mirabilis jalaba* (Nyctaginaceae), were grown individually in 2-liter pots containing a 50 : 50 mixture of common potting soil and vermiculite. Plants were grown in a greenhouse and collections of volatiles were made in November 1992. Two separate GSAs were used to collect volatiles from 6-week-old red and yellow four o'clock's, each containing one plant. At approximately 1130 hr, a GCA was placed over each plant and purified air at 5 liter/min was introduced into each chamber to purge the system. Volatile collections were made continuously using the AVCS starting at 1300 hr for 1 hr at a sampling rate of 1 liter/min, then continuing with samples taken at 2-hr intervals for a total period of 14 hr, ending at 0400 hr the following morning (total of eight samples). Each sample represented 20% of all chemicals present in the chamber during the period of each collection (5 liters/min total airflow entering at the top with 1 liter/min being sampled and the remaining 4 liters being vented through the bottom). The number of flowers completely opened or partially opened were counted at approximately 1700 and 1900 hr each day of collection. This process was continued for six consecutive days ($N = 6$) using both new red and yellow plants for each collection. In addition to the physical samples, environmental data (temperature, relative humidity, and light intensity) were collected continuously prior, during, and after each experiment using the EDMS.

Analysis of Volatiles. A Varian model 3400 GC and Finnigan ITD MS, equipped with a CTC-A200S 200 sample liquid automatic injector was used for analysis of plant volatiles. Typically, 10 μl of extract was injected into a septum programmable injector (SPI) for direct capillary cool on-column injection at 60°C. Zero grade helium (99.998%) was used as the carrier gas at a linear flow velocity of 20 cm/sec, and the temperature program was initially isothermal at 60°C for 5 min, then temperature programmed at 20°/min to 180°C. Capillary gas chromatography (CGC) was done using a combination of the two fused silica columns: first a 10-m × 0.25-mm-ID trimethylsilane deactivated fused silica retention gap column (Quadrex, New Haven, Connecticut) connected in series to the analytical capillary column, then a 30-m × 0.25-mm-ID Supelcowax 10 (bonded Carbowax) with a 0.25-μm-film thickness, also purchased from Quadrex, using GlasSeal connectors (Supelco Inc., Bellefonte, Pennsylvania). This system permitted the on-column injection of samples without concentration in 5–100 μl of solvent (Grob, 1982; Murphy, 1989). The detector end of the analytical column was coupled to the source of a Finnigan MAT Ion Trap Detector mass spectrometer (ITD-MS) or a Varian flame ionization detector (FID). GC-MS spectra were obtained using electron impact (EI) and analysis of the spectra data was done using the Finnigan-MAT Trapmaster software. The

GC/FID chromatographic peak quantitation was processed using the Perkin-Elmer/Nelson TurboChrom3 software.

RESULTS AND DISCUSSION

Several major modifications in the design of systems previously described (Heath et al., 1992; Heath and Manukian, 1992) for the collection of volatiles from plants were incorporated into the system presented in this paper. During initial attempts to increase the diameter of the carbon filter disks from 9.5 cm to 15.25 cm for this larger system, we observed that the multiple layers of the 1.27-cm-thick carbon filter media would separate, acting as individual filters and thus the purity of air entering the chamber was unacceptable. By fusing the multiple layers of this charcoal media together using extreme pressures, the resulting single fused filter disk produced an excellent means of purifying air in addition to circumventing the problems associated when trying to handle the multiple, thinner disks.

The open-ended system previously used required large volumes of air to ensure that the air velocity through the test chamber was sufficient to prevent intrusion of unpurified ambient air into the chamber. The development of the GCA provides an easy method of incorporating multiple collector traps and simultaneously resolving the problem of ambient air intrusion into the purified air chamber by restricting the open end of this chamber to a minimum. The degree of air purification within the chamber is shown by comparing typical gas chromatograms of the volatiles released during the 1800- to 2000-hr (peak release) period and absence of volatiles observed during the 0200- to 0400-hr (nominal background) period (Figure 5). As seen in the gas chromatograms, impurities from the air inside the chamber are minimal during the nominal background periods and the amount of material released from a plant, which is in the low nanogram (< 10 ng) range, is easily detected.

In the course of our evaluation of the system, we used four o'clocks as the plant model. The major compound released was identified as 3,7-dimethyl 1,3,7-octatriene (ocimene) based on mass spectroscopy (GC-MS). Confirmation of the identity of ocimene was based on comparison of mass spectra and retention time of the collected natural material with synthetic standard material (Ocimene # 15-1353/Lot # SK061188, International Flavors & Fragrances Inc., New York, New York, CAS# 502-99-8). Several other compounds also were identified in some of the volatile collections from four o'clocks. These included: (Z)-3-hexenyl acetate, 7-methyl 3-methylene 1,6-octadiene (myrcene), benzaldehyde, and indole. None of these compounds were found consistently and their occurrence was not related to a particular time period.

The average percent of ocimene released from four o'clocks during the

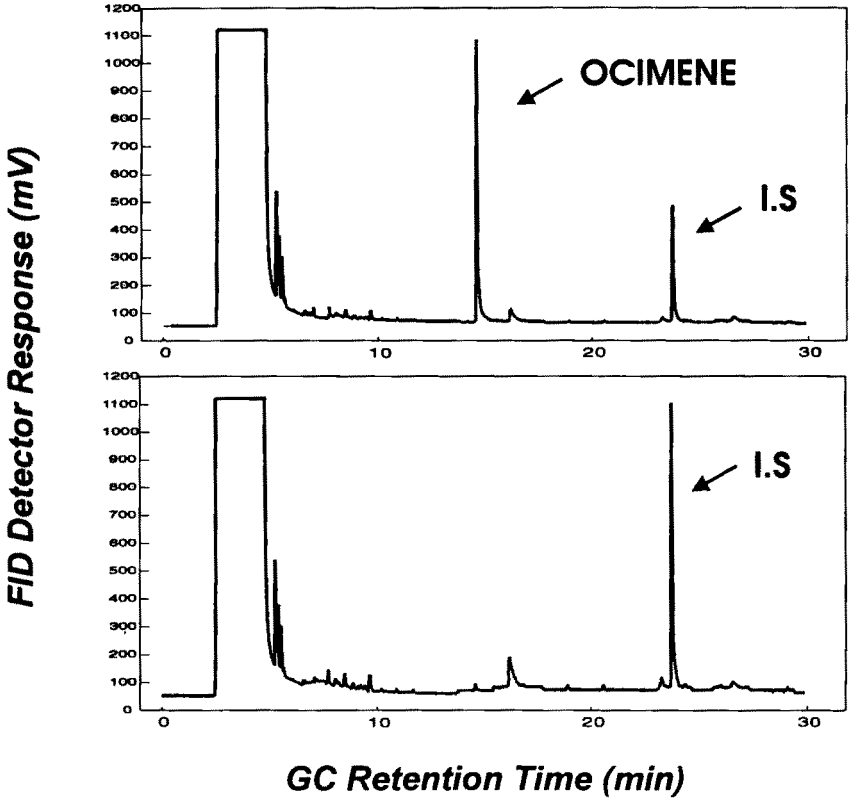

FIG. 5. Upper trace is a typical gas chromatogram obtained of volatiles released from four o'clocks during 1800–2000 hr. Lower trace is a typical gas chromatogram obtained when no volatiles are released from four o'clocks during 0200–0400 hr. The internal standard (IS) is 20 ng of *S*-C13 : Ac.

various time periods is shown in Figure 6. Onset of chemical release occurred during the 1400- to 1600-hr time period, and the greatest amount of ocimene was released between 1800 and 2000 hr. Release of ocimene then decreased during the 2000- to 2400-hr time period. Very little ocimene was released after 2400 hr. Determination of the amount of ocimene released per flower was calculated for the 1800- to 2000-hr time period. Based on the number of fully open and partially opened flowers, we were able to determine that yellow four o'clocks released 80.9 (±7.3 SD) ng ocimene/flower/hr and that the red four o'clocks released 51.9 (±7.0 SD) ng ocimene/flower/hr. The amount of ocimene released by yellow flowered four o'clock's was significantly more than

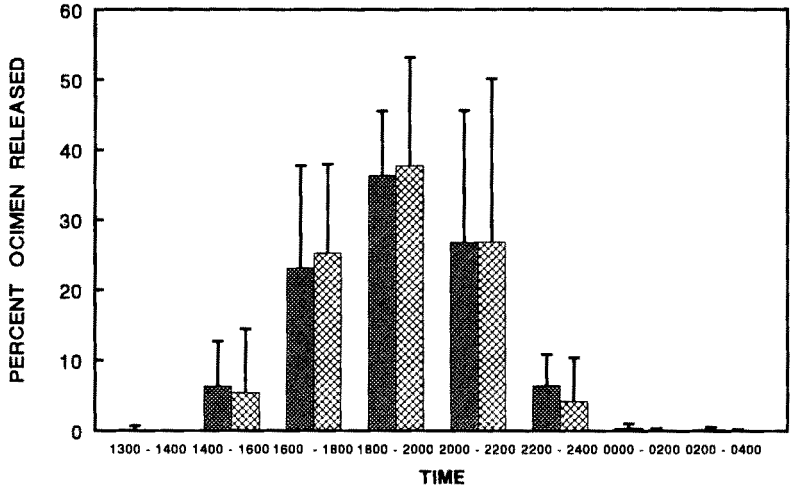

FIG. 6. Average percent ($N = 6$) of ocimene released by four o'clocks at different time periods. Cross-hatch bars are percent ocimene released from red flowers and slanted striped bars are percent ocimene released from yellow flowers. Error bars indicate SD.

that released by yellow flowered four o'clocks was significantly more than that released from red flowered four o'clocks ($t = 6.42$, $df = 10$, $P = 0.0001$). Although the timing of release for ocimene was similar for both the red and yellow flowering plants, the yellow flowers released more material than the red flowers.

Examination of environmental data recorded during the six experiments indicated a high degree of variability in light intensity, temperature, and humidity. This largely was due to the dynamic weather changes experienced daily in Florida during this time of year. A representative data set of continuous measurements of temperature, humidity, and light intensity obtained during an experiment is shown in Figure 7. Considerable fluctuation in light intensity occurs between 1200 and 1700 hr. These fluctuations reflect the sensitivity and the responsiveness of the light sensor and EDMS to varying cloud coverage that occurred during a relatively sunny day. Light intensity decreased rapidly after 1700 hr with darkness (<2 lux) occurring at approximately 1840 hr. Temperature was fairly stable, with an overall drop in temperature throughout the test. Relative humidity, being inversely related to temperature, showed an increase as both light intensity and temperature dropped after 1430 hr.

The system described here affords the simultaneous identification of volatile chemicals released from plants and an automated record of environmental conditions that occurred during the investigation. Automation allows for continuous

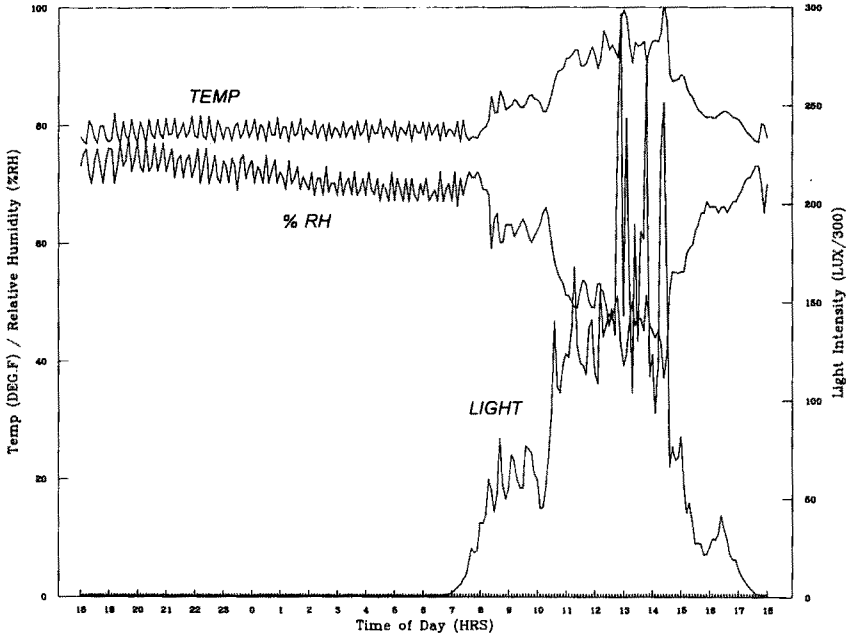

FIG. 7. Plot of environmental conditions recorded from the EDMS during a 24-hr period covering a typical volatile collection. Temperature, relative humidity, and light intensity are measured at a frequency of 20 KHz and are averaged and recorded every 6 min.

around-the-clock experimentation without requiring the presence of a human operator. This, in turn, should increase research productivity by decreasing the time taken to obtain experimental data and minimize the chance of contaminating the test chamber atmosphere or artificially inducing stress upon the plant through contact. Use of the described system offers a myriad of research opportunities including the determination of volatiles released from different varieties of crops, volatiles released from plants due to environmental stress and larval feeding damage, and determination of the effect of plants on pheromone released from pest insects.

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HOW GOATS LEARN TO DISTINGUISH BETWEEN NOVEL FOODS THAT DIFFER IN POSTINGESTIVE CONSEQUENCES

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Abstract—To better understand some of the mechanisms that control selection of novel foods differing in postingestive consequences, we offered goats current season's (CSG) and older (OG) growth twigs from the shrub blackbrush (*Coleogyne ramosissima*). CSG is higher than OG in nitrogen (1.04% v. 0.74%) and it is more digestible in vitro in goat rumen fluid (48% v. 38%). Nevertheless, goats acquire a preference for OG because CSG contains much higher levels of a condensed tannin that causes a learned food aversion. When CSG and OG were offered to goat naive to blackbrush, the goats did not choose either OG or CSG exclusively, but when they finally (1) ate more CSG than OG within a meal (averages of 44 g and 16 g, respectively) and (2) ate enough CSG within the meal to acquire an aversion (average of 44 g), they ingested less CSG than OG from then onward. Accordingly, the change in food selection resulting from postingestive feedback was influenced by the amount of each food ingested within a meal. This was further shown when we varied the amounts of CSG and OG that goats ingested within a meal, and then gave them by gavage the toxin lithium chloride (LiCl). They subsequently ate less of the food eaten in the greatest amount, regardless of whether it was CSG or OG. The salience of the flavor (i.e., taste and odor) of CSG and OG also played a role in the acquired aversion to CSG. Salience evidently was due to a flavor common to both OG and CSG that was more concentrated in CSG. We conclude that the relative amounts of different foods ingested within a meal, and the salience of the flavors of those foods, are both important variables that cause goats to distinguish between novel foods that differ in postingestive consequences.

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Key Words—Toxin, food selection, food aversion, secondary metabolite, nutrition, palatability, lithium chloride, ruminants, goat, *Capra* sp.

INTRODUCTION

Secondary metabolites defend plants from herbivory, but the causal mechanisms underlying plant defense are poorly understood in marine (Hay and Fenical, 1988) and in terrestrial (Feeny, 1992) ecosystems. Some argue that toxicosis is the basis for plant defense (Freeland and Janzen, 1974; Berenbaum, 1986; Bryant et al., 1991), while others contend that deterrence (i.e., rejection based on odor and taste only) is often independent of toxicosis (Bernays and Chapman, 1987; Bernays and Cornelius, 1992). Studies designed to separate the effects of toxicosis from deterrence show that ruminants decrease intake of foods paired with toxicity (Olsen and Ralphs, 1986; Lane et al., 1990; Provenza et al., 1990, Pfister et al., 1990; duToit et al., 1991; Launchbaugh et al., 1993) and with nutrient deficits (Rodgers and Egan, 1975; Egan and Rodgers, 1978), and they increase intake of the foods as toxicity diminishes and nutritional value improves (Burritt and Provenza, 1992). The increases in intake were caused by positive postingestive feedback derived from nutritional value the food provided, and the decreases in intake resulted from aversive postingestive feedback caused by toxicosis and nutrient deficits, not taste and odor alone (Provenza et al. 1992, Provenza, 1994a, b).

Our objective was to understand some of the variables that control selection of novel foods differing in postingestive consequences. We reasoned that insights into acquired preferences for and aversions to novel foods might be gained by analysis of food selection within meals. Combining this with an understanding of the way ontogeny (Provenza, 1994a, b) and variation among individuals (Provenza and Cincotta, 1993) affect food selection may help to clarify our views of an apparently complex process: How ruminants learn to distinguish between nutritious and toxic foods given a diverse array of plant species, individuals, growth stages, and parts that vary in nutritional value and chemical defenses (Provenza and Balph, 1990).

The shrub blackbrush (*Coleogyne ramosissima*), studied by Provenza et al. [1990], provided an opportunity to examine the response of goats, on a meal-by-meal basis, to foods that differed in postingestive consequences. Blackbrush, a small, evergreen shrub that averages less than 1 m in height, grows in dense stands on millions of hectares in the southwestern United States. In the absence of browsing, blackbrush twigs (i.e., older growth, OG) grow little, but branches produce current season's growth (CSG) when apical meristems are removed by browsing (Provenza et al., 1983a). CSG and OG differ in various characteristics. CSG is less "woody" than OG and its bark is red while that of OG is grey.

CSG is higher than OG in nitrogen (1.04% vs. 0.74%) and it is more digestible in vitro (48% vs. 38%) (Provenza et al., 1983b). Nevertheless, goats acquire a strong preference for OG because CSG contains much higher levels of a condensed tannin that causes a learned food aversion in goats (Provenza et al., 1990). In the research reported in this paper, we studied the responses of individually penned goats to CSG and OG because that provided a degree of experimental control not possible with free-ranging goats. We conducted four experiments.

METHODS AND MATERIALS

Experiment 1: Novel CSG and OG. Goats introduced to blackbrush-dominated rangelands learn to avoid CSG and to eat OG even though both foods are novel (Provenza and Malechek, 1984; Provenza et al., 1990). This experiment was designed to determine how long it takes goats to learn to distinguish between CSG and OG.

During October 1989, 10 goats (average body weight 21 kg) naive to blackbrush were offered a choice between CSG and OG in two separate food containers from 0900 until 1600 hr daily for three days. Blackbrush twigs were harvested daily and chopped into 1- to 3-cm lengths in a wood chipper. CSG and OG were offered ad libitum throughout the day, but goats were without food from 1600 hr one day until 0900 hr the next. They had access to water ad libitum. The amount of each food ingested was measured daily. The repeated measures analysis of variance had 10 blocks (goats) crossed with two foods (Winer, 1971).

Experiment 2: Hourly Monitoring of CSG and OG. Goats rapidly acquired an aversion to CSG in the first experiment, but how they distinguished between OG and CSG on day 1 is not clear from the first experiment. They may have initially ingested CSG, experienced aversive postingestive feedback, and then switched to OG, or they may have ingested more CSG than OG, experienced aversive feedback, and then switched to OG. To discover which of these feeding patterns was involved, we conducted a second experiment in which we observed the sequence of feeding events, and the amounts of CSG and OG ingested by goats on an hourly basis for three days.

During September 1990, 12 goats (average body weight 17 kg) naive to blackbrush were offered 500 g of CSG and 500 g of OG in separate food containers. The foods were available from 0930 to 1730 hr on day 1, from 0815 to 1820 hr on day 2, and from 0800 to 1400 hr on day 3. The foods were weighed at hourly intervals; about 15–30 min per hour were required to weigh the food. No food was available for the remainder of the day. Thus, goats had food for a total of 5, 8 and 5 hr, respectively, on days 1, 2, and 3. Goats had access to water ad libitum.

We performed two analyses on these data. For one analysis, 12 goats (blocks) were crossed with two foods. A repeated measures analysis was used because the data were analyzed across hours (Winer, 1971). The data were analyzed separately for each day. The other analysis involved the intake of OG and CSG for the first hour when a goat ingested >30 g of CSG and the intake of CSG and OG for the next three hourly meals >30 g. The consumption (CSG + OG) of different animals ranged from 32 to 94 g. For this analysis, 12 goats (blocks) were crossed with two foods and repeated across four meals.

Experiment 3: Equal CSG and OG. The data from experiment 2 suggested that salience (i.e., a greater tendency of the flavor of CSG than OG to be associated with malaise) and differences in the relative amount of food ingested in a meal (i.e., a greater amount of CSG than OG) might both play a role in the goats' acquisition of an aversion to CSG. To test hypotheses concerning salience and amount of food ingested, we allowed goats to ingest the same amount of CSG and OG. We reasoned that the acquisition of an aversion to CSG under these conditions would be consistent with the salience hypothesis. The acquisition of an aversion to both CSG and OG would be consistent with the amount-ingested hypothesis.

The experiment was conducted in November 1990 and involved 12 goats (average body weight 25 kg) naive to blackbrush. All animals were given 50 g of CSG and 50 g of OG from 1530 to 1630 hr on day 1; each animal received 1300 g of alfalfa pellets at 1730 hr. On day 2, goats were offered 100 g of CSG and 100 g of OG from 1630 until 1700 hr and were then fed alfalfa pellets. On day 3, all goats had access to 200 g of CSG and 200 g of OG from 1630 until 1730 hr. We measured the amount of CSG and OG ingested each day. The data were analyzed as a repeated measures with 12 blocks (goats) crossed with two foods and repeated across three days (Winer, 1971).

In addition, we determined the relationship between mass (g) and volume (cc) for both CSG and OG. The analysis of variance had two treatments, and twig type was nested within treatments.

Experiment 4: Different ratios of CSG and OG. We divided goats into five groups and offered them different proportions of CSG and OG on a volumetric basis: (1) 10:90, (2) 30:70, (3) 50:50, (4) 70:30, and (5) 90:10. After eating a small amount of CSG and OG, the goats were given lithium chloride (LiCl), a compound that causes learned food aversions in ruminants (Provenza et al., 1994c). We reasoned that if the volume of food ingested affected the acquisition of the aversion, goats should eat less OG than CSG in cases (1) and (2), should eat less CSG than OG in cases (4) and (5), and should avoid OG and CSG equally in case (3). If some salient characteristic of the odor and taste of CSG also influenced the aversion, goats in all treatments should eat less CSG than OG.

During December 1992, 37 goats (average body weight 27 kg) naive to

blackbrush were divided into five groups and were offered (for 30 min) the following volumes (cc) of CSG/OG mixed together: (1) 11:103 (10:90; n = 7), (2) 34:80 (30:70; n = 7), (3) 57:57 (50:50; n = 9), (4) 80:34 (30:70; n = 7), and (5) 103:11 (90:10; n = 7). These mixtures were equivalent, on a mass (g) basis for CSG/OG, to the following ratios: (1) 4:35, (2) 10:27, (3) 17:19, (4) 12:23, and (5) 30:4. Goats were offered only a small amount of CSG and OG to minimize aversive effects of tannin, and to ensure that both foods were eaten quickly. After eating the OG/CSG mixture, all goats received LiCl (150 mg/kg body wt in 100 ml water; duToit et al. 1991) by gavage. On the next day, goats were offered a choice between CSG (200 g) and OG (200 g) for 1 hr.

The volume of CSG ingested by each goat, as a percentage of the total volume consumed (OG + CSG), was used as the dependent variable in the least-squares regression analysis. The regression equation was derived using the raw data from each of the five treatment groups (i.e., 10, 30, 50, 70, 90%). In an attempt to better estimate the slope and the intercept for the regression equation, the data from days 1 and 2 of experiment 3 were expressed on a volumetric basis for each goat and included in the analysis. This provided a greater sample size in the range of values from 40 to 60% CSG ingested in a meal. Neither the slope nor the intercept of the equation generated from the data in experiment 4 changed significantly ($P > 0.10$) as a result of adding the data from experiment 3 to the regression model. The 11 data points from experiment 3 were not included in the analysis of variance of the data from experiment 4 because they did not fit the qualitative categories (i.e., 10, 30, 50, 70, 90%) exactly.

RESULTS AND DISCUSSION

Experiment 1. Goats ingested more OG than CSG on the first day of the experiment (Figure 1). Intake of OG increased on day 2 and again on day 3, but intake of CSG was similar on all days. This caused an interaction between food and day ($P < 0.001$).

Goats ate both OG and CSG on day 1, but they had consumed more OG by 1600 hr, which indicates most goats acquired an aversion to CSG on the first day. Goats ingested < 100 g of CSG on all three days, evidently because aversive feedback from the tannin caused them to limit intake of CSG. Nonetheless, it is important to note in this and the other experiments that goats did not eliminate CSG from their diets, and it is likely that if the concentration of the condensed tannin decreased, goats would have increased intake of CSG, as occurs in sheep when the toxin concentration of a nutritious food decreases (Launchbaugh et al. 1993).

The increase in intake of OG on day 2 and again on day 3 is similar to the

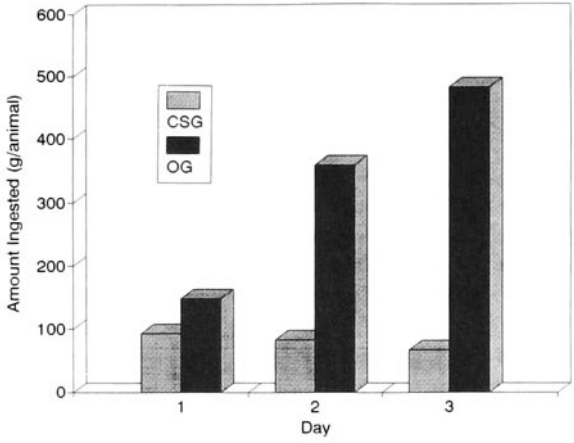

FIG. 1. Average intake (g) of current season's (CSG) and older (OG) growth blackbrush (SEM = 19) by goats from 0900 to 1600 hr for three consecutive days during experiment 1. Means separated by more than 54 g differ ($LSD_{0.05}$).

response of sheep to novel foods (Provenza et al., 1994a). The reluctance of sheep to eat new foods apparently does not depend on the nutritional quality of the food, or on the level of food deprivation, but on fear of the novel food (neophobia).

Experiment 2. During the first hour of day 1, goats ingested significantly more CSG than OG, but during the last hour of day 1 they ingested significantly more OG than CSG (Figure 2), which resulted in a significant food × hour interaction ($P < 0.05$). On day 2, goats ingested more OG than CSG throughout the day ($P < 0.01$; Figure 3). The same pattern was evident on day 3 ($P < 0.001$).

Goats' choice of food changed dramatically after they ate more than 30 g of CSG within 1 hr. Thereafter, when goats ate a meal of >30 g, they ate less CSG than OG (Figure 4). As a result of this change in food selection, the interaction between food and time was significant ($P < 0.001$).

There was no pattern concerning the time when a goat ate its first large meal of CSG. Six goats ate >30 g of CSG during the morning of the first day, five ate >30 g during the afternoon of the first day, and one ate >30 g on the morning of the second day. The sequence of CSG and OG consumption also varied. Seven goats initially ate a greater proportion of CSG, and five initially consumed more OG.

Goats ate both CSG and OG, and after they ate a substantial amount of CSG relative to OG (average of 44 g and 16 g), their intake of CSG declined (Figure 4). When goats eat two or more novel foods and experience malaise, it

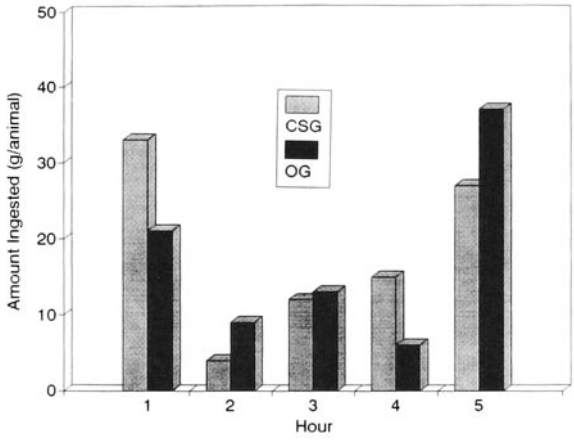

FIG. 2. Average intake (g) of current season's (CSG) and older (OG) growth blackbrush (SEM = 3.8) by goats from 0930 until 1730 hr on day 1 of experiment 2. Means separated by more than 8 g differ ($LSD_{0.05}$).

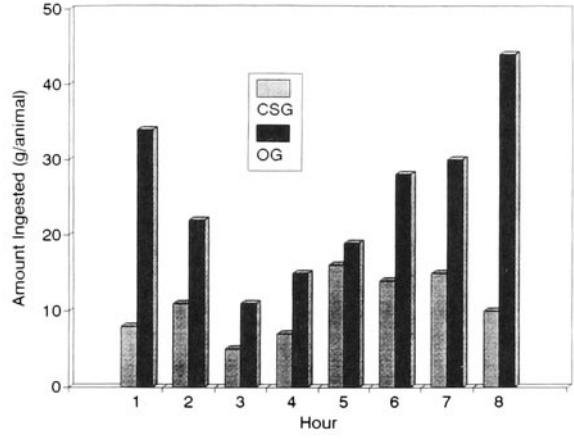

FIG. 3. Average intake (g) of current season's (CSG) and older (OG) growth blackbrush (SEM = 4.3) by goats from 0815 until 1820 hr on day 2 of Experiment 2. Means separated by more than 8 g differ ($LSD_{0.05}$).

is not clear which food(s) will be avoided in the following meal. As Garcia (1989) states, the critical feature involves two tastes (each with its potentiated odor) competing for association with one nauseous feedback in the gut. Thus, there are at least four explanations, which are not mutually exclusive, for the way in which flavor (taste and odor) might control the behavior of goats.

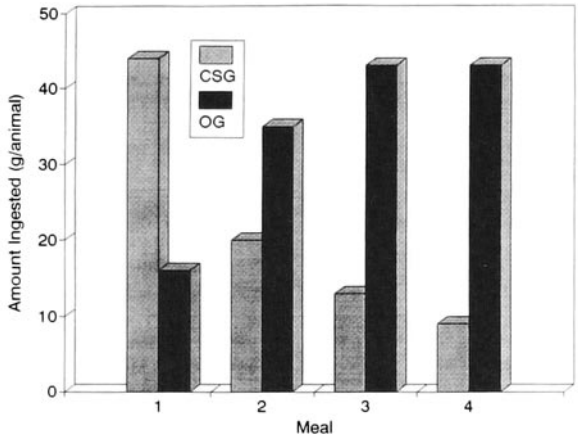

FIG. 4. Average intake (g) of current season's (CSG) and older (OG) growth blackbrush (SEM = 4.4) by goats when they ate a meal of more than 30 g of CSG, and for the ensuing three large meals whenever they occurred during days 1–3 of experiment 2. Means separated by more than 9 g differ ($LSD_{0.05}$).

First, novelty might be important. For instance, Revusky and Bedarf (1967) successively paired two flavors, one novel, with a single toxin dose. The novel flavor became much more aversive, whether it was first or second. Moreover, Cannon et al. [1985] found that only 10 min of exposure to one novel solution (saccharin) was enough to cause rats to prefer saccharin to a completely novel solution (coffee) following toxicosis. Some goats initially ate more OG than CSG, which could make OG more familiar than CSG. Thus, the greater novelty of CSG to OG might account for the learned aversion to CSG by some of the goats, but it can not account for the aversion to CSG by the other goats that ate CSG prior to OG.

Learned safety might also be a factor. For example, Kalat and Rozin (1973) showed that a rat that drank the same solution twice prior to poisoning learned less aversion than when it received the solution only at the second presentation. Some goats ingested more CSG than OG more than once before they acquired an aversion to CSG, which according to this hypothesis should have meant that their aversion to CSG was less than their aversion to OG. Nevertheless, all goats acquired an aversion to CSG, so this explanation can not account for the learned aversion to CSG.

Salience, in this case the tendency of a novel food to be associated with malaise, often influences acquired aversions more than does temporal proximity of food ingestion to poisoning (Kalet and Rozin, 1970, 1971). For instance, Cannon et al. (1985) paired exposure to saccharin, followed by exposure to

either a low or a high concentration of a quinine solution, with a single toxin dose. They found that subsequent consumption of saccharin was a positive function of the concentration of the quinine solution drunk prior to toxicosis. Accordingly, if some characteristic of the flavor of CSG is more salient than that of OG, it could explain why goats acquired a stronger aversion to CSG.

Finally, the amount of food ingested might influence aversions to CSG. For example, the strength of a rat's aversion to saccharin is a direct function of the amount of saccharin consumed prior to poisoning (Bond and DiGiusto, 1975). If this also occurs in goats, then an aversion to CSG might have resulted when a goat consumed enough CSG to experience malaise. OG consumption may not matter if it were a relatively small proportion of the meal.

Experiment 3. Goats ingested similar amounts of CSG and OG on day 1, but they ate less CSG than OG on day 2 and day 3 (Figure 5). This caused an interaction between food and day ($P < 0.05$).

The relationship between mass and volume differed for CSG and OG ($P < 0.0001$). CSG was less dense (0.25 g/cc; SEM = 0.006) than OG (0.35 g/cc; SEM = 0.006).

Goats ate similar amounts of CSG (39 g) and OG (45 g) on day 1 of this experiment ($P > 0.05$), but on days 2 and 3 they ate less CSG than OG. Thus, these results are in agreement with the hypothesis that the aversion was caused by something salient about the flavor of CSG, and they are not consistent with the amount-ingested hypothesis. Nevertheless, the data do not rule out the amount-ingested hypothesis because when goats ate an average of 39 g of CSG

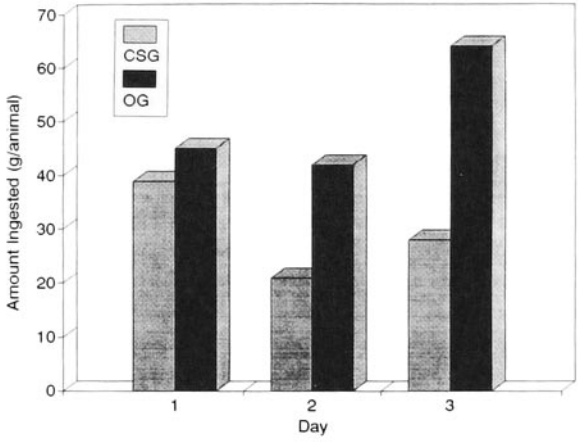

FIG. 5. Average intake (g) of current season's (CSG) and older (OG) growth blackbrush (SEM = 6.0) by goats during experiment 3. Means separated by more than 12 g differ ($LSD_{0.05}$).

and 45 g of OG on day 1 of the experiment, they consumed a larger volume of CSG (159 cc) than of OG (130 cc). Thus, we conducted a more definitive test of these two hypotheses in experiment 4 in which we accounted for the volumes of CSG and OG ingested by goats, and we included a wider range of volumes, similar to those ingested by goats in experiment 2 (Figures 2–4).

Experiment 4. Virtually all of the goats (34 of 37) ingested all of the CSG/OG mixture in 15 min when it was offered on day 1 of experiment 4, but during the 1-hr test on day 2 only 15 of 37 goats ate blackbrush (four goats in 10:90 CSG/OG, two goats in 30:70, two goats in 50:50, three goats in 70:30, and four goats in 90:10). Nevertheless, there were differences ($P < 0.001$) among treatments in the percentage of CSG ingested by goats on day 2 (Table 1).

There was an inverse relationship ($r = -0.73$; $P < 0.0001$) between the percentage of CSG ingested on day 2 and that ingested on day 1 for experiments 3 and 4 (Figure 6). The intercept of the regression equation was 74 ($P < 0.0001$; SE = 7.5) and the slope was $-0.89X$ ($P < 0.0001$; SE = 0.17). The equation suggests that on day 2 the average goat in treatment (1) ate a meal of 65% CSG and 35% OG and that the average goat in treatment (5) ate only OG. When a quadratic term was added to the model, the term was not significant ($P > 0.10$). Any goat that ate a meal of more than 6 cc during testing was included in the regression analysis.

The inverse relationship between the percentage of CSG ingested on day 1 and that ingested on day 2 and the fact that the slope of the line (-0.89) did not differ from that of the hypothetical line (-1.0) depicted in Figure 6 indicate that the volume of food ingested played a role in the acquisition of an aversion by goats. These data are consistent with the outcome of Experiment 2 in which

TABLE 1. RELATIONSHIP BETWEEN CURRENT SEASON'S GROWTH (CSG) INGESTED ON DAY 1 AND ON DAY 2 OF EXPERIMENT 4[a]

CSG Ingested		
% of meal, day 1	% of meal, day 2[b]	SE
10	56[a]	5
30	20[b]	6
50	17[b]	7
70	10[bc]	7
90	5[c]	5

[a] Goats in different treatments consumed meals that varied from 10% to 90%, on a volumetric basis, of CSG and older growth (OG) on day 1. Immediately after ingesting the meal, goats were gavaged with the toxin lithium chloride. On day 2, they were given a choice between CSG and OG.
[b] abc, means in the same column with different letters differ significantly ($LSD_{0.05}$).

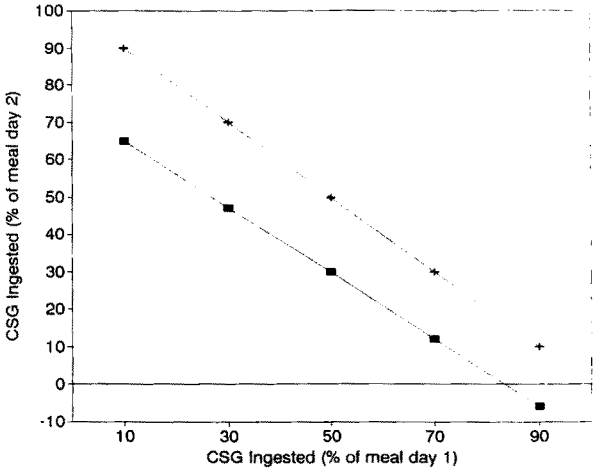

FIG. 6. The relationship between current season's growth (CSG) ingested on day 1 of experiments 3 and 4 and that ingested on day 2 (lower line). Goats in different treatments consumed meals that varied from 10% to 90%, on volumetric basis, of CSG and older growth (OG) on day 1; immediately after ingesting the meal, goats were gavaged with the toxin lithium chloride. On day 2, they were given a choice between CSG and OG. The "volume of food ingested" played a role in the acquisition of an aversion to CSG in goats because there was a significant inverse relationship ($r = -0.73$) between the percentage of CSG ingested on day 1 and that ingested on day 2. The slope of the regression line is $-0.89X$ ($P < 0.0001$; SE = 0.17) and the intercept is 74 ($P < 0.0001$; SE = 7.5). The upper line is the expected relationship if only volume of food ingested controlled the ingestion of OG and CSG. "Salience" also played a role in the goats' acquisition of an aversion to CSG because the intercept for the lower line is significantly less ($P < 0.01$) than that for the upper line (see text for further discussion).

goats ingested a meal of 79% CSG and 21% OG (on a volumetric basis), and thereafter ate meals composed primarily of OG (Figure 4).

The hypothetical (upper) line in Figure 6 represents the expected relationship if only volume of food ingested controlled the ingestion of OG and CSG by goats. Clearly, salience also influenced the goats' aversion to CSG because the intercept of the lower line (74) was significantly less than that of the hypothetical line (100). Thus, these data are consistent with the results of experiment 3 (Figure 5).

We did not attempt to characterize the salient properties of the flavor, but CSG had a stronger (more salient) odor than did OG. If the odor, and perhaps the taste as well, were caused by a flavor common to both OG and CSG, but more concentrated in CSG, then goats that ate primarily OG (treatment 1) prior to receiving LiCl should have eaten less CSG during testing because CSG was

the food with the highest concentration of the flavor. Likewise, goats that ate primarily CSG (treatment 5) should have subsequently ingested OG, the food with the lowest concentration of the flavor. The data are consistent with this hypothesis.

Lambs generalized food aversions on the basis of a salient flavor when they were offered barley with a low and a high concentration of either a sweet flavor (sodium saccharin) or a bitter flavor (aluminum sulfate). The lambs initially consumed the same amounts of barley, regardless of flavor intensity, but after they ate the flavored barley and received a mild dose of LiCl, they subsequently preferred the barley with the lower concentration of either flavor (Launchbaugh et al., 1993).

More than half of the goats (22 of 37) did not eat either OG or CSG during testing in experiment 4 because of the novelty of the food and the dosage of LiCl. Animals form much stronger aversions to novel foods than to familiar foods (Revusky and Bedarf, 1967; Cannon et al., 1985; Burritt and Provenza, 1989, 1991). Moreover, the higher the dose of LiCl, the stronger the aversion (duToit et al., 1991). Administering LiCl by gavage produced a much stronger aversion in goats than did ingesting the condensed tannin in CSG (e.g., Figure 4), because when the toxin is given by gavage (i.e., LiCl), goats could not limit intake of the food to minimize aversive feedback.

CONCLUSIONS

When CSG and OG were offered to goats naive to blackbrush, the goats did not exclusively select either OG or CSG, but when they finally ate enough CSG to experience malaise, they ingested less CSG than OG from that point onward. The change in food selection resulting from postingestive feedback occurred within hours and was influenced both by volume of food ingested and by salience. The greater the volume of food ingested and the more salient the flavor of the food, the stronger was the acquired aversion. This was true whether the aversion was caused by the tannin in CSG (Figure 4) or by LiCl (Figure 6).

On several occasions, we have observed food selection by goats when they were first introduced to blackbrush-dominated rangelands. The goats sampled all foods in the area during the first few hours, including potentially toxic plants like *Juniperus osteosperma* (bark and green leaves), *Gutierrezia microcephala* (a forb), and *Marrubium vulgare* (a forb), as well as more nutritious shrubs like *Prunus fasciculata* and *Purshia tridentata*. They also sampled both CSG and OG from blackbrush. Within a few days, goats limit intake of the potentially toxic foods and ingest meals composed primarily of blackbrush OG and *Prunus fasciculata*. We do not know how much of each food goats ingest during the

first few days, but we suspect that the amount is low initially and gradually increases (or decreases), depending on ensuing postingestive feedback. It would be an interesting extension of the present experiments to determine how the goats' aversions and preferences are acquired in that more complex situation.

Postingestive feedback and the senses of taste, smell, and sight are interrelated through affective and cognitive processes (Garcia, 1989). Taste plays a prominent role in both processes. Affective processes integrate the taste of food and its postingestive consequences, and changes in the intake of food items depend on the degree to which postingestive consequences are aversive or positive. The net result is a change in incentive to eat a particular food. Cognitive processes involve use of the senses of smell, sight, and higher cortical centers to seek foods that provide positive consequences and to avoid foods that cause internal malaise. The net result is a change in behavior.

Animals do not make conscious decisions concerning acquired food aversions based on the amount of food ingested or its salience, because postingestive feedback does not involve cognitive processes. This is illustrated by the fact that animals (Garcia et al., 1985; Garcia, 1989), including sheep (Provenza et al., 1994b), in deep anesthesia still acquire food aversions. Thus, when a goat eats CSG and OG, and subsequently acquires an aversion to CSG, the decrease in incentive to eat CSG occurs whether or not the goat is conscious. The resulting feedback is a function of the match between the animal's physiology at the time it ingests the food and the chemical characteristics of the food.

Several variables have been shown to control the interrelationship between affective and cognitive processes in both monogastrics and ruminants. For instance, acquired aversions are dependent: (1) on temporal contiguity between food ingestion and toxicosis (Cannon et al., 1985; Provenza et al., 1993b), (2) on food novelty (Revusky and Bedarf, 1967; Burritt and Provenza, 1991), (3) on the intensity of a particular flavor (Cannon et al., 1985; Launchbaugh et al., 1993), (4) on the volume of food ingested (Bond and DiGiusto, 1975; this study), (5) on prior experience with illness (Cannon et al., 1975; Launchbaugh et al., 1993), and (6) on prior experience with a salient flavor (Kalat and Rozin, 1970, 1971; Launchbaugh and Provenza, 1993; this study). Thus, the results of these and other studies (reviewed by Garcia, 1989; Provenza, 1994a, b) suggest that deterrence (i.e., taste and odor of foods) and toxicosis are intimately related via feedback mechanisms.

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RESPONSE OF MALE EYE-SPOTTED BUD MOTH, *Spilonota ocellana* (LEPIDOPTERA: TORTRICIDAE), TO DIFFERENT PHEROMONE BLENDS IN NORTH AMERICA AND THE NETHERLANDS[1]

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Abstract—Response of male eye-spotted bud moth, *Spilonota ocellana* (Denis and Schiffermüller), to different ratios of synthetic sex pheromone components, (Z)-8-tetradecenyl acetate (Z8–14:OAc) and (Z)-8-tetradecenyl alcohol (Z8–14:OH), were compared in four North American locations and in one location in The Netherlands. In British Columbia, Nova Scotia, Michigan, and The Netherlands, a 99:1 blend of Z8–14:OAc and Z8–14:OH captured significantly more male *S. ocellana* than Z8–14:OAc alone or binary blends containing 10–50% Z8–14:OH. In Ontario, where population sizes were low compared to the other four locations, trends in trap catches were similar, and there was no indication that male *S. ocellana* responded differently to the tested pheromone blends. A 99:1 blend of Z8–14:OAc and Z8–14:OH should

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be most effective in pheromone-based control programs of *S. ocellana* in North America and in The Netherlands. Our results confirm earlier studies that a 99:1 blend of Z8–14:OAc and Z8–14:OH captures significantly more male *S. ocellana* than Z8–14:OAc alone. However, our finding that a 99:1 blend of Z8–14:OAc and Z8–14:OH is significantly more attractive than binary blends containing 10–50% Z8–14:OH differs from previous findings in Germany and Switzerland.

Key Words—Lepidoptera, Tortricidae, *Spilonota ocellana*, eye-spotted bud moth, sex pheromone, (Z)-8-tetradecenyl acetate and (Z)-8-tetradecenyl alcohol.

INTRODUCTION

The eye-spotted bud moth, *Spilonota ocellana* (Denis and Schiffermüller), a pest of apples, cherries, and blueberries, was first recorded in eastern North America in 1841 (Gilliatt, 1932). Since its introduction from Europe, its North American range has extended to include most temperate fruit-growing regions from Nova Scotia to British Columbia (Gilliatt, 1932; MacLellan, 1978). In apple, *S. ocellana* has a history of outbreaks in North America (MacLellan, 1978), but wide-spectrum insecticides used against the codling moth, *Cydia pomonella* L., often control *S. ocellana* populations (Madsen and Downing, 1968). As growers implement noninsecticidal controls for codling moth, such as pheromone-based mating disruption, *S. ocellana* damage may increase, as reported in the Okanagan Valley of British Columbia (Judd and Gardiner, 1991) and The Netherlands (Van Deventer et al., 1992).

In British Columbia, a 99:1 blend of Z8–14:OAc and Z8–14:OH was recommended for pheromone-based monitoring of *S. ocellana* (McBrien et al., 1991), but in Germany and Switzerland, male *S. ocellana* were best attracted to binary blends containing 91–50% Z8–14:OAc (Witzgall et al., 1991). Before pheromone-based monitoring and mating disruption programs for *S. ocellana* can be developed, the responses of male *S. ocellana* to different ratios of Z8–14:OAc and Z8–14:OH should be examined throughout North America and Europe. Regional variation in pheromone blends as documented for other species of Tortricidae, e.g., the oblique-banded leaf roller, *Choristoneura rosaceana* (Harris) (Thomson et al., 1991), and the fruit-tree leaf roller, *Archips argyrospilus* (Walker) (Deland et al., 1993), may also exist in *S. ocellana*. We report the response of male *S. ocellana* to Z8–14:OAc alone and to four blends of Z8–14:OAc and Z8–14:OH containing 1–50% Z8–14:OH at four North American locations and at one location in The Netherlands.

METHODS AND MATERIALS

Field Experiments. Z8–14 : OH and Z8–14 : OAc were synthesized (isomeric purity >98%; chemical purity >97%) by standard acetylenic methods, using a procedure similar to that used to prepare Z10–14 : OAc (Hendry et al., 1975). Further purification of Z8–14 : OAc was achieved by argentation chromatography resulting in >99% isomeric purity and >98% chemical purity as determined by capillary GC. GC analyses using both flame ionization (FID) and electroantennographic detection (EAD) (McBrien et al., 1991) showed that synthetic Z8–14 : OAc did not contain detectable amounts of alcohol and vice versa. None of the chemical contaminants elicited antennal responses in GC-EAD recordings.

Field tests were conducted in apple orchards in five widely separated geographic locations: Winfield, British Columbia; Clarksburg, Ontario; Kentville, Nova Scotia; Fennville, Michigan; and near Kesteren, The Netherlands. Wing traps (Phero Tech Inc., Delta, British Columbia V4G 1E9) were set up in five complete randomized blocks, with 15 m between traps within blocks, and at least 25 m between blocks. Traps were suspended 1.5 m above ground in the outer 0.5 m of foliage, at least 10 m from every border row. Red rubber septa (Aldrich Chemical Co. Inc., Milwaukee, Wisconsin 53233) were loaded with 100 μg of synthetic pheromone, either Z8–14 : OAc alone or in binary combination with Z8–14 : OH. The five pheromone blends tested were 100 : 0, 99 : 1, 90 : 10, 80 : 20, and 50 : 50 μg/μg of Z8–14 : OAc and Z8–14 : OH. All septa were loaded with pheromone at the Agriculture Canada Research Station in Summerland, British Columbia, and then shipped to each location. Before placement in the field, each septum was pinned to the inner side of the trap top. The number of male *S. ocellana* captured after 14 days was counted. Specimens captured in pheromone-baited traps were randomly selected from each location and returned to Summerland for species verification.

Data Analyses. Due to the small number of male *S. ocellana* captured in Ontario, these data were excluded from statistical analyses. All statistical tests were performed using SAS software (SAS Institute Inc., 1985, Cary, North Carolina), and in all cases, $\alpha = 0.05$. Trap catches were transformed by log $(x + 1)$ to stabilize variance. Responses to the three binary blends representing a progressive series of treatments with decreasing Z8–14 : OAc concentration from 99 to 80% were subjected to regression analysis (Chew, 1976; Perry, 1986), and results were compared between locations by a three-way ANOVA. Trap catches for Z8–14 : OAc alone were compared to each of the binary blends by a two-way ANOVA followed by a Dunnett's test. The percentage of mean total trap catch represented by each treatment for the five locations was plotted against the percentage of Z8–14 : OAc in the synthetic blend.

RESULTS AND DISCUSSION

All *Spilonota* captured in pheromone-baited traps were identified as *S. ocellana*. In British Columbia, Nova Scotia, Michigan, and The Netherlands, there was a significant positive relationship ($P < 0.05$) between trap catch and Z8–14:OAc concentration for binary blends containing 80–99% Z8–14:OAc (Figure 1) (British Columbia: $y = 0.020x - 0.808$, $r^2 = 0.82$; Nova Scotia: $y = 0.027x - 1.031$, $r^2 = 0.95$; Michigan: $y = 0.025x - 1.078$, $r^2 = 0.72$; The Netherlands: $y = 0.021x - 0.668$, $r^2 = 0.94$). The significant slopes of each regression line indicate that the three treatments are significantly different in their effects (Chew, 1976). The responses of male *S. ocellana* to binary blends of Z8–14:OAc and Z8–14:OH with 80–99% Z8–14:OAc were similar in all four locations ($F_{0.05,3,36} = 0.57$, $P > 0.05$). The 50:50 blend of Z8–14:OAc and Z8–14:OH was excluded from regression analysis because the relationship between trap catch and Z8–14:OAc concentration becomes asymptotic in the region between 50% and 80% Z8–14:OAc (Figure 2), suggesting that there is a threshold value below which trap catch changes very little in response to changing Z8–14:OAc concentration.

In the four locations, the numbers of male *S. ocellana* captured by the binary blends were significantly higher than for Z8–14:OAc alone ($F_{0.05,4,16} \geq 7.31$, $P < 0.05$, followed by Dunnett's test), with the exception of the 50:50 blend in Michigan, which was not more attractive than Z8–14:OAc alone (Figure 2). Mean trap catch with the 99:1 blend was 6.6–11.2 times greater than

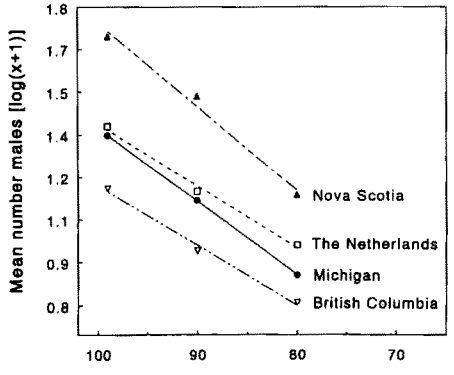

FIG. 1. Relationship between numbers of male *S. ocellana* captured in wing traps and three concentrations of Z8–14:OAc (99, 90, and 80%) in binary combination with Z8–14:OH for a total loading of 100 μg. All linear regression lines are significant ($P < 0.05$).

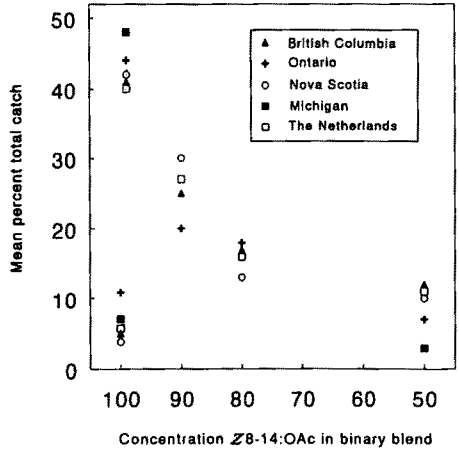

FIG. 2. Mean percentage of the total number of male *S. ocellana* captured in each treatment (n = 5 for each location). Traps were baited with Z8–14:OAc alone or in binary combination with Z8–14:OH for a total loading of 100 μg. Michigan and The Netherlands are identical for treatments containing 90 and 80% Z8–14:OAc. The dotted line joins the mean percentage of all five sites.

for Z8–14:OAc alone, confirming results of earlier studies (McBrien et al., 1991; Witzgall et al., 1991) showing that the addition of 1% Z8–14:OH to Z8–14:OAc significantly increases trap catch of male *S. ocellana*.

Trends in trap catches were similar in all five locations, with a 99:1 blend of Z8–14:OAc and Z8–14:OH capturing most male *S. ocellana*. These results differ from those obtained in Switzerland and Germany, where most male *S. ocellana* were captured with blends of 10:1 to 1:1 Z8–14:OAc and Z8–14:OH (Witzgall et al., 1991). This suggests there may be geographic variation in pheromone response of male *S. ocellana*, as has been reported for many other Lepidoptera (Cardé and Baker, 1984; Löfstedt, 1990).

In North America, a pheromone-based monitoring and mating disruption program for *S. ocellana* should probably be developed using a 99:1 blend of Z8–14:OAc and Z8–14:OH. Further study of regional variation in the pheromone biology of *S. ocellana* in Europe should be conducted before pheromone-based monitoring or control programs are implemented. The fact that *S. ocellana* in North America respond similarly to those in The Netherlands suggests the North American population may have originated from a similar area of Europe.

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EFFECT OF ODOR DERIVED FROM LION FAECES ON BEHAVIOR OF WILD RABBITS

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Abstract—A synthetic repellent derived from lion feces was tested in both pen and field bioassay trials. The chemical suppressed the feeding of rabbits on carrots for approximately one month. The application of the chemical to rabbit burrows under field conditions showed that it decreased rabbit numbers in the treated warrens and that this effect could still be detected after five months. It is suggested that this chemical could be a timely environmentally acceptable addition to the armamentarium of control measures required to reduce the recent increase in rabbit numbers occurring in Britain.

Key Words—Rabbit, *Oryctolagus cuniculus*, odor, lion feces, repellent, control.

INTRODUCTION

Rabbits were the major vertebrate pest of agricultural crops in Great Britain prior to 1953 when myxomatosis reduced their numbers by an estimated 99.9% (Thompson, 1953; Armour and Thompson, 1955). Since then their numbers have gradually increased due to the rabbits' increased resistance to the myxoma virus and to the virus becoming less virulent (Ross and Sanders, 1984; Fenner and Chapple, 1965). The increase in population (Trout et al., 1986) has also occurred in spite of the use of a range of conventional control measures, e.g., gassing, snaring, shooting, fencing, and destruction of preferred habitats (Buckley, 1935; Lockley, 1940; Southern, 1948; Thompson and Armour, 1951; Phillips, 1955; McKillop and Wilson, 1987; Boag, 1987) until by 1986 it was

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evident that rabbits caused between £90,000,000 and £120,000,000 of damage in Britain alone (Mills, 1986).

Alternative control measures have until now either proved to be of little use, e.g., acoustic scaring devices (Wilson and McKillop, 1986) and warren ripping (Parker, et al., 1976) or are environmentally unacceptable or illegal in Great Britain, e.g., poisoning (Richards, et al., 1986) or introducing new diseases, i.e., rabbit hemorrhagic diseases (RHD) (Xu and Chen, 1989).

The use of predator odors to deter lagomorphs from damaging crops and trees has received little attention, but results of investigations using mustelid scent gland chemicals showed promise against snowshoe hares (Sullivan and Crump 1984). Van Haaften (1963) reported that lion feces could protect crops against rabbits, hares, and red and roe deer for up to three months and suggested producing a repellent from synthesizing the active ingredients. In 1988 such a repellent was patented by Dalgety PLC (Baines et al., 1988) and preliminary trials indicated that the substance affected both rabbits and roe deer (Boag and Mlotkiewicz, 1991; Mlotkiewicz, 1994).

The purpose of the present investigation was to study the effects of an odor derived from lion feces on the behavior of rabbits in pens and under natural conditions.

METHODS AND MATERIALS

Pen Bioassay. A bioassay was conducted at Rossie Priory Estate, Perthshire, Scotland in an enclosure (25 × 12.5 m) set in a grass field. The enclosure had solid wooden sides to a height of ca. 1 m and rabbit-proof netting between 1 and 2 m. The whole enclosure was covered with bird netting to exclude predators. The experiment within the enclosure consisted of eight feeding bowls ca. 22 cm diameter × 10 cm deep placed 3 m from each other at one end of the pen. In each bowl a 1.5-cm-diameter × 6-cm-high plastic stoppered sampling tube, which had 0.5-cm-diameter holes drilled in it, was fixed upright in the center of the bowl in which cotton wool was placed. In four of the bowls, the cotton wool in the sampling tube was impregnated with 2.5 cc of the repellent, while in the other four distilled water was used. The positions of the bowls were randomly chosen and changed each time the food was changed. Five hundred grams of fresh carrots were weighed and placed in each bowl twice a week, any remaining carrot from each bowl was removed and individually weighed. A shelter was constructed at the end of an enclosure away from the trial for the four wild rabbits that were introduced on March 14, 1992. The experiment was started on March 21 and terminated on May 5.

Field Bioassays. Two comparative trials were conducted at Redmyre Farm, Ordnance Grid Reference No. NO280340. Four relatively similar rabbit war-

rens, within 300 m of each other were chosen in a large grass field. The two warrens that were selected at random to be treated with the repellent in 1991 were the same as those treated in 1990, while the other two warrens were left as controls. The numbers of rabbits at each warren were counted twice weekly for the four weeks prior to the application of the chemical on April 23, 1990, and April 24, 1991, then twice weekly for the next eight weeks and once weekly for another eight weeks (with the exception of July 13, 1990).

The repellent supplied by Dalgety PLC was diluted 50:50 by volume with distilled water and put in a knapsack sprayer. At each instance approximately 5 ml of the diluted liquid was administered 30–40 cm into each burrow entrance.

RESULTS

Pen Bioassay. The mixture of chemicals comprising the repellent was very effective in suppressing the feeding of the rabbits on the chopped carrots (Table 1). At no time during the experiment were all of the untreated control carrots eaten, although the amount varied between a mean of 427 g on March 25 to 300 g on April 15. No carrots were eaten from the bowls with the repellent present during the first week and it was not until April 8, over two weeks later, that the carrots were regularly taken. By April 22, approximately 50% of the weight of the control carrots were being taken from the bowls with the repellent present, and by May 5 no significant differences could be found between the amount of carrots eaten by the rabbits from the two groups.

TABLE 1. EFFECT OF REPELLENT ON MEAN WEIGHT OF CARROTS EATEN BY RABBITS (START DATE MARCH 22, 1992)

Date	Treated	Untreated control[a]
25.03.92	0	427***
28.03.92	0	378**
01.04.92	9	316***
04.04.92	0	311**
08.04.92	102	359**
11.04.92	70	301***
15.04.92	141	300*
18.04.92	95	334**
22.04.92	184	398*
25.04.92	273	355*
28.04.92	318	390
01.05.92	374	411
05.05.92	404	406

[a] *, **, *** $P < 0.05$, < 0.01, < 0.001 with respect to untreated controls.

Field Bioassays. In the field trials the number of rabbits seen at the treated and untreated warrens prior to treatment were not equal (Table 2). The ratio between treated and untreated in 1990 was 1 : 1.15 and the figure for 1991 was 1 : 1.47. These figures indicate that the rabbit population in the treated warrens 1 and 2 had a smaller rabbit population than the controls in warrens 3 and 4. This suggestion is supported by the numbers of rabbits shot at these warrens during a long-term investigation into rabbit parasites (Boag, 1985), which extended between 1977 and 1989. Rabbits collected at warrens 1 and 2 over that period totaled 64, while 84 were shot at warrens 3 and 4, giving a treated–untreated ratio of 1 : 1.32, which is between those of 1 : 1.5 and 1 : 1.47 for the rabbits counted prior to the experiments starting in 1990 and 1991.

The differences in the initial populations observed in early April 1990 and 1991 can probably be explained by the fact that the warrens were gassed during the winter of 1989–1990 and that breeding took place earlier in 1991 than in

TABLE 2. MEAN NUMBERS OF RABBITS COUNTED EACH WEEK AT WARRENS TREATED AND NOT TREATED WITH SYNTHETIC REPELLENT

1990			1991		
Date	Treated	Untreated	Date	Treated	Untreated
01.04.90	5.5	6.0	31.03.91	11.5	16.0
08.04.90	3.0	4.0	07.04.91	11.5	24.5
05.04.90	6.5	6.5	14.04.91	22.0	24.0
22.04.90	4.5	6.0	21.04.91	20.0	31.0
23.04.90	Repellent added		24.04.91	Repellent added	
29.04.90	1.0	20.0	28.04.91	0	32.0
06.05.90	1.0	18.0	05.05.91	6.5	41.0
13.05.90	1.5	18.5	12.05.91	4.5	48.5
20.05.90	4.0	26.5	19.05.91	7.0	52.0
27.05.90	7.0	31.5	26.05.91	9.0	55.0
03.06.90	7.0	31.0	02.06.91	7.5	55.5
10.06.90	6.0	33.0	09.06.91	6.0	70.0
17.06.90	5.0	35.0	16.06.91	7.5	44.5
24.06.90	8.5	31.0	23.06.91	5.0	40.5
01.07.90	9.5	36.0	30.06.91	7.5	42.0
08.08.90	8.0	25.0	07.07.91	7.5	42.0
15.07.90	10.0	33.5	14.07.91	8.5	49.0
22.07.90	12.0	39.0	21.07.91	10.0	44.0
29.07.90	9.0	37.0	28.07.91	9.0	24.5
05.08.90	13.0	32.5	04.08.91	8.5	21.5
13.09.90	12.0	32.0	12.08.91	5.0	14.5
18.09.90	16.0	34.0	17.08.91	5.0	12.0

1990. This meant that very few young were observed until the end of April 1990 whereas they were seen in March 1991.

The succeeding fluctuations in rabbit numbers also showed some variation in the control populations between years. In 1990 rabbit numbers increased and stayed high while those in 1991 also rose, to a level twice that recorded in 1990, but subsequently decreased markedly until they were significantly less than those in 1990.

The addition of the repellent significantly reduced the rabbits observed in both 1990 and 1991, and it was not until the third week in May that adults returned. Numbers gradually increased until the first week in August 1990 and the third week of August 1991; thereafter in both years numbers remained relatively constant or declined slightly. However, even by the middle of September the numbers recorded at the treated warrens were less than 50% of those found at the untreated control warrens.

DISCUSSION

The importance of different odors on communication between and within rabbit communities has received a great deal of attention. Studies have shown rabbit odors to affect territorial, sexual, and social status of rabbits (Mykytowycz et al., 1976; Goodrich and Mykytowycz, 1972; Hesterman et al., 1976; Hudson and Vodermayer, 1992) but the effect of the odor of predators has received little attention. The repellent effects of odors from weasel, stoat and fox on rodents has been recorded (Stoddart, 1976; Gorman, 1984; Dickman and Doncaster, 1984), and it has been shown that these and others could be used to reduce the feeding damage done by herbivores (Sullivan and Crump, 1984). The results of the present pen bioassay would suggest that the synthetic repellent derived from lion feces significantly affected the feeding of rabbits on carrots for over one month, which is similar to the six-week protection offered by a component of weasel scent (Sullivan and Crump, 1984). However, Sullivan et al. (1985) have suggested that this period could be extended if the odors were in weatherproof controlled-release devices. Apart from the possibility of using repellents to directly protect crops, they could, in the case of burrowing pests, be used to selectively manage them by humanely eradicating them from their harborages. The field bioassay trials showed that the effect of treating rabbit burrows was successful in reducing rabbit numbers. Even after five months, rabbit populations had not recovered to the level that would be expected by comparing them with the controls even after correcting for the inequality in numbers of rabbits before the experiment started.

The results of both pen and field trials suggest that the synthetic repellent derived from lion feces could have a role in both protecting crops and be a

humane, environmentally acceptable chemical that can be used to manage wild rabbit populations.

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OXIDATIVE RESPONSES IN SOYBEAN FOLIAGE TO HERBIVORY BY BEAN LEAF BEETLE AND THREE-CORNERED ALFALFA HOPPER

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Abstract—Variation in induced responses in soybean is shown to be dependent, in part, upon herbivore species. Herbivory by the phloem-feeding three-cornered alfalfa hopper caused increases in the activities of several oxidative enzymes including lipoxygenases, peroxidases, ascorbate oxidase, and polyphenol oxidase. Bean leaf beetle defoliation caused increased lipoxygenase activity, but had little effect upon peroxidase, polyphenol oxidase, ascorbate oxidase, or trypsin inhibitor levels in either field or greenhouse studies. In one field experiment, prior herbivory by the bean leaf beetle subsequently reduced the suitability of foliage to the corn earworm *Helicoverpa zea*. The contribution of these findings to emerging theories of insect-plant interactions is discussed.

Key Words—Soybean, lipoxygenase, peroxidase, polyphenol oxidase, trypsin inhibitor, ascorbate oxidase, oxidative stress, Lepidoptera, Noctuidae, *Helicoverpa zea*, corn earworm, *Cerotoma trifurcata*, bean leaf beetle, Coleoptera, Chrysomelidae, *Spissistilus festinus*, three-cornered alfalfa hopper, Homoptera, Membracidae, induced resistance, interspecific competition.

INTRODUCTION

There is growing recognition that an integral component of plant defense consists of chemicals that impose oxidative stress upon invading organisms (e.g., Berenbaum, 1978; Hildebrand et al., 1986a,b, 1989; Felton et al., 1989; Duffey and Felton, 1991; Lee and Berenbaum, 1989; Aucoin et al., 1990; Zangerl,

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1990; Zangerl and Berenbaum, 1990). Oxidative stress results from the formation of reactive oxygen species (i.e., superoxide, singlet oxygen, hydroxyl radical, hydrogen peroxide) during photoactivation of thiophenes (Iyengar et al., 1987) and furanocoumarins (Joshi and Pathak, 1983; Lee and Berenbaum, 1989, 1990) or during the autooxidation of prooxidant chemicals such as quercetin (Hodnick et al., 1989; Ahmad and Pardini, 1990), gossypol (Grankvist, 1989), and caffeic acids (Hanham et al., 1983), The oxygen species, particularly hydroxyl radical and singlet oxygen, are highly reactive and induce lipid peroxidation and DNA and protein damage (Halliwell, 1991). The formation of reactive oxygen species in plant tissues in response to pathogens and/or wounding has recently been reported (Zacheo and Bleve-Zacheo, 1988; Apostol et al., 1989; Vianello and Macri, 1991; Rubinstein, 1992; Vera-Estrella et al., 1992; Legendre et al., 1993). These responses may be defensive and/or involved in repair processes.

Oxidative stress also accrues from plant oxidative enzyme activity. Polyphenol oxidases and peroxidases oxidize phenolics in damaged tissues to form reactive, electrophilic quinones capable of irreversible binding to proteins (Gaspar et al., 1981; Pierpoint, 1983). The quinones formed from the oxidation of chlorogenic acid adversely affect noctuid larval growth (Felton et al., 1989, 1992b; Duffey and Felton, 1991; Felton and Duffey, 1991). Furthermore, secondary oxidation–reductions of the substituted quinones form reactive oxygen species (Cheynier and Van Hulst, 1988). Gossypol, a terpenoid aldehyde in cotton, is a constitutive product of peroxidase activity implicated in defense against *Helicoverpa zea* (Bell et al., 1987). Lipoxygenase catalyzes the hydroperoxidation of *cis*,*cis*-penta-1,4-diene moieties of unsaturated fatty acids such as linoleic acid (Hildebrand et al., 1988). The hydroperoxides and resulting secondary products such as malondialdehyde can cause membrane deterioration, protein damage, and may inhibit arthropod growth (Shukle and Murdock, 1983; Mohri et al., 1990; Duffey and Felton, 1991). Reactive oxygen species may also be formed during lipoxygenase-catalyzed reactions (Kanofsky and Axelrod, 1986; Chamulitrat et al., 1991). The oxidation of ascorbate to dehydroascorbic acid by plant ascorbate oxidase also generates reactive oxygen species that damage proteins or membrane lipids (Nishimura et al., 1989; Baysal et al., 1989; Felton and Summers, 1993). Thus, the action of polyphenol oxidase, peroxidase, lipoxygenase, and ascorbate oxidase form a combination of reactive, electrophilic products such as quinones, hydroperoxides, and secondary oxygen species. Indeed, the herbivore midgut can be likened to a "free radical time bomb" because of its inundation by a diversity and quantity of reactive radical species.

As part of our ongoing research of oxidative plant responses, this investigation was initiated to determine the oxidative response of soybean to herbivory by a defoliating insect, the bean leaf beetle, *Cerotoma trifurcata* (Coleoptera:

Chrysomelidae), and by a phloem-feeding insect, the three-cornered alfalfa hopper *Spissistilus festinus* (Homoptera: Membracidae). Specifically, the effect of herbivory on foliar lipoxygenases, polyphenol oxidase, ascorbate oxidase, peroxidases, and protease inhibitor levels was determined under greenhouse and field conditions. The impact of bean leaf beetle herbivory on larval growth rates of the corn earworm, *Helicoverpa zea* (Lepidoptera: Noctuidae), also was assessed under field conditions.

METHODS AND MATERIALS

Insects and Plants. Adults of the bean leaf beetle (BLB) and three-cornered alfalfa hopper (TCAH) were collected in soybean fields from Foreman, Arkansas, and brought to Fayetteville, Arkansas, for use in greenhouse and field studies. Eggs of *H. zea* were obtained from the University of Arkansas Insect Rearing Facility. Larvae were maintained on artificial diet (Chippendale, 1970) until their use on soybean plants.

The soybean cultivar Forrest (Maturity Group V; Fehr and Caviness, 1977) was used in all studies. For greenhouse studies, plants were grown in one-gallon containers in screen mesh cages 12 × 12 × 24 in. (L × W × H). For field studies plants were grown in 6 × 6 × 6 ft mesh cages with two 4-ft rows of 8 plants/ft.

Plant Response to Herbivory. To determine the response of soybean to herbivory by BLB, beetles were placed on greenhouse-grown V3 stage plants at densities of 1, 3, and 5/plant. V3 stage plants are vegetative stage with three nodes on main stem. Control plants were not treated with beetles. After 72 hr of feeding, the fully expanded trifoliate from the uppermost node was removed for chemical analyses as described below. In most cases, this trifoliate was damaged by feeding. Five plants per treatment were tested, and the experiment was replicated three times. Treatments were set up as a randomized complete block design.

The above experiment was repeated with field-grown plants. At the V3 stage, three bean leaf beetles were placed on each plant in the treatment cages. Beetles were not placed on plants in the control cages. After 72 hr, eight plants per cage were randomly removed and the expanded trifoliate from the uppermost node was used in the analyses described below. All plants in treatment cages showed evidence of beetle herbivory. Three cages were used per treatment with a cage representing a replicate. Treatments were set up as a randomized complete block experiment.

To determine the response of soybean to herbivory by TCAH, the experiments described above were carried out identically with the exception of TCAH replacing BLB. In greenhouse studies, treatments were one or two TCAH per

plant plus an untreated control. After 72 hr, plants showing evidence of TCAH herbivory, as indexed by main stem girdling, were used in the chemical analyses. Five plants per treatment were tested, and the experiment was repeated three times using a randomized complete block experimental design.

The experiment with TCAH was repeated with field-grown plants. At the V3 stage, one TCAH was placed on each plant, and the control plants were left untreated. After 72 hr, eight plants (with main stem girdling) were removed from each cage for analyses, and the expanded trifoliate from the uppermost node was used in chemical assays described below. Again, three cages per treatment were used with cages set up in a randomized complete block design.

Response of Helicoverpa zea to Previous Herbivory by Bean Leaf Beetle. To assess the response of *H. zea* larvae to previous wounding by BLB, plants were grown under cages in the field as described above. At the V7–8 stage, three BLB adults were placed on each soybean plant in the beetle treatment cages. Control cages received no beetles. Three cages for each treatment were used with a cage comprising a replicate. Eight plants were randomly removed from each cage for chemical analyses. The fully expanded trifoliate from the uppermost node was removed from each plant for analyses. One third-stage *H. zea* larva was placed on each soybean plant in both control and beetle treatment cages. Larvae weighing between 9.5 mg and 10.5 mg were selected from the laboratory colony for placement on the plants. Approximately 50 larvae were placed in each cage, depending upon final plant density. After five days, larvae were recovered from the plants and weighed to the nearest 0.1 mg. For purposes of statistical computation of relative growth rates (Waldbauer, 1968), it was assumed that the initial weights of larvae were 10.0 mg. Recovery rates of larvae were ca. 10–18% in each cage.

Chemical Assays of Plant Natural Products. Foliage was excised from plants and homogenized in 0.1 M potassium phosphate, pH 7.0, containing 1% polyvinylpolypyrrolidone and 0.5 mM EDTA. The resulting slurry was centrifuged for 20 min at 10,000*g*. The supernatant was used immediately as the enzyme source. Linoleic acid was used as a substrate for lipoxygenase, and the rate of change in absorbance at 234 nm was measured (Grayburn et al., 1991). In one experiment with V7–8 plants, lipoxygenase was assayed at pH 5.5, 7.0, and 8.5 due to the presence of multiple lipoxygenase isozymes with different pH optima (Grayburn et al., 1991).

Ascorbate oxidase was measured by following the decrease in ascorbate absorbance at 265 nm (Esaka et al., 1989). Peroxidase activity was assessed with guaiacol and hydrogen peroxide by measuring the increase in absorbance at 470 nm due to guaiacol polymerization (MacAdam et al., 1992). Polyphenol oxidase was assayed with chlorogenic acid by measuring the increase in absorbance at 470 nm following Ryan et al. (1982). Protease inhibitor levels were estimated as trypsin inhibitor activity with bovine trypsin (Broadway and Colvin,

1992) in experiments with BLB. Soybean trypsin inhibitor (Sigma Chemical Co., St. Louis, Missouri) was used to develop a standard curve. A SLM-Aminco 3000 Array Spectrophotometer with kinetic software was used in all analyses.

Statistics. Data were analyzed by one-way randomized complete blocks ANOVA, and means were separated by least significance difference (LSD) CoSTAT software (CoHort Software, Berkeley, California).

RESULTS

Plant Response to Herbivory by Bean Leaf Beetle. In greenhouse studies, herbivory by 1 BLB/plant for 72 hr did not result in significant changes in lipoxygenase, peroxidase, polyphenol oxidase, ascorbate oxidase, or protease inhibitor levels (Table 1; $P > 0.05$). When BLB densities were 3/plant, lipoxygenase levels were greatly increased by 5.3-fold ($P < 0.05$). The levels of the other plant defense proteins were unaffected at this density. When plants were treated with 5 BLB/plant, lipoxygenase was 7.1-fold higher in the wounded plants ($P < 0.01$), and ascorbate oxidase showed a modest but significant increase of 25% ($P < 0.05$).

Results were similar in experiments with field-grown plants (Table 1). At a density of 3 BLB/plant, ascorbate oxidase, peroxidase, polyphenol oxidase, and protease inhibitor levels were unaffected by beetle herbivory ($P > 0.05$).

TABLE 1. EFFECT OF BEAN LEAF BEETLE (BLB) DENSITY ON PLANT PROTEINS IN V3 STAGE FORREST SOYBEAN[a]

Treatment	LOX (pH 7.0)	POD	PPO	AOX	PI
Greenhouse					
Control	117.4a	239.7a	24.48a	319a	12.48a
1 BLB	197.5a	185.2a	24.25a	372ab	11.68a
3 BLB	627.2b	199.9a	26.89a	350ab	11.18a
5 BLB	828.5c	214.7a	24.90a	398b	15.69a
LSD	189.6	58.5	4.60	55	5.90
Field					
Control	230.7a	108.6a	25.87a	543a	11.54a
3 BLB	447.3b	110.0a	29.56a	518a	10.30a
LSD	84.6	10.8	5.74	56	3.12

[a]Means in columns not followed by the same letter are significantly different at $P < 0.05$; and LSD values at $P = 0.05$. LOX = lipoxygenase activity expressed as nmol/min/gm foliage; POD = peroxidase expressed as Δ OD470/min/g; PPO = polyphenol oxidase expressed as Δ OD470/min/g; AOX = ascorbate oxidase expressed as nmol/min/g; PI = protease inhibitor expressed as μg/g foliage.

Lipoxygenase levels were significantly increased nearly twofold following beetle damage ($P < 0.05$).

Plant Response to Herbivory by Three-Cornered Alfalfa Hopper. The response of soybean to TCAH was different compared to BLB herbivory. In general, TCAH herbivory results in a more complex oxidative response. In greenhouse studies, 1 adult TCAH/plant did not affect the levels of lipoxygenase, peroxidase, or polyphenol oxidase but caused a 7.4-fold increase in ascorbate oxidase (Table 2; $P < 0.02$). At two TCAH, all of the oxidative enzymes were significantly affected, with lipoxygenase having a 5.0-fold increase ($P < 0.01$), peroxidase a 1.4-fold increase ($P < 0.05$), polyphenol oxidase an increase of 1.6-fold ($P < 0.05$), and ascorbate oxidase an increase of 2.9-fold ($P < 05$).

In field studies, TCAH at 1 adult/plant caused significant increases in all oxidative enzymes tested (Table 2). Lipoxygenase was increased by 2.3-fold ($P < 0.05$), peroxidase was 2.1-fold higher ($P < 0.05$), polyphenol oxidase was 1.7-fold higher ($P < 0.05$), and ascorbate oxidase was 3.1-fold higher ($P < 0.05$).

Response of Helicoverpa zea to Previous Herbivory by Bean Leaf Beetle. Lipoxygenase levels in untreated plants were substantially lower at the V7–8 stages (Table 3) than plants at the V3 stage (Table 1). Herbivory by BLB for five days resulted in large increases in lipoxygenase isozymes in V7–8 plants. When lipoxygenase was assayed at pH 5.5, BLB damage resulted in a 7.2-fold increase in activity (Table 3; $P < 0.01$), and at pH 7.0, lipoxygenase was 17.5-

TABLE 2. EFFECT OF THREE-CORNERED ALFALFA HOPPER (TCAH) DENSITY ON OXIDATIVE ENZYMES IN V3 FORREST SOYBEAN[a]

Treatment	LOX (pH 7.0)	POD	PPO	AOX
Greenhouse				
Control	160a	143a	15.0a	250a
1 TCAH	199a	193ab	15.1a	1860c
2 TCAH	804b	202b	24.0b	730b
LSD	75	58	2.2	91
Field				
Control	102a	148a	8.5a	1040a
1 TCAH	237b	309b	14.0b	3170b
LSD	62	34	2.5	183

[a]Means in columns not followed by the same letter are significantly different at $P < 0.05$; and LSD values at $P = 0.05$. LOX = lipoxygenase activity expressed as nmol/min/g foliage; POD = peroxidase expressed as Δ OD470/min/g; PPO = polyphenol oxidase expressed as Δ OD470/min/g; AOX = ascorbate oxidase expressed as nmol/min/g.

TABLE 3. EFFECT OF BEAN LEAF BEETLE (BLB) DAMAGE ON LIPOXYGENASES AND RELATIVE GROWTH OF THIRD-STAGE *Helicoverpa zea* LARVAE ON V7–8 STAGE FIELD PLANTS[a]

Treatment	LOX (pH 5.5)	LOX (pH 7.0)	LOX (pH 8.5)	RGR
Control	68.0a	26.0a	ND	0.231b
3 BLB	486.0b	454.0b	63.0	0.087a
LSD	104.2	132.7	26.0	0.049

[a] Means in columns not followed by the same letter are significantly different at $P < 0.05$; and LSD values at $P = 0.05$. LOX = lipoxygenase activity expressed as nmol/min/g foliage; ND = not detectable; RGR = relative growth rate of *H. zea* expressed as mg/day/mg larva.

fold higher ($P < 0.01$) in the BLB treatment. In undamaged V7–8 plants, lipoxygenase isozymes were not detectable at pH 8.5, but damaged plants showed an activity of 63 nmol hydroperoxide formed/min/g foliage.

Larval growth rates were significantly affected by previous BLB damage (Table 3). The relative growth rate of *H. zea* was reduced by over 62% on beetle-damaged plants ($P < 0.05$). The recovery rates for larvae from plants were not significantly different between treatments ($P > 0.05$). Twenty-one larvae from the initial 150 were recovered from the control treatment, and 23 larvae were recovered from the initial 150 larvae from beetle-treated plants.

DISCUSSION

Previous studies on soybean defensive responses to herbivory have focused primarily on phenolic biosynthesis (Chiang et al., 1987; Neupane and Norris, 1991a,b), protease inhibitor induction (Kraemer et al., 1987), or lipoxygenases (Hildebrand et al., 1988). Chiang et al. (1987) found that inducible resistance in the genotype PI 227687 to the Mexican bean beetle was positively correlated with total phenolic content and increased phenylalanine ammonia lyase and tyrosine ammonia lyase activities. These enzymes are positively associated with biosynthesis of phenolics such as flavonoids (Chiang et al., 1987). Induced feeding nonpreference by *Trichoplusia ni* was positively correlated with total phenolic glyceollins (Neupane and Norris, 1991a,b). Kraemer et al. (1987) showed that herbivory by Mexican bean beetle caused large increases in protease inhibitor levels. Two types of trypsin inhibitors, differing in molecular weight, were induced. The smaller molecular weight inhibitor was found only in damaged plants. In our studies, bean leaf beetle feeding did not significantly affect total trypsin inhibitor levels during the course of the experiment.

Spider mite damage in soybean increased lipoxygenase levels and lipid peroxidation (Hildebrand et al. 1986a,b, 1989). In our studies, foliar-feeding BLB and phloem-feeding TCAH also caused enhanced levels of lipoxygenases. Herbivory by the phloem-feeding TCAH produced a broader range of induced responses than that by the leaf-feeding BLB. TCAH caused elevated lipoxygenase, peroxidase, ascorbate oxidase, and polyphenol oxidase activities that were not observed in plants wounded by BLB. The oxidative products of these enzymatic activities may pose considerable oxidative stress to herbivores. Quinones, hydroperoxides, aldehydes, etc., formed by plant oxidases form irreversible chemical bonds with nucleophiles such as the $-SH$, $-NH$, $\alpha-NH_2$, and $\epsilon-NH_2$ groups of proteins and thus limit amino acid bioavailability (Duffey and Felton, 1991; Felton et al., 1992a,b). These reactive products can severely limit the bioavailability of key amino acids such as cysteine, lysine, and histidine (Duffey and Felton, 1991; Felton et al., 1992a,b). Dietary prooxidants can also cause direct injury to the herbivore by damage to lipids, proteins, and DNA (Ahmad, 1992). Furthermore, the loss of reduced ascorbic acid by ascorbate oxidase may exacerbate the oxidative damage caused by quinones, peroxides, etc. (Felton and Summers, 1993). The oxidized form of ascorbic acid, dehydroascorbic acid, is deleterious to membrane proteins and lipids (Nishimura et al., 1989; Baysal et al., 1989). Thus the redox status of the host plant may prove to be an important factor in determining host suitability to herbivores.

Oxidative processes may also be involved in defensive signal transduction and elicitation (Farmer and Ryan, 1990; Enyedi et al., 1992). For example, several products of the lipoxygenase pathway in plants such as (13*S*)-hydroperoxylinolenic acid, phytodienoic acid, and jasmonic acid are potent inducers of protease inhibitors in tomato, tobacco, and alfalfa (Farmer and Ryan, 1992). Hildebrand (1992) reported that methyl jasmonate induces large increases in lipoxygenase in tobacco. Grimes et al. (1992) found that low levels of atmospheric methyl jasmonate induced the accumulation of lipoxygenase in vacuoles and plastids.

Moreover, oxidative processes may be involved in chemical mediation of interspecific competition among herbivores sharing the same food plant. Previous herbivory by BLB significantly reduced the growth rate of *H. zea* larvae on soybean leaves (Table 3). Host-plant mediation of interspecific competition among herbivores has frequently been observed (e.g., Karban, 1986; Harrison and Karban, 1986; Faeth, 1986; Karban and English-Loeb, 1990); however, a chemical basis for this mediation has not often been shown (Tallamy and Krischik, 1989; Felton et al., 1992a).

These data provide support for our contention that induced oxidative responses are important components of plant–herbivore relationships. The production of oxidative products and active oxygen species is widely recognized as an important aspect of animal defense against pathogens (Rubinstein, 1992).

Rubinstein (1992) recently pointed out that animals and plants share many similar mechanisms for avoiding predation and disease. Thus, a new theory is emerging that implicates the production of oxygen radicals and oxidants as important components of plant defense against infection by microbes (Sutherland, 1991). We suggest that this theory may extend to plant–herbivore relationships, but it will require more causative evidence to be affirmed.

The relative importance of induced oxidative responses to antiherbivore defense is dependent upon multiple plant-related factors (e.g., resource availability, genotype, phenological, temporal, spatial, etc.) and herbivore-related factors (e.g., spatial and temporal distributions, feeding patterns, mouthpart form, type, and microbial contamination, saliva, etc.). In summary, we suggest that induced defenses in soybean are a concatenation of responses involving induction of multiple proteins, including phenylalanine ammonia lyase, tyrosine ammonia lyase, protease inhibitors, lipoxygenases, ascorbate oxidase, polyphenol oxidase, and peroxidases that systematically impair the growth and development of herbivores (Chiang et al., 1987; Kraemer et al., 1987; Hildebrand et al., 1989).

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POTENTIAL ROLE OF LIPOXYGENASES IN DEFENSE AGAINST INSECT HERBIVORY

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Abstract—The potential role of the plant enzyme lipoxygenase in host resistance against the corn earworm *Helicoverpa zea* was examined. Lipoxygenase is present in most of the common host plants of *H. zea*, with highest activity in the leguminous hosts such as soybean and redbean. Treatment of dietary proteins with linoleic acid and lipoxygenase significantly reduced the nutritive quality of soybean protein and soy foliar protein. Larval growth was reduced from 24 to 63% depending upon treatment. Feeding by *H. zea* on soybean plants caused damage-induced increases in foliar lipoxygenase and lipid peroxidation products. Larvae feeding on previously wounded plant tissue demonstrated decreased growth rates compared to larvae feeding on unwounded tissue. Midgut epithelium from larvae feeding on wounded tissues showed evidence of oxidative damage as indicated by significant increases in lipid peroxidation products and losses in free primary amines. The potential role of oxidative and nutritional stress as a plant defensive response to herbivory is discussed.

Key Words—*Helicoverpa zea*, Lepidoptera, Noctuidae, lipoxygenase, lipid peroxidation, resistance, herbivory, soybean, tomato, cotton, oxidative stress, induced defense.

INTRODUCTION

The phagocytic cells of animals produce reactive oxygen species and other oxidants as a defensive response to invading organisms (Baggliolini and Wymann, 1990). Recent research indicates that plants also produce reactive

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oxygen species and reactive oxidants in an analogous manner as a putative defense against pathogens and herbivores (Hildebrand et al., 1986a,b; Zacheo and Bleve-Zacheo, 1988; Apostol et al., 1989; Dillworth, et al., 1991; Doke et al., 1991; Montalbini, 1991; Felton et al., 1992a,b; 1994; Orlandi et al., 1992; Rubinstein, 1992; Vera-Estrella et al., 1992; Felton and Summers, 1993; Jiang and Miles, 1993; Legendre et al., 1993; Davis et al., 1993; Bi et al., 1994).

The oxidative status of host-plant tissues may undergo a rapid shift from a reduced state to a more oxidative state in response to invasive pests. Oxidative shifts may result from increased activities of oxidative enzymes such as lipoxygenase (Hildebrand et al., 1989; Croft et al., 1990), peroxidase (Bronner et al., 1991), or polyphenol oxidase (Felton et al., 1992a). The plant's oxidative status is enhanced during the generation of reactive oxygen species such as hydroxyl radical, hydrogen peroxide, and superoxide anion that accompany certain plant–pathogen interactions (Doke et al., 1991; Sutherland, 1991; Popham and Novacky, 1991). Reactive oxygen species arise from the enzymatic activities of NAD(P)H oxidase, peroxidases, polyamine oxidases, uric acid oxidase, and xanthine oxidase (Choudhuri, 1988; Vianello and Macri, 1991; Montalbini, 1991). Enhanced oxidative status of plant tissues may proceed from a loss of chemical antioxidants such as carotenoids (Hildebrand et al., 1986a), ascorbate (Milo and Santini, 1966), glutathione and related thiols (Polle and Rennenberg), 1992), and/or decreases in antioxidant enzymes such as catalase, glutathione reductase, and superoxide dismutase (Choudhuri, 1988).

In several instances, plant resistance to herbivores has been correlated with an enhanced oxidative state of plant tissues. The larval growth rate of the noctuid, *Helicoverpa zea*, was highly correlated with foliar polyphenol oxidase activity in tomato *Lycopersicon esculentum* (Felton et al., 1989). The mastication of foliage by larvae disrupts tissue integrity and the enzyme polyphenol oxidase is then allowed to interact with the substrate, chlorogenic acid (Felton et al., 1989). Polyphenol oxidase oxidizes chlorogenic acid to form the corresponding orthoquinone in the digestive system of lepidopteran larvae (Felton and Duffey, 1991b, Felton et al., 1989, 1992a). The quinone forms covalent bonds with proteins or amino acids, and thus limits amino acid bioavailability for the herbivore (Felton et al., 1989, 1992b; Felton and Duffey, 1991b). Spider mite resistance in soybean was highly correlated with enhanced lipid peroxidation and a loss of carotenoids due to lipoxygenases (Hildebrand et al., 1986b).

Lipoxygenases (EC 1.13.1.12) may be important mediators of both insect and phytopathogen resistance (Shukle and Murdock, 1983; Hildebrand et al., 1988; Croft et al., 1990; Gardner, 1991; Ohta et al., 1991). Lipoxygenases are ubiquitous enzymes that catalyze the hydroperoxidation of polyunsaturated lipids possessing *cis,cis*-pentadiene moieties (Hildebrand et al., 1988). The major substrates in plant tissues are linoleic and linolenic acids (Hildebrand et al.,

1988). The hydroperoxide products may be chemically and/or enzymatically degrade to an array of reactive aldehydes, γ-ketols, and epoxides (Gardner, 1991). Moreover, reactive oxygen species (e.g., singlet oxygen, hydroxyl radicals, superoxide anion) and peroxyl, acyl, and carbon-centered radicals are formed (Kanofsky and Axelrod, 1986; Chamulitrat et al., 1991). These reactive products may directly damage insect tissues, indirectly impair insect growth through damage to essential nutrients (e.g., linoleic acid, cholesterol, amino acids, β-carotene), and/or act as feeding repellents (Shukle and Murdock, 1983; Mohri et al., 1990; Duffey and Felton, 1991).

The aim of this investigation was to evaluate the potential for lipoxygenases to inhibit the growth of *H. zea*. The ability of lipoxygenase and the free fatty acid, linoleic acid, to reduce the nutritional quality of soy foliar protein was assessed. Additionally, the induction of lipoxygenase activity in soybean foliage following insect herbivory was studied. The role of dietary oxidative stress in plant defense against herbivory is discussed

METHODS AND MATERIALS

Insects and Plants. Eggs of *H. zea* were obtained from the University of Arkansas Insect Rearing Facility. Larvae were maintained on artificial diet (Chippendale, 1970) unless otherwise noted. Seeds of *Lycopersicon esculentum* (var. Castlemart), *Lycopersicon hirsutum* (LA 286), *Glycine max* (var. Forrest), *Phaseolus vulgaris* (a commercial redbean), and *Gossypium hirsutum* (var. DPL 50) were planted in one-gallon containers in the greenhouse.

Assay of Lipoxygenase, Peroxidase, and Lipid Peroxidation. To assay for foliar lipoxygenase, leaf tissue was homogenized in 0.1 M potassium phosphate, pH 7.0, containing 1% polyvinylpolypyrrolidone. The resulting slurry was centrifuged for 20 min at 10,000*g*. The supernatant was used immediately as the enzyme source. Linoleic acid was used as a substrate and the rate of change in absorbance at 234 nm was measured (Grayburn et al., 1991). In soybean foliage, lipoxygenase was assayed at pH 5.5, 7.0, and 8.5 due to the presence of multiple lipoxygenase isozymes with different pH optima (Grayburn et al., 1991).

To assay for peroxidase, the supernatant prepared as described above was used as the enzyme source with the exception of adding 0.5 mM EDTA. Peroxidase was assayed with 0.4 mM hydrogen peroxide and 3 mM guaiacol and the absorbance monitored at 436 nm as described by MacAdam and Sharp (1992).

To estimate lipid peroxidation in foliage, the thiobarbituric acid assay was used (Stewart and Bewley, 1980). Leaf tissue was homogenized in 0.1% TCA and 1% SDS followed by centrifugation at 5000*g* for 15 min. Aliquots of the supernatant were incubated in two volumes of 0.5% thiobarbituric acid in 20%

TCA at 95°C for 60 min. Samples were then centrifuged at 10,000*g* for 15 min. The absorbance of the supernatant was determined following Stewart and Bewley (1980).

Assay of Lipoxygenase in Larval Midgut Lumen. To determine if lipoxygenase remains active in the larval midgut, a newly molted fifth instar was placed on a three-node stage plant for 24 hr. The following plant species were tested: *L. esculentum*, *L. hirsutum*, *G. hirsutum*, *P. vulgaris*, and *G. max*. Additionally, larvae were placed on bolls from maturing *G. hirsutum*. Lipoxygenase was assayed from these plant tissues at pH 7.0 as described above. Control insects were maintained on artificial diet. A total of seven replicates per plant species were tested. After 24 hr, leaflets with feeding damage were excised and assayed for lipoxygenase activity. Midguts were removed and lumen contents were separated from midgut wall and peritrophic membrane. Lumen contents were mixed with an equal weight of 0.1 M potassium phosphate buffer, pH 7.0, and centrifuged at 10,000*g* for 20 min. Supernatant was used immediately as the enzyme preparation. Additionally, lumen activity was tested at pH 8.5.

Effect of Midgut Enzymes on Lipoxygenase Activity. To determine if midgut enzymes affect foliar lipoxygenase activity, a foliar protein extract from *L. hirsutum* was incubated with larval midgut tissue. Midguts were pooled from 15 fifth instars and prepared as described by Felton and Duffey (1991a). Ten grams of foliage from *L. hirsutum* was homogenized and prepared for lipoxygenase assay as described above. Ammonium sulfate was slowly added to the enzyme preparation to 80% saturation at 4°C. The sample was centrifuged, the pellet was resuspended in H_2O, and the ammonium sulfate was removed from the supernatant via a desalting column (PD-10; Pharmacia LKB, Uppsala, Sweden). The desalted protein extract was used immediately.

Midgut preparations were mixed with the plant protein extract and immediately assayed for lipoxygenase activity at pH 8.5 (0.05 mM potassium phosphate). This pH was chosen because it is within the range normally found in the midgut lumen. The following concentrations of midgut protein were mixed with 100 μg of plant protein: 17, 34, 51, and 68 μg. A control containing no midgut protein was also tested. Protein was measured by the method of Stoscheck (1990) with the addition of 1% soluble polyvinylpolypyrrolidone to the Coomassie dye reagent. Bovine serum albumin was used as a standard for midgut protein and dialyzed D-ribulose 1,5-diphosphate carboxylase (Sigma Chemical. Co., St. Louis, Missouri) for a plant protein standard. To determine if protease activity in the midgut affected lipoxygenase, 50 μg soybean trypsin inhibitor (Kunitz, Type 1-S; Sigma Chemical) was mixed with 51 μg of midgut protein prior to testing its effect on lipoxygenase. The experiment was replicated three times.

Induction of Lipoxygenase. To determine if feeding by *H. zea* larvae causes

an increase in the activity of foliar lipoxygenases, one fourth-instar was placed on each V3 stage soybean plant grown in the greenhouse. Each plant was covered with a screen cage to prevent larval escape. Five plants were treated with larvae and five control plants were treated identically except that larvae were excluded. After three days, fully expanded leaflets were excised from the uppermost node and assayed for peroxidase and lipoxygenase at pH 5.5, 7.0, and 8.5. Lipid peroxidation products were measured as described above following Stewart and Bewley (1980). The experiment was replicated three times.

To determine if ingestion of foliage from wounded plants affected larval growth rates and midgut oxidative stress, two trifoliates also were excised from each wounded and unwounded plant and offered to a newly molted fifth-instar *H. zea* for 48 hr. Larval weight gain and amount of leaf eaten were determined and relative growth rates and consumption rates were computed following Waldbauer (1968). A total of 20 larvae was tested per treatment. After weighing larvae, midguts were extirpated, washed free of lumen contents, and homogenized. Lipid peroxidation of the midgut was determined following Stewart and Bewley (1980). Free amine content of the midgut protein was determined following the procedures of Fields (1972).

Effects of Lipoxygenase on Dietary and Larval Growth. The effect of linoleic acid oxidation by lipoxygenase on protein quality was assessed in two separate experiments. In the first experiment designed to assess the effect of lipoxygenase activity on soy foliar protein quality, 600 g fresh weight leaf tissue was removed from ca. 200 V4–6 soybean plants (cv. Forrest) grown in the greenhouse. Tissue was homogenized in 2 liters of ice-cold 0.1 M potassium phosphate buffer, pH 7.0, containing 0.5 mM EDTA with 1% polyvinylpolypyrrolidone. The homogenate was filtered through Miracloth (Calbiochem, San Diego, California) and centrifuged for 30 min at 10,000*g*. The supernatant was removed and kept on ice while ammonium sulfate was slowly added to 80% saturation. The preparation was held on ice for 60 min and then centrifuged at 10,000*g* for 30 min. The pelleted protein was resuspended in 0.1 M potassium phosphate, pH 8.0, at 1 g protein/100 ml buffer and divided into six equal suspensions. To simulate the effect of linoleic acid oxidation on protein quality, three of the suspensions received 0.5 mM linoleic acid. The three linoleic acid-treated protein suspensions and three controls were stirred for 2 hr at 25°C. Following the incubation, suspensions were dialyzed at 6000–8000 mol wt cutoff for 48 hr against repeated exchanges of deionized water.

The samples were frozen, freeze-dried, and incorporated into artificial diet. A 100-g preparation of artificial diet contained the following: 1 g soy foliar protein, 5.215 g cellulose, 0.685 g Vanderzant vitamins, 2.400 g agar, 0.200 g wheat germ oil, 3.370 g dextrose, 2.750 g wheat germ, 0.900 g Wesson salts, 0.425 g alginic acid, 0.365 g ascorbate, 0.180 g cholesterol, 0.090 g choline

chloride, 0.010 g streptomycin, 0.120 g aureomycin, 0.200 g methyl paraben, 0.90 g sorbic acid, and 82 ml distilled water.

The free amine content (primarily ϵ-NH_2 of lysine) of the treated proteins was determined following procedure of Fields (1972). Protein content was determined following the modification of Stoscheck (1990) described above with dialyzed D-ribulose 1,5-diphosphate carboxylase (Sigma Chemical) as a protein standard.

Neonate *H. zea* larvae were individually placed in 18.5-ml clear plastic cups containing diet made with appropriately treated protein. Twenty larvae were tested per treatment, and the experiment was replicated three times. Larvae were weighed to the nearest 0.1 mg after 16 days.

In a second experiment to assess the effect of purified soybean lipoxygenase on soybean protein quality, 1 g soybean protein (ICN Biomedicals Inc., Costa Mesa, California) was incubated in a 100-ml buffered solution (pH 8.0, 0.1 M potassium phosphate) with 2 mM linoleic acid, 100 μl Tween 20, and 100 mmol/min of purified soybean lipoxygenase (Type V-affinity purified; Sigma Chemical) for 30 min with mechanical stirring. The control treatment was treated identically with the exception that lipoxygenase was not added. Following the incubation, suspensions were dialyzed at 6000–8000 mol wt cutoff for 48 hr against repeated exchanges of deionized water.

The samples were frozen, freeze-dried, and incorporated into artificial diet as described above. Bioassays were conducted as described and the experiment was replicated three times.

Statistics. Data were analyzed by ANOVA using CoStat Software (Berkeley, California) and means were separated using a studentized *t*-test.

RESULTS

Assay of Lipoxygenase in Larval Midgut Lumen. Preliminary data indicated that maximal lipoxygenase activity for most plant species tested occurred at pH 7.0, with the exception of the soybean cultivar Forrest. Subsequently all measurements of plant activity were made at pH 7.0. The levels of lipoxygenase varied considerably among the various host plants tested (Table 1). Activity ranged from a low of 5 nmol/min/g in the cotton to a high of 1458 nmol/min/g in red bean. The activity of lipoxygenase in the wild tomato *L. hirsutum* (LA 286) was more than 25 times greater than the activity in commercial tomato.

Lipoxygenase activity could not be detected in the midgut lumen contents of larvae regardless of the dietary host plant species or tissue ingested. Activity also was tested at pH 8.5, to determine if different lipoxygenase isozymes may retain activity in the midgut; however, activity also was not detectable at the higher pH.

Effect of Midgut Enzymes on Lipoxygenase Activity. Midgut preparations significantly ($P < 0.001$) reduced lipoxygenase activity in the plant protein extracts from *L. hirsutum* (Figure 1). The addition of soybean trypsin inhibitor to the mixture effectively mitigated the negative effect of midgut tissue on lipoxygenase. In the absence of midgut tissue, the inhibitor had no effect on lipoxygenase activities. These data suggest that the digestive trypsinlike enzymes, are in large part responsible for digesting lipoxygenase and its subsequent loss in activity in the midgut lumen.

Induction of Lipoxygenase and Effect on Larvae. Feeding by *H. zea* larvae on soybean foliage for 72 hr increased lipoxygenase activity by 2.27× when assayed at pH 7.0 and a 1.72× when tested at pH 8.5 ($P < 0.02$) compared

TABLE 1. LIPOXYGENASE ACTIVITY IN MIDGUT LUMEN AND LARVAL DIET

Diet source[a]	Plant activity[b]	Lumen activity[b]
L. esculentum (Castlemart)	26b	ND
L. hirsutum (LA 286)	672d	ND
G. hirsutum (DPL 50)	5a	ND
Boll	4a	ND
P. vulgaris	1,458e	ND
G. max (Forrest)	354c	ND
Artificial diet	ND	ND

[a] Larvae were placed on the corresponding plant species for 24 hr prior to assay of lumen activity. Larvae were fed leaves or bolls of *G. hirsutum*.
[b] LOX activity reported as nmol/min/g tissue. ND = not detectable. Means in columns not followed by the same letter are significantly different at $P < 0.05$.

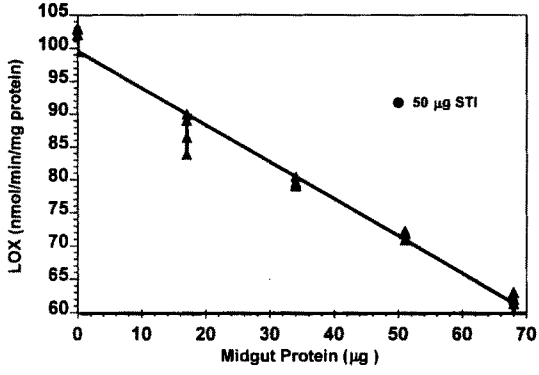

FIG. 1. Effect of midgut enzymes on foliar lipoxygenase activity in *Lycopersicon hirsutum*. STI = soybean trypsin inhibitor.

to the undamaged control foliage (Table 2). Apparently, the lipoxygenase isozymes most active at pH 5.5 were less inducible as the difference between treatments was not significant ($P > 0.05$). Lipid peroxidation products in wounded foliage were 17.3% higher ($P < 0.0009$) than in unwounded foliage. Peroxidase activity was unaffected by larval feeding ($P > 0.05$).

Larval growth rates were reduced by more than 27% ($P < 0.01$) when larvae fed on previously damaged plant foliage compared to control foliage (Table 3). However, relative consumption rates of larvae were unaffected by treatment ($P > 0.05$). Accompanying the decrease in larval growth rate was a 46% increase in lipid peroxidation in midgut tissues and a 9.1% decrease in the free amine content of midgut protein (Table 3). Both assays provided an index for oxidative stress and indicated that larvae ingesting wounded foliage are exposed to increased oxidative stress. The oxidative stress may be attributable to not only lipoxygenase activity, but also may be due to other biochemical changes in the redox status of the host plant.

Effects of Lipoxygenase on Dietary Protein and Larval Growth. The iso-

TABLE 2. EFFECT OF WOUNDING ON FOLIAR CHEMISTRY[a]

	LOX[b]				
Treatment	pH 5.5	pH 7.0	pH 8.5	POD[c]	TBARS[d]
Control	382a	176a	76a	44.0a	100.0a
H. zea	591a	400b	131b	45.0a	117.3b

[a]Means in columns not followed by the same letter are statistically different at $P < 0.05$.
[b]LOX = lipoxygenase activity expressed as nmol/min/g foliage.
[c]POD = peroxidase activity expressed as Δ OD 436 nm/min/g foliage.
[d]TBARS = foliar lipid peroxidation products measured as thiobarbituric acid reactive substances following Stewart and Bewley (1980). Expressed as relative percent.

TABLE 3. EFFECT OF INGESTION OF DAMAGED SOYBEAN FOLIAGE ON LARVAE[a]

Diet treatment	Midgut TBARS[b]	Free amines[c]	RGR[d]	RCR[e]
Undamaged Foliage	100a	100b	0.37b	1.33a
Damaged Foliage	146b	90.9a	0.27a	1.55a

[a]Means in columns not followed by the same letter are statistically different at $P < 0.05$.
[b]TBARS = lipid peroxidation products measured as thiobarbituric acid reactive substances following Stewart and Bewley (1980). Expressed as relative percent.
[c]Free amines = relative amount in midgut epithelium.
[d]RGR = relative growth rate of 5th instar larvae expressed as mg/day/mg larva.
[e]RCR = relative consumption rate expressed as mg biomass ingested/day/mg larva.

lated protein fractions had a lipoxygenase activity of 23.1 nmol/min/mg protein when tested with linoleic acid as substrate (Table 4). In the absence of the added linoleic acid, activity was not detectable, indicating that endogenous free fatty acids had been removed from the protein fraction. The oxidation of linoleic acid by lipoxygenase resulted in a decrease in the nutritional quality of foliar protein as indicated by a 4.3% loss of free amines ($P < 0.01$) and greater than 60% reduction in larval growth ($P < 0.01$) on diet containing the treated protein. It should be noted that the linoleic hydroperoxides formed by lipoxygenases during the protein incubation would likely undergo chemical and enzymatic degradation to other reactive molecules (e.g., aldehydes) that may, in large part, be responsible for the reduction in protein quality.

When soybean protein was treated with purified lipoxygenase, the growth of larvae was reduced by 24.4% ($P < 0.05$; Table 5); however, the effect was less than the treatment with soy foliar protein. This may reflect that fewer secondary reactions of the lipid hydroperoxides may have occurred due to the absence of further enzymatic degradation.

TABLE 4. EFFECTS OF LIPOXYGENASE ACTIVITY ON NUTRITIONAL QUALITY OF SOY FOLIAR PROTEIN AND LARVAL GROWTH[a]

Treatment	LOX activity[b]	Relative free amines	Larval Growth[c]
Control	ND	100b	163.2b
+Linoleic acid	23.1	95.7a	61.0a

[a]Means in columns not followed by the same letter are significantly different at $P < 0.01$.
[b]LOX activity = expressed as nmol/min/mg protein; control has no detectable (ND) activity in the absence of added linoleic acid.
[c]Larval growth = weight in mg after 16 days on artificial diet.

TABLE 5. EFFECTS OF PURIFIED SOYBEAN LIPOXYGENASE ON NUTRITIONAL QUALITY OF SOY PROTEIN AND LARVAL GROWTH

Treatment	Larval growth[a]
Soy protein + 2 mM linoleic acid	158.5b
Soy protein + 2 mM linoleic acid + soybean lipoxygenase[b]	119.8a

[a]Larval growth = weight in mg after 16 days on artificial diet.
[b]Soy protein treated with lipoxygenase at 100 μmol/min/g protein.

DISCUSSION

Lipoxygenases may be components of induced resistance to herbivores in soybean. Larvae ingest increased amounts of lipid peroxidation products when feeding upon foliage from previously wounded plants (Table 2). The resultant increase in lipid peroxidation products also has been noted with mite damage (e.g., Hildebrand et al., 1986b). Induced resistance in soybean to Mexican bean beetle and loopers has been correlated with increased phenolic content and phenylalanine ammonia lyase and tyrosine ammonia lyase activities (Chiang et al., 1987; Nuepane and Norris, 1991a,b). These enzymes are the initial enzymes in the biosynthesis of phenolics. However, the phenolic isoflavonoids, glyceollins and coumestrol, may not be the major components of induced resistance to insects in soybean (e.g., Mexican bean beetle, velvetbean caterpillar, soybean looper). Recent tests conducted with these flavonoids indicate they are comparatively nontoxic at concentrations well above their natural occurrence (Hart et al., 1983; Rose et al., 1988; Burden and Norris, 1992; Slansky and Wheeler, 1992). Thus induced resistance in soybean is most likely a multicomponent plant response and not associated with a single biosynthetic pathway.

Lin and Kogan (1990) reported that prior herbivory by soybean loopers caused reductions in relative growth rates and developmental times of Mexican bean beetles and soybean loopers. They reported that induced resistance inhibited the relative growth rates of loopers by 3.4% and beetles by 10.7%. The magnitude of the induced resistance to *H. zea* observed in our study was much greater (i.e., ~27% reduction in relative growth rate) and may be due to plant genotypic differences and/or herbivore differences. Lin and Kogan (1990) indicated that loopers reduced leaf area by 26%, but the levels of damage in our studies were consistently less (unpublished data). Certain herbivores such as *H. zea* may be more proficient at eliciting induced resistance than others. Preliminary data in our laboratory indicate that other defoliating herbivores may be less effective than *H. zea* at eliciting lipoxygenases, despite producing comparable levels of defoliation.

Lipoxygenases may function as plant defense proteins by affecting insect growth and development in a variety of direct and indirect manners. The products of lipoxygenase may be repellent to insect feeding and operate as antixenosis bases of resistance. Mohri et al. (1990) showed that the products, linoleic acid hydroperoxide and hexanal, acted as feeding repellents to several beetle species. Older soybean leaves have lower levels of lipoxygenase (Hildebrand et al., 1988; our unpublished data) and are the preferred feeding sites for *H. zea* larvae throughout the growing season (Nault et al., 1992). However, in this study the effects of wounding and the associated induction of lipoxygenase did not significantly affect larval consumption rates (Table 3).

The products of lipid peroxidation may be toxic and function in antibiosis-

based resistance. The initial products of lipoxygenase (LOX) activity are fatty acid hydroperoxides; however, the hydroperoxides may chemically and/or enzymatically degrade to reactive aldehydes, γ-ketols, and epoxides (Gardner, 1991). Many of these products can form Schiff base adducts with proteins or act as potent alkylating agents of macromolecules (Gardner, 1979). Chemical changes caused by the action of lipid hydroperoxides on proteins include lipid–protein adducts, amino acid damage, protein scission, and protein–protein cross-links (Gardner, 1979). Thus, dietary nutrients such as amino acids and carotenoids may be destroyed by LOX products (e.g., Hildebrand and Kito, 1984; Hildebrand et al., 1986a,b). Additionally, endogenous membrane proteins and lipids can be damaged by ingestion of these oxidative products as indicated in Table 3. Moreover, reactive oxygen species (e.g., singlet oxygen, hydroxyl radicals, superoxide anion) and peroxyl, acyl, and carbon-centered radicals are formed during lipoxygenase reactions (Kanofsky and Axelrod, 1986; Chamulitrat et al., 1991). Free radicals are implicated in numerous pathologies associated with protein, lipid, and DNA damage (Halliwell, 1991). Our data indicate lipoxygenase activity is rapidly degraded in the midgut (Table 1, Figure 1), and consequently, the toxic effects of lipoxygenase are likely due to the ingestion of preformed lipid peroxidation products that accumulate in wounded foliar tissue (see Table 2). This is in contrast to foliar polyphenol oxidase and ascorbate oxidase, which remain active in the midgut of *H. zea* during feeding (Felton et al., 1989; Felton and Summers, 1993).

Lipoxygenases also may be involved in indirect forms of pest resistance in plants such as interplant communication (Hildebrand et al., 1988; Farmer and Ryan, 1990). Lipoxygenase is required for the biosynthesis of jasmonic acid, a compound that has been shown to promote cell senescence and rapidly induce the synthesis of new proteins (Enyedi et al., 1992). Jasmonic acid and its methyl ester, methyl jasmonate, induce the synthesis of several plant defensive proteins or chemicals including protease inhibitors, phenylalanine ammonia lyase, and alkaloids (Enyedi et al., 1992). Moreover, jasmonic acid affects its own biosynthesis because low concentrations of gaseous methyl jasmonate in soybean plants induce the accumulation of a 94-kDa storage protein, believed to be a lipoxygenase (Tranbarger et al., 1991; Enyedi et al., 1992). Grimes et al. (1992) confirmed that methyl jasmonate induces the accumulation of lipoxygenase in soybean seedlings. Recently, Hildebrand (1992) found that application of methyl jasmonate to tobacco leaves induces a 10-fold increase in lipoxygenase activity.

It is becoming clear that induced resistance in soybean is a complex phenomenon and is associated with the induction and activation of several diverse biochemical pathways (Chiang et al., 1987; Kraemer et al., 1987; Hildebrand et al., 1988; Kogan and Fischer, 1991; Liu et al., 1992). Specifically, our findings provide further evidence that lipoxygenase is an important component of the induced response (Felton et al., 1994; Bi et al., 1994). The roles of

lipoxygenases in wound repair and defense apparently evolved in primitive organisms and have been maintained in both higher plants and animals (Hildebrand et al., 1988). We suggest that lipoxygenases function in plant defense by initiating oxidative and nutritional stress to herbivores (Bi et al., 1994).

The importance of oxidative stress in animal–parasite associations is well established (Baggliolini and Wymann, 1990). Recently, there has been a burst of research indicating that oxidative stress is an important mediator of many host plant–pathogen interactions (Zacheo and Bleve-Zacheo, 1988; Doke et al., 1991; Popham and Novacky, 1991; Sutherland, 1991; Orlandi et al., 1992; Vera-Estrella, 1992; Davis et al., 1993). However, our understanding of oxidative stress as a mediating factor in insect–plant interactions is in its infancy (Ahmad, 1992). Appreciation of oxidative stress in entomological research has been hampered by the misconception that oxidative stress is a slow-acting process and may be relatively insignificant for animals possessing short life-spans. Given the presence of elaborate antioxidant mechanisms in all aerobic organisms including truly short-lived microbes, it would seem that oxidative stress is an important phenomenon regardless of life-span. It is argued here that there are at least three potential roles for oxidative stress in antiherbivore defense: (1) direct oxidative injury to the herbivore; (2) indirect injury to the herbivore through oxidative damage to dietary lipids, proteins, vitamins, antioxidants, etc.; and (3) signal transduction for eliciting plant defensive systems. The most immediate research need is for confirmation that the causal bases of "oxidative" resistance is truly due to oxidative stresses rather than epiphenomena or secondary effects. Nevertheless, research studies are beginning to reveal that plants and animals share remarkably similar mechanisms of defense through production of activated oxygen and reactive oxidants (Rubinstein, 1992).

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INFLUENCE OF MUSTELID SEMIOCHEMICALS ON POPULATION DYNAMICS OF THE DEER MOUSE (*Peromyscus maniculatus*)

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Abstract—The influence of mustelid anal-gland compounds on population dynamics of the deer mouse (*Peromyscus maniculatus*) was investigated. Densities of deer mice were not found to be significantly different between treated and control areas. However, on average, numbers of deer mice were lower on treated areas following the July treatments of 1991 and 1992. Survival rates appeared to be lower and more erratic on treated than control areas in 1991; however, there were no statistically significant differences. Reproduction appeared to be unaffected by the odors. We concluded that deer mice did not display a behavioral response to weasel odors. We speculated that decreases in densities were owing to predation by weasels, which were attracted to the odors on treated sites. We also concluded that the use of weasel odors as a biological control method for voles (*Microtus* spp.) would likely have little impact on deer mice (a nontarget species).

Key Words—Semiochemicals, deer mouse, weasel, *Mustela* spp., interspecific communication, *Peromyscus maniculatus*, biological control, vole, *Microtus* spp.

INTRODUCTION

Certain predator odors originating from feces, urine, or scent (anal) gland secretions elicit an innate fear response when detected by prey species (Gorman,

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1984; Kavaliers, 1988; Jedrzejewski and Jedrzejewska, 1990). These odors carry information and are defined as semiochemicals.

Several species of small mammals inflict serious feeding damage to forest and agricultural crops in North America, Europe, and Asia (Hansson and Nilsson, 1975; Green, 1978; Byers, 1984; Hansson, 1985). To date, natural and synthetic predator odors have produced significant avoidance responses in several pest species and reduced damage to forest and agricultural crops, on an experimental basis. Such pests include snowshoe hares (*Lepus americanus*) (Sullivan and Crump, 1984, 1986a; Sullivan et al., 1985a), black-tailed deer (*Odocoileus hemionus columbianus*) (Müller-Schwarze, 1972; Melchiors and Leslie, 1984; Sullivan et al., 1985b) montane (*Microtus montanus*) and meadow (*M. pennsylvanicus*) voles (Sullivan et al., 1988b), and northern pocket gophers (*Thomomys talpoides*) (Sullivan and Crump, 1986b; Sullivan et al., 1988c, 1990a).

There is also evidence of chemical information exchanges within a species. Synthetic compounds from the anal-gland secretion of the ferret (*Mustela furo*) and urine of the red fox (*Vulpes vulpes*) have acted as signaling pheromones and have attracted conspecifics to treated areas (Whitten et al., 1980; Clapperton et al., 1988, 1989). The additional predators attracted to the odors, which they assume other members of their species have deposited as territory or other behavioral marks, would enhance the small mammal pest repellent strategy by direct predation on target rodents. The increased presence of predators should also serve to reinforce the avoidance response by those rodents that have detected the odor and are actively avoiding the protected area. This biological control method takes advantage of both the innate fear response of the prey animals and the attraction of predators to synthetic odors.

Voles in the genus *Microtus* cause considerable feeding damage to young coniferous plantations and agricultural crops (Sullivan et al., 1988a,b). Weasels of the genus *Mustela* are principal predators of voles. Therefore, the use of synthetic weasel odor has considerable potential to control these pest rodents. However, the impact of these odors on nontarget, alternative prey species is not known. Biological control is a favored practice as it claims to be species-specific, unlike toxicants, which are indiscriminate in the range of species affected. This research intended to study the responses of small mammals, particularly voles, to weasel odors. Unfortunately, vole numbers were very low, and hence our efforts concentrated on determining the response of a nontarget species, the deer mouse (*Peromyscus maniculatus*) to a predator odor. The deer mouse is part of the weasel's diet, but voles are preferred (Raymond et al., 1990). In times of low numbers of voles, it has been documented that female weasels will still concentrate their foraging on voles, whereas male weasels will forage for more abundant, alternative prey species (Raymond et al., 1990).

This study provided the field evaluation of a pest management strategy

based on synthetic weasel odor for large-scale use in forest plantations. Two hypotheses were tested in determining the response of prey (deer mice) and predator (short-tail *Mustela erminea*, long-tail *M. frenata*, and least *M. nivalis* weasels) to mustelid semiochemicals: H_1—Abundance of deer mice will decline in areas treated with weasel odor relative to control areas. H_2—Demographic parameters of deer mice, such as survival and reproduction, will also decline in treated areas relative to control areas.

METHODS AND MATERIALS

Study Area. The study area was located in the Southern Morice Forest District of the Prince Rupert Forest Region, near Houston, British Columbia (54°3′N, 127°25′W). Monitoring of deer mouse populations was conducted on nine (six treatment and three control) coniferous plantations on the Northwood Pulp and Timber Ltd Forest License. All sites were located in either the SBS_{dk} (dry-cool subzone) or the SBS_{mk} (moist-cool subzone) of the SBS (Sub-Boreal Spruce) Biogeoclimatic Zone, or the $ESSF_{mc}$ (moist-cold subzone) of the ESSF (Engelmann Spruce Subalpine Fir) Biogeoclimatic Zone (Meidinger and Pojar, 1991). Hybrid white spruce (*Picea engelmannii* × *glauca*) and subalpine fir (*Abies lasiocarpa*) are the dominant climax tree species in the SBS. In the ESSF, Engelmann spruce (*Picea engelmannii*) and subalpine fir are the dominant climax tree species.

The blocks used for study ranged in size from 53 to 116 ha and had an average elevation of 762–1000 m. The blocks were clear-cut harvested between 1988 and 1990 and were prepared for planting with broadcast burns (controlled burning of postharvest debris). The blocks were planted between 1989 and 1991, mostly with lodgepole pine (*Pinus contorta*) and to a lesser extent with white (*Picea glauca*) and hybrid spruce. Fireweed (*Epilobium angustifolium*) was present on all sites.

Field Procedures. Each plantation had one 1-ha (7 × 7) checkerboard sampling grid with a Longworth live-trap at each of the 49 stations set at 14.3-m intervals. Trapping sessions lasted three days; traps were set in the late afternoon of day 1, checked in the morning of day 2, rechecked in the late afternoon of day 2, and checked in the morning of day 3, then locked open between trapping sessions. Traps were baited with whole oats and a slice of carrot or apple: coarse brown cotton was supplied as bedding. Small rodents that were trapped (long-tail voles *Microtus longicaudus*, meadow voles *M. pennsylvanicus*, boreal red-back voles *Clethrionomys gapperi*, deer mice *Peromyscus maniculatus*, and northwestern chipmunks *Eutamius amoenus*) were given a numbered fingerling fish tag in their right ear, weighed on a Pesola spring scale, had their sex and breeding condition recorded, and point of capture noted. These

populations were monitored at two- to three-week intervals from May to October in 1991 and 1992.

Mustelid Semiochemicals. The six treatment areas (A, B, C, D, E, and F) were covered manually with mustelid semiochemicals at approximately 160 devices/ha over a 25-ha square unit. This strategy used the formulation of mustelid semiochemicals produced by Phero Tech Inc. Delta, British Columbia. The compounds were encapsulated in 6-cm × 0.4-cm polyvinylchloride (PVC) cylinders. These devices consist of a 0.5-mm-thick sheath and a core plastisol formulated with 2% active ingredient. This type of device was determined to yield the best controlled release of the odor (Sullivan et al., 1990b). The active ingredient was a 1 : 1 mixture of 2-propylthietane and 3-propyl-1,2-dithiolane after Crump (1978, 1980, 1982). Release of the chemical from this type of device has been documented to persist for six months and has been tested successfully over winter (Sullivan et al., 1988a).

Experimental Design. For experiment 1, grids A, B, and C served as summer treatment grids, which received a weasel odor treatment on July 18, 1991. Grids D, E, and F served as control areas for these treatment areas in 1991.

For experiment 2, on September 26, 1991 weasel odor was applied to grids D, E, and F. Only temporal controls (pretreatment period) were available for the September treatment of 1991.

For experiment 3, grids A, B, and C were treated with weasel odors on July 19, 1992. Three new areas, grids G, H, and I, served as controls in 1992 for the July treatment.

For experiment 4, grids D, E, and F were treated on September 26, 1992 and grids G, H, and I served as controls for the September treatment.

Demographic Analysis. For the purpose of demographic analysis, body weight was used to classify deer mice into adult and immature age classes. Any animal weighing 19 g or over was classed as an adult and any animal below this weight was classed as immature. This was based on the weight at which greater than 50% of the animals were in breeding condition. Breeding condition was classified as males with scrotal testes and females with large nipples (lactating). New animals found on the trapped areas were grouped to include juveniles born to resident females (recruits) as well as immigrants (both adults and immature deer mice).

In all cases, population density of deer mice has been expressed as minimum number known to be alive (MNA) (Krebs, 1966). Jolly-Seber estimates (Seber, 1982) are presented in Appendix 1. In general, the MNA and Jolly-Seber estimates are highly correlated. However, the Jolly-Seber estimate becomes unreliable and impossible to calculate when population size falls to a very low level and no tagged mice are recaptured. It is necessary to use the MNA estimate under these conditions (Krebs et al., 1986). MNA provides sufficiently accurate

enumeration of a population when trappability (susceptibility to capture) is 70% or higher (Hilborn et al., 1976), and this level was achieved in this study.

Statistical Analysis. An analysis of variance (ANOVA) was performed to investigate differences in numbers of deer mice between treated and control areas. The level of significance was set at $\alpha = 0.05$. The mean number of animals on each grid, for the pretreatment period, was subtracted from the mean number of animals on each grid for the posttreatment period. The analysis of variance was performed on the mean differences obtained from each grid from pre- to posttreatment periods. The mean of only two estimates was used from each grid for the posttreatment values of the September treated areas.

An analysis of variance (ANOVA) was performed to determine differences in percentages of male and female adult deer mice in breeding condition between treatment and control areas for the summer treatments of 1991 and 1992. Arcsine transformations were performed on the percentage data to alter the binomially distributed proportions to resultant data that have an underlying distribution that is normal. No analyses of reproductive condition were made for the fall treatments of 1991 and 1992 as this occurred after the breeding season.

Minimum survival rates were calculated per 14-day period and reflect disappearance of deer mice from the trappable population. Thus, both mortality and emigration were included in these survival estimates. An analysis of variance (ANOVA) was performed to determine differences in mean survival rates of deer mice between treatment and control areas. Arcsine transformations were performed on these proportions to fit the data to a normal distribution. The data were analyzed using mean differences between pre- and posttreatment periods, in the same manner as described for numbers of deer mice.

RESULTS

Trappability Estimates. Trappability estimates for deer mice during the course of this study are presented in Table 1. Average trappability for males was 82.57 and 83.38 for females in 1991. In 1992, average trappability for males was 78.76 and 85.96 for females.

Experiment 1

Deer Mouse Populations. All grids showed an increasing number of animals during the pretreatment phase of the study (Figure 1). After the summer treatment (July 18), numbers on grid A increased slightly and then began to decrease until late September. Numbers of animals on grid B fluctuated erratically, but showed a decreasing trend, and there was a pronounced decrease in numbers on grid C. Overall, numbers on the July treatment grids barely rose above 20 animals/ha. Mean numbers of deer mice were 1.83 animals/ha higher

TABLE 1. TRAPPABILITY ESTIMATES FOR *Peromyscus maniculatus* ON 6 TREATMENT AND 3 CONTROL AREAS FOR THIS STUDY[a]

	1991		1992	
	♂♂	♀♀	♂♂	♀♀
Treatment				
A	75.0	83.0	92.1	85.8
	(18)	(11)	(10)	(4)
B	69.8	86.7	88.6	71.4
	(17)	(6)	(11)	(7)
C	91.0	87.5	87.5	85.7
	(13)	(8)	(4)	(7)
D	77.0	65.5	76.6	79.9
	(26)	(15)	(17)	(13)
E	100.0	90.9	50.0	94.4
	(7)	(11)	(4)	(6)
F	82.6	86.7	51.9	76.8
	(24)	(19)	(9)	(14)
Control				
G			94.4	100.0
			(6)	(5)
H			84.4	100.0
			(8)	(2)
I			83.3	79.6
			(9)	(9)

[a]Sample size in parentheses. Minimum unweighted trappability eliminates first and last captures and provides only 1 value for each individual regardless of how long it lives.

in the posttreatment period from pretreatment levels. Two of the control grids, D and F, continued to increase in numbers, with estimates of over 35 animals/ha. Numbers on grid E declined after the first week in July and continued to decrease through August until the late fall. Mean numbers of deer mice on control areas were 8 animals/ha higher in the posttreatment period compared to pretreatment levels. There was no significant difference in mean numbers of animals between treated and control areas (ANOVA, $P = 0.2718$).

New Animals. The numbers of new animals (recruited juveniles and immigrants) entering each population were quite similar during the pretreatment period of the study (Figure 1). New animals entering grids A, B, and C averaged 17 males and 12 females during the pretreatment period. The control areas, D, E, and F, averaged 19.7 new males and 17.3 new females. During the posttreatment period, treated grids A, B, and C averaged 11.3 new males and 7.7 new females. Control areas D, E, and F averaged 7.7 new males and 8.3 new females. Numbers of new animals decreased from pretreatment to posttreatment periods

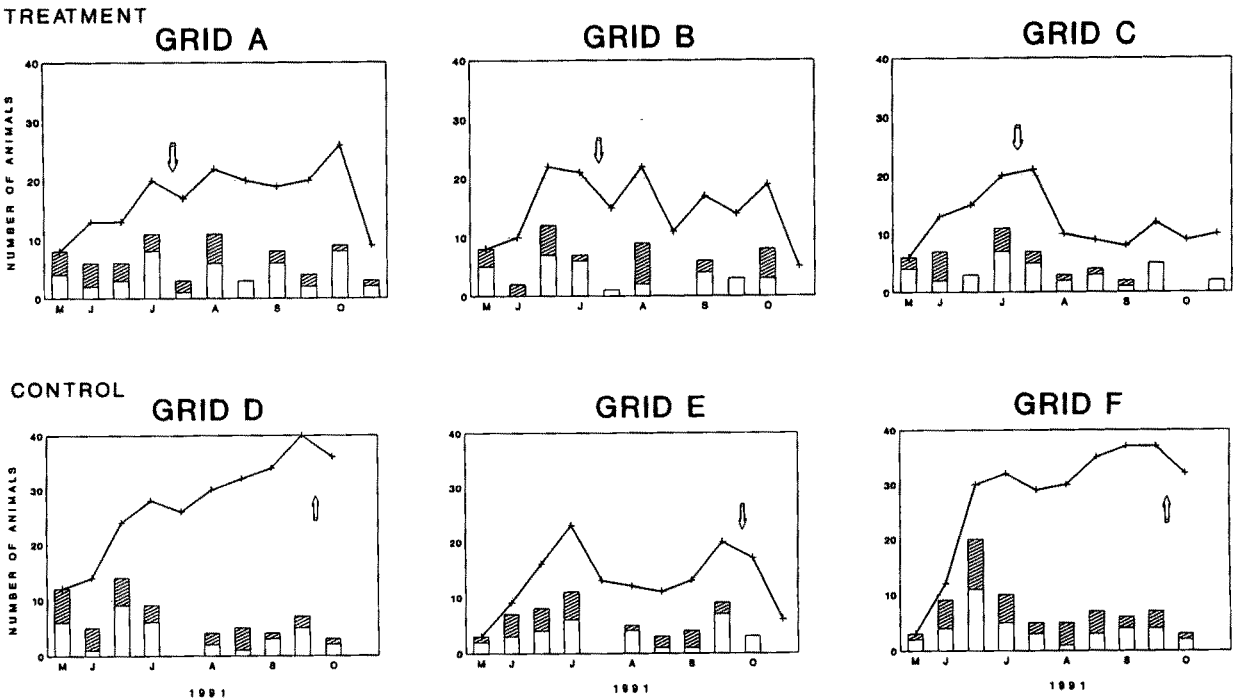

FIG. 1. MNA estimates per hectare for deer mice from May until October 1991 and numbers of new deer mice joining treatment and control populations of experiments 1 and 2.

on both treatment and controls. However, numbers of new males on treatment areas were not significantly different from numbers of new males on control areas (ANOVA, $P = 0.1511$). Numbers of new females were significantly lower on control areas (ANOVA, $P = 0.0405$). There were no significant differences between numbers of males or females entering treated (ANOVA, $P = 0.6934$) or control areas (ANOVA, $P = 0.2638$).

Survival Rates. Survival rates on treatment grids A, B, and C fluctuated throughout the season (Figure 2). Survival rates on grid A ranged from 0.25 to 1.00 for males and 0.17 to 0.83 for females over the entire season (Figure 2). Grid B survival rates ranged from 0.42 to 1.00 for males and from 0.00 to 1.00 for females. Rates on grid C ranged from 0.36 to 1.00 for males and from 0.00 to 1.00 for females. Rates were lowest on grid C shortly after treatment. The survival rates on two controls, grids D and F, were less variable. Rates on grid D ranged from 0.73 to 1.00 and from 0.50 to 1.00 for males and females, respectively. Rates on grid F ranged from 0.67 to 1.00 for males and females. Survival rates on control grid E were lower than the other two control grids and ranged from 0.33 to 1.00 for males and from 0.29 to 1.00 for females. There were no significant differences in survival rates between treatment and control areas for males (ANOVA, $P = 0.5059$), or females (ANOVA, $P = 0.1095$). The survival rates of males were not significantly different from survival rates of females on treatment (ANOVA, $P = 0.3495$), or control areas (ANOVA, $P = 0.7804$).

Reproductive Condition. The percentages of male and female adults in breeding condition from May 25 to September 10, 1991, were examined (Table 2). This period is within the normal breeding season of deer mice (Millar *et al.*, 1979). The mean percentages of adults in breeding condition on treatment grids A, B, and C were not significantly different from control areas, D, E, and F for males (ANOVA, $P = 0.5839$), or females (ANOVA, $P = 0.2462$). There were no significant differences between males and females in breeding condition on treatment (ANOVA, $P = 0.1124$), or control areas (ANOVA, $P = 0.0512$).

Experiment 2

Deer Mouse Populations. Numbers of deer mice decreased for the last sampling period on grids D, E, and F (Figure 1). However, only two trapping sessions were completed after weasel odors were dispersed in September. The last session in October had very poor trapping conditions ($-18°C$ with no snow cover). In October, few animals were caught on any of the grids, and subsequent data analysis was based on very low sample sizes. There was no significant difference in mean numbers of deer mice between pre- and posttreatment periods for the September treatment areas (ANOVA, $P = 0.8362$).

New Animals. Numbers of new animals entering grids D, E, and F are

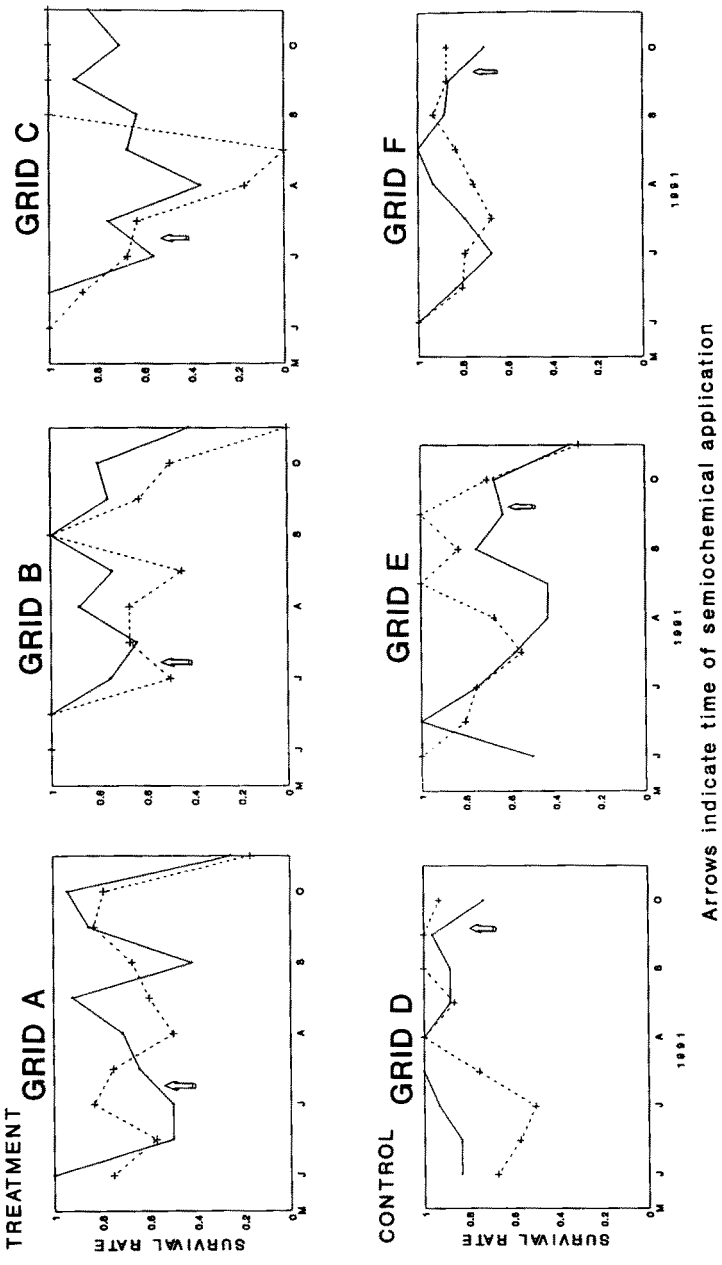

FIG. 2. Minimum survival rates of male and female deer mice from May to October 1991 of experiments 1 and 2.

TABLE 2. MEAN PERCENTAGES (±SE) OF ADULT MALE AND FEMALE *Peromyscus maniculatus* IN BREEDING CONDITION FROM MAY 27 TO SEPTEMBER 10, 1991 FOR EXPERIMENT 1 (Sample Size in Parentheses)

	Pre-treatment		Post-treatment	
Grid	♂♂	♀♀	♂♂	♀♀
Treatment				
A	89.6 ± 6.3 (16)	83.9 ± 11.8 (22)	66.7 ± 23.6 (9)	87.5 ± 12.5 (12)
B	90.6 ± 6.0 (23)	79.5 ± 7.4 (19)	27.1 ± 10.4 (16)	41.7 ± 20.9 (11)
C	95.0 ± 5.0 (17)	66.7 ± 23.6 (17)	50.0 ± 50.0 (3)	100.0 ± 0.0 (3)
Control				
D	91.7 ± 12.5 (19)	88.8 ± 6.6 (15)	33.3 ± 33.3 (4)	33.3 ± 33.3 (3)
E	87.5 ± 12.5 (15)	100.0 ± 0.0 (15)	25.0 ± 17.7 (12)	79.2 ± 12.5 (9)
F	95.8 ± 4.2 (24)	70.0 ± 23.8 (25)	42.5 ± 21.7 (17)	54.2 ± 20.8 (12)

shown in Figure 1. There were only two posttreatment values; therefore, sample size was very low for statistical analysis. Mean numbers of new animals during the posttreatment were not significantly different from pretreatment levels (ANOVA, males $P = 0.8442$, females $P = 0.2028$). There were no significant differences between numbers of males and females entering the areas (ANOVA, $P = 0.3550$).

Survival Rates. The mean survival rates of deer mice for the four sampling periods before treatment were compared to mean survival rates for the two sampling periods after the September treatment (Figure 2). There were no significant differences in survival rates between pre- and posttreatment periods for males (ANOVA, $P = 0.4781$) or females (ANOVA, $P = 0.4321$). Survival rates did not differ between males and females (ANOVA, $P = 0.5416$).

Experiment 3

Deer Mouse Populations. In 1992 there was an initial increase in numbers of deer mice on all grids (Figure 3). After the July treatment, the numbers decreased on treatment grids A and B, but continued to increase on treatment grid C, and reached a maximum of 18 animals/ha. Numbers on grid A did not continue to decrease and instead showed an increase throughout the fall reaching

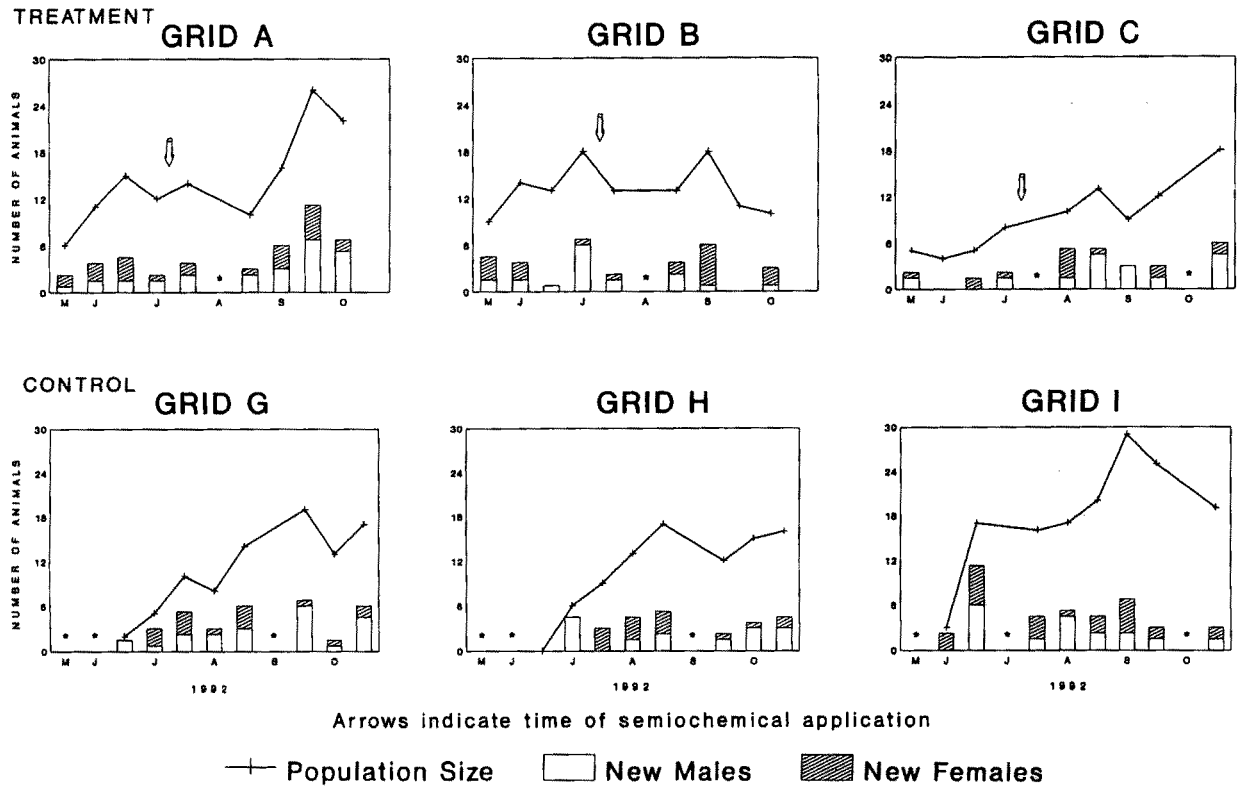

FIG. 3. MNA estimates for deer mice from May until October 1992 and numbers of new deer mice joining treatment and control populations of experiment 3.

a maximum of 26 animals/ha. Numbers on grid B fluctuated but remained relatively constant with pretreatment and posttreatment maximums of 18 animals/ha. Numbers on the three control grids, G, H, and I, continued to increase throughout the summer. Deer mice on grid G reached a maximum of 19 animals/ha and then decreased slightly in October. Grid H showed a maximum of 17 animals/ha in late August, and numbers slowly decreased throughout the fall. Deer mouse abundance reached a peak of 29 animals/ha in September on grid I, and then numbers dropped in October. No significant difference was found in mean numbers of deer mice between treated and control areas (ANOVA, $P = 0.0699$).

New Animals. There were no trends in the numbers of new animals entering the populations (Figure 3). In the pretreatment period, grids A, B, and C averaged 8 new males and 7.3 new females. During the posttreatment period, treated grids averaged 13 new males and 10.3 new females. Control areas, G, H, and I, averaged 5.7 new males and 4.3 new females in the pretreatment period. During the posttreatment period, the controls averaged 13 new males and 12.3 new females. The control areas, G, H, and I, were not sampled as often as the treatment areas during the pretreatment period, but mean numbers of new animals showed no significant differences between treatment and control areas for males (ANOVA, $P = 0.5165$) or females (ANOVA, $P = 0.9097$). There were no significant differences between numbers of males and females entering treated areas (ANOVA, $P = 0.7578$) or control areas (ANOVA, $P = 0.5812$).

Survival Rates. Survival rates for the July treatment areas did not show any trends (Figure 4). Rates on grid A ranged from 0.48 to 1.00 for males and females over the whole season. Survival rates for males and females on grid B ranged from 0.38 to 1.00 and 0.60 to 1.00, respectively. Survival rates on grid C ranged from 0.00 to 0.93 for males and from 0.63 to 1.00 for females. The survival rates on control G ranged from 0.50 to 1.00 for males and from 0.48 to 1.00 for females. Rates on control H ranged from 0.67 to 1.00 for males and from 0.40 to 1.00 for females. Survival rates on control I ranged from 0.62 to 1.00 and 0.50 to 1.00 for males and females, respectively.

Statistical analyses could not be performed between treatment and control areas due to the limited sampling on control areas in the pretreatment period. Survival rates on treated areas, A, B, and C, were not significantly different between pre- and posttreatment periods for males (ANOVA, $P = 0.5798$). Survival rates on treated areas were significantly different between pre- and posttreatment periods for females (ANOVA, $P = 0.0473$). Survival rates were not significantly different between males and females (ANOVA, $P = 0.3344$).

Reproductive Condition. Mean percentages of male and female adults in breeding condition are presented in Table 3. Mean percentages of adults in breeding condition on treatment grids A, B, and C were not significantly dif-

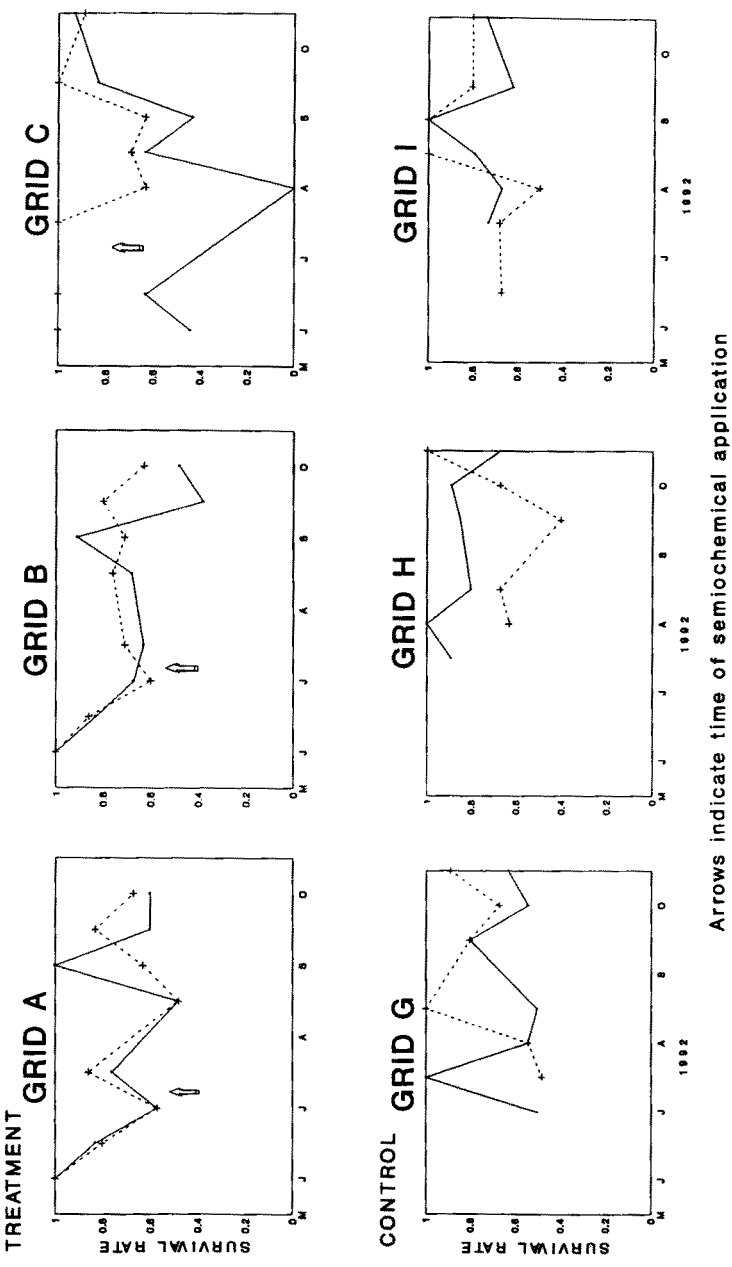

FIG. 4. Minimum survival rates of male and female deer mice from May to October 1992 for study areas for experiment 3.

TABLE 3. MEAN PERCENTAGES (±SE) OF ADULT MALE AND FEMALE *Peromyscus maniculatus* IN BREEDING CONDITION FROM MAY 25 TO SEPTEMBER 11, 1992 FOR EXPERIMENT 3 (SAMPLE SIZE IN PARENTHESES)

	Pre-treatment		Post-treatment	
Grid	♂♂	♀♀	♂♂	♀♀
Treatment				
A	87.5 ± 7.9 (18)	41.7 ± 14.4 (13)	66.7 ± 33.3 (5)	52.8 ± 12.1 (9)
B	95.8 ± 4.2 (20)	40.0 ± 24.5 (21)	33.3 ± 33.3 (9)	63.9 ± 7.4 (9)
C	88.9 ± 11.1 (8)	50.0 ± 14.4 (8)		75.0 ± 25.0 (4)
Control				
G	75.0 ± 25.0 (3)	100.0 ± 0.0 (1)	50.0 ± 50.0 (5)	83.3 ± 16.7 (5)
H			0.0 ± 0.0 (3)	61.1 ± 20.0 (8)
I		58.4 ± 8.4 (7)	50.0 ± 50.0 (3)	88.9 ± 11.1 (8)

ferent from mean percentages of adults in breeding condition on control grids G, H, and I for males (ANOVA, $P = 0.3836$) or for females (ANOVA, $P = 0.5171$). Mean percentages of males in breeding condition were significantly lower than females on treated areas (ANOVA, $P = 0.0141$). Males did not differ from females on control areas (ANOVA, $P = 0.0807$).

Experiment 4

Deer Mouse Populations. Numbers on treatment grids D, E, and F continued to increase throughout the summer and early fall, reaching maximums of 26, 18, and 29 animals/ha, respectively (Figure 5). All three areas showed decreases in numbers after the September treatment. However, only two samples were made after the September treatment. Treated grids D, E, and F averaged 18.25 animals/ha before the treatment and 21.5 animals/ha after treatment. Control areas G, H, and I averaged 15.3 animals/ha and 17.5 animals/ha for the same periods. No significant differences were found in mean numbers of animals between treated and control areas (ANOVA, $P = 0.4933$).

New Animals. There were no trends in the numbers of new animals entering the population (Figure 5). In the four sampling periods prior to treatment, grids D, E, and F averaged 14.7 new males and 8.7 new females. After treatment

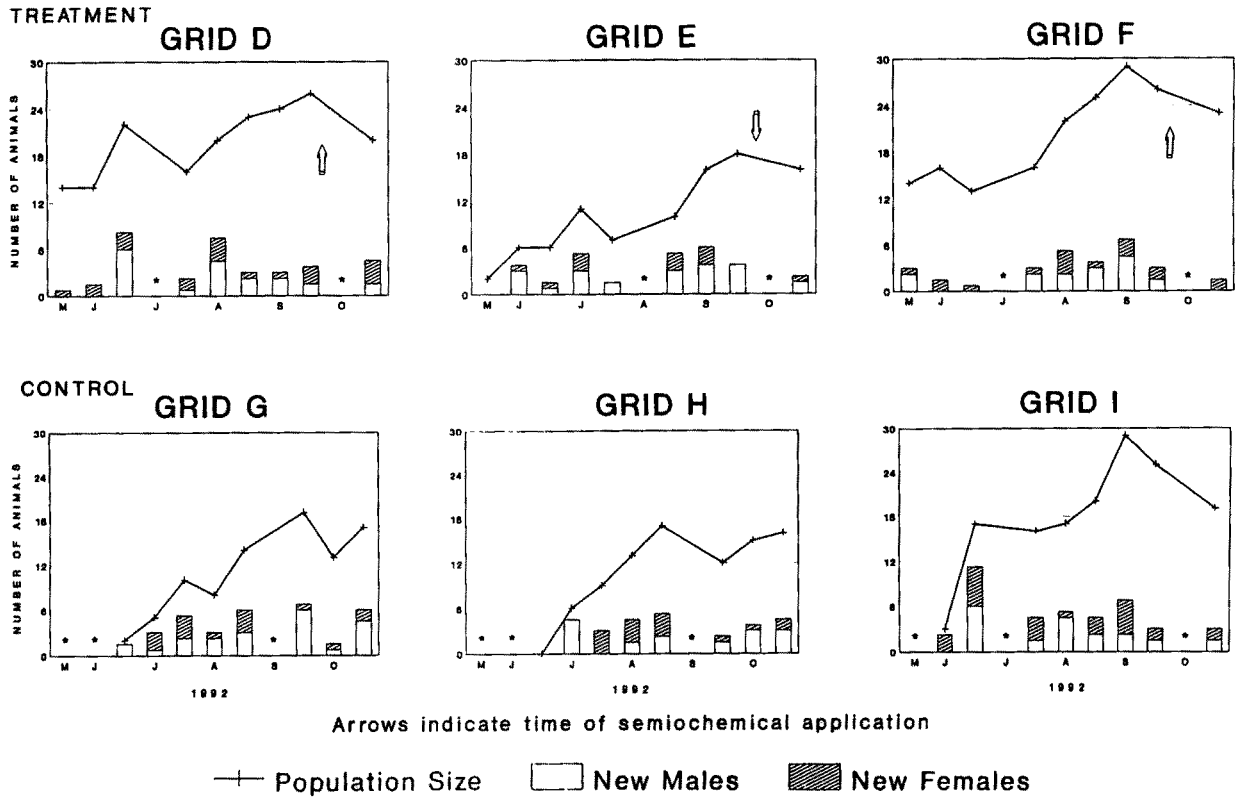

FIG. 5. MNA estimates for deer mice from May until October 1992 and numbers of new deer mice joining treatment and control populations of experiment 4.

they averaged 4.3 new males and 4 new females. Controls G, H, and I averaged 13 new males and 12.3 new females in the pretreatment period, and 6.3 new males and 3.3 new females during the posttreatment period. No significant differences were found between treatment and controls for males (ANOVA, $P = 0.3761$) or females (ANOVA, $P = 0.2648$). Numbers of new males were not significantly different from numbers of new females on treated (ANOVA, $P = 0.3414$) or control areas (ANOVA, $P = 0.3274$).

Survival Rates. Survival rates on grid D ranged from 0.42 to 0.93 for males and from 0.50 to 1.00 for females, during the 1992 season (Figure 6). Rates on grid E ranged from 0.34 to 0.80 and from 0.67 to 1.00 for males and females, respectively. Grid F had survival rates ranging from 0.43 to 1.00 and from 0.75 to 1.00 for males and females, respectively. Survival rates on control G ranged from 0.50 to 1.00 for males from 0.48 to 1.00 for females. Rates on control H ranged from 0.67 to 1.00 for males and from 0.40 to 1.00 for females. Survival rates on control I ranged from 0.62 to 1.00 and 0.50 to 1.00 for males and females, respectively. Survival rates on the control grids, G, H, and I, were not significantly different from those on treatment grids for males (ANOVA, $P = 0.5384$) or females (ANOVA, $P = 0.8277$). Survival rates did not differ between males and females on treated (ANOVA, $P = 0.8762$) or control areas (ANOVA, $P = 0.1434$).

DISCUSSION

The data from 1991 suggest that numbers of deer mice were affected by the weasel semiochemicals. Numbers of new animals entering treated areas fell slightly, but remained relatively constant from pre- to posttreatment periods. Fewer new animals entered control areas during the posttreatment period. However, only numbers of females were significantly different between treatment and control areas. If new animals continued to move onto treated areas, this suggests that residents were disappearing. Deer mice may have been leaving the treated areas as an avoidance response. However, the simple evidence of new animals continuing to enter these areas contradicts such an avoidance behavior. Deer mice may have suffered decreased survival, and this may have resulted in habitat becoming available for new immigrants. The control areas did not appear to experience these openings. Therefore, immigration to these areas was reduced after the spring influx of animals occupying the sites. Numbers of new animals entering the population remained similar on treatment areas before and after treatment. However, the population did not show a steady increase, as would be expected. Instead, population size stabilized or decreased on treated sites. Mean numbers of deer mice on treated areas increased by an average of only

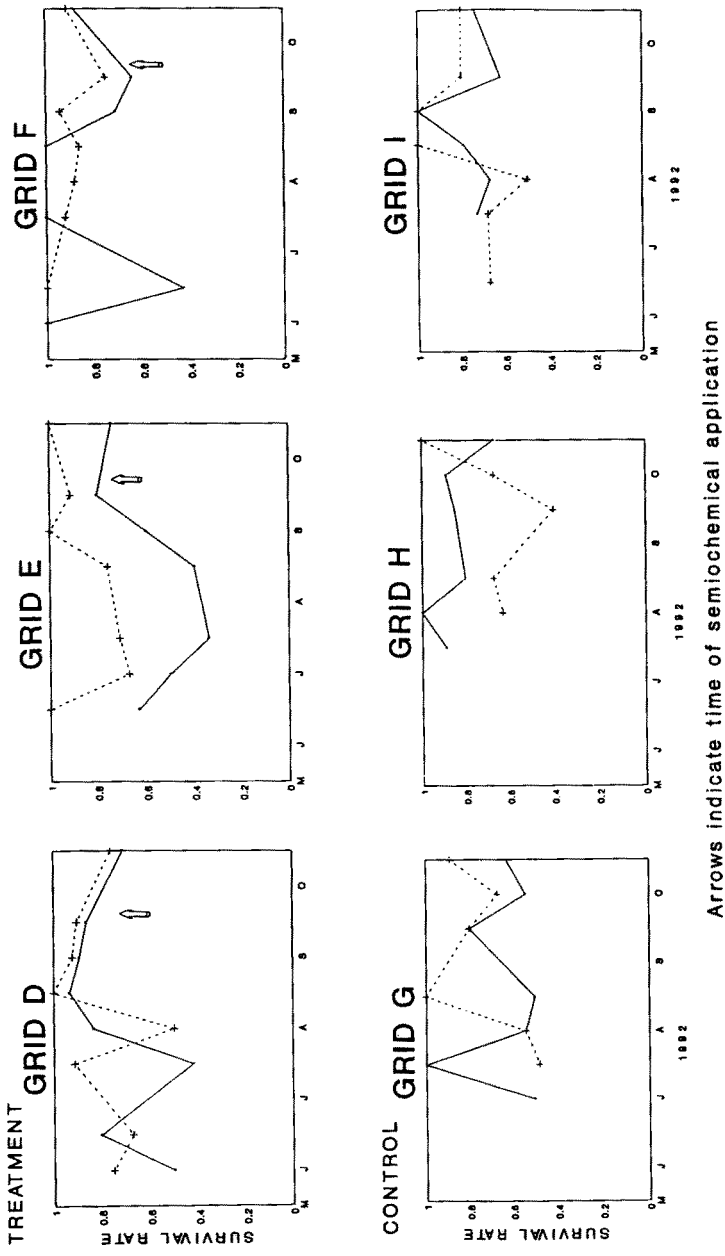

FIG. 6. Minimum survival rates of male and female deer mice from May to October 1992 for study areas of experiment 4.

1.83 animals/ha from pretreatment to posttreatment periods. Mean numbers of deer mice on control areas increased by an average of 8 animals/ha from pretreatment to posttreatment periods, even though numbers of new animals were only approximately half that of treated areas. Survival rates were more erratic and lower on the treated areas than on the control areas. However, there were no statistical differences between treated and control sites.

One possible explanation for the observed differences is that deer mice on treated areas experienced some kind of physiological stress caused by the weasel odors. Kavaliers (1988, 1990) found that deer mice and white-footed mice (*P. leucopus*) displayed an analgesic response when exposed to odors of a natural predator (short-tail weasel) in the laboratory. This analgesic response was assessed by exposing mice to the sound and scent of a weasel for various time periods, and then placing the mice on a 50°C hot plate and timing the latency period of a foot-licking response. The analgesia prepares the mice for flight or fight and reduces their receptiveness to adverse stimuli (Kavaliers, 1988, 1990).

Our personal observations and some of our results suggest that the physiological stress observed in laboratory situations does not manifest itself as a behavioral response in the field. Some deer mice were observed to enter traps freshly soiled with weasel scent from a trapped weasel. This lack of an avoidance response is not completely unexpected. Studies by Stoddart (1976, 1982) suggest that wood mice (*Apodemus sylvaticus*), an Old World species similar to deer mice, were indiscriminate in their avoidance of traps soiled with weasel scent. Sullivan et al. (1988b) give evidence of deer mice and chipmunks entering traps treated with synthetic weasel scent. Hirsch and Bolles (1980) also concluded that deer mice failed to discriminate between a predator and nonpredator in some situations. The fact that new animals continued to enter treated areas also suggests lack of avoidance.

Reproduction of deer mice did not appear to be affected by the predator odor. There were no significant differences noted in percentages of animals in breeding condition before and after treatment, between treatment and control areas. Although deer mice may respond with a neurological stress response to the scent of weasels (Kavaliers, 1988, 1990), it appears that this response does not affect physiological aspects (such as reproduction) or manifest itself as an observable demographic response in the field.

A second possibility for deer mice populations being lower on treated areas is that weasels were attracted to these areas. Vole numbers were very low, and as a result, the weasels preyed upon less preferred, but more abundant, prey species, the deer mouse. Low and fluctuating survival rates on treated areas suggest that general mortality and perhaps predation was higher on the treated areas compared with controls. These results support the conjecture that these semiochemicals are capable of attracting additional predators and thereby

increasing predation in an area (Sullivan et al., 1988a; Whitten et al., 1980; Clapperton et al., 1988, 1989).

Several species of voles have been shown to be adversely affected by weasel odors. Ylönen (1989) demonstrated that reproduction could be suppressed in bank voles (*Clethrionomys glareolus*) exposed to weasel scent. Jedrzejewski and Jedrzejewska (1990) showed how bank voles altered their movements and spatial distribution after a weasel had visited the area. Stoddart (1976, 1980) and Sullivan et al. (1988b) demonstrated that voles of the genus Microtus were discriminatory in avoiding traps soiled with weasel odor.

Use of weasel odors would likely decrease vole numbers via physiological and behavioral responses. Possible increased numbers of predators on treated areas also would decrease survival of voles and reinforce their avoidance response. Voles are the preferred prey species of weasels, and, given abundant vole numbers, weasels attracted to the treated areas would likely prey on voles more heavily than on deer mice.

The data from 1992 do not strongly support conclusions made from the 1991 results. There were some indications of lower or fluctuating survival rates on the July treated areas and lower numbers of deer mice. However, no clear trends were visible. One possible explanation for the lack of response in 1992 is that the weasel semiochemical was in some way not as effective. The weasel semiochemical was exposed to extremely hot and dry conditions for an abnormally long period of time (40 consecutive days without rain). The release devices may have been unable to protect the chemical mixture from drying up soon after its dispersal. As a result, the semiochemical may have become inactive in a very short period of time after application.

CONCLUSIONS

During periods of high vole numbers, and expected damage to tree seedlings, weasel semiochemicals may be a useful method of biological control. Our results suggest that deer mice do not respond behaviorally to the odors. Thus, weasel semiochemicals have the potential of providing plantation protection from voles, which has a biological rationale (the combination of an avoidance response and increased predation pressure), without significant impact on nontarget species (deer mice). This result only enhances the desire to use semiochemicals rather than toxicants. With nontarget species not appearing to be strongly affected, this would maintain community stability in an area. While vole numbers would be controlled, many small rodent species would still inhabit these areas, and a prey base would still be available to maintain utilization of these areas by many predator species.

APPENDIX 1
Jolly-Saber Density Estimates with 2 SE. MNA Estimates in Parentheses

	Grid A	Grid B	Grid C
1991			
May	0.0 + 0.0 (8)	0.0 + 0.0 (8)	0.0 + 0.0 (6)
June	13.0 + 2.4 (13)	10.0 + 1.1 (10)	13.0 + 0.0 (13)
	13.8 + 5.5 (13)	22.0 + 0.0 (22)	15.0 + 1.9 (15)
July	21.0 + 6.2 (20)	21.8 + 5.5 (21)	20.0 + 4.0 (20)
	19.6 + 7.6 (17)	16.1 + 6.2 (15)	24.0 + 9.9 (21)
Aug	22.0 + 5.3 (22)	23.7 + 7.7 (22)	11.4 + 7.2 (10)
	22.3 + 7.8 (20)	11.0 + 5.5 (11)	9.0 + 4.1 (9)
Sept	19.0 + 5.5 (19)	17.8 + 6.6 (17)	8.0 + 3.9 (8)
	20.2 + 5.8 (20)	15.1 + 6.4 (14)	12.0 + 3.9 (12)
Oct	26.0 + 5.8 (26)	19.0 + 5.3 (19)	9.1 + 4.3 (9)
	42.0 + 8249 (9)	7.0 + 9.8 (5)	10.0 + 4.2 (10)
1992			
May	6.0 + 4.8 (6)	9.0 + 4.8 (9)	5.0 + 2.7 (5)
June	11.0 + 4.1 (11)	14.3 + 3.9 (14)	4.0 + 2.8 (4)
	16.1 + 6.0 (15)	14.2 + 5.0 (13)	5.0 + 3.4 (5)
July	12.4 + 5.2 (12)	19.6 + 7.2 (18)	10.7 + 10.0 (8)
Aug	19.8 + 14.2 (14)	13.8 + 5.9 (13)	12.2 + 10.3 (10)
	10.4 + 5.4 (10)	13.0 + 4.7 (13)	13.0 + 3.9 (13)
Sept	16.0 + 4.5 (16)	18.0 + 4.7 (18)	9.0 + 3.8 (9)
	26.0 + 9.1 (26)	13.0 + 7.4 (11)	12.0 + 5.6 (12)
Oct	0.0 + 0.0 (22)	0.0 + 0.0 (10)	0.0 + 0.0 (18)
1991	Grid D	Grid E	Grid F
May	0.0 + 0.0 (12)	0.0 + 0.0 (3)	0.0 + 0.0 (3)
June	14.6 + 4.4 (14)	9.0 + 2.8 (9)	12.8 + 6.2 (12)
	27.0 + 8.6 (24)	16.0 + 3.1 (16)	31.1 + 5.8 (30)
July	30.2 + 7.1 (28)	23.0 + 4.2 (23)	34.6 + 7.3 (32)
	26.0 + 5.8 (26)	13.0 + 5.3 (13)	29.6 + 6.7 (29)
Aug	31.9 + 6.8 (30)	12.0 + 4.6 (12)	31.1 + 7.2 (30)
	33.3 + 6.9 (32)	11.3 + 5.0 (11)	35.9 + 7.3 (35)
Sept	35.9 + 7.4 (34)	13.3 + 4.9 (13)	37.3 + 7.3 (37)
	41.3 + 7.2 (40)	20.8 + 5.9 (20)	38.0 + 8.0 (37)
Oct	40.3 + 10.9 (36)	19.2 + 8.4 (17)	62.3 + 45.6 (32)
	13.0 + 2599 (13)	8.0 + 10.0 (6)	10.0 + 1999 (10)
1992			
May	18.9 + 12.7 (14)	3.0 + 489 (2)	18.0 + 10.8 (14)
June	15.6 + 7.6 (14)	6.0 + 2.0 (6)	16.0 + 6.0 (16)
July	29.9 + 15.5 (22)	6.8 + 4.6 (6)	13.1 + 5.8 (13)
Aug	16.6 + 6.9 (16)	14.3 + 12.1 (11)	17.1 + 6.5 (16)
	21.3 + 7.2 (20)	7.0 + 3.9 (7)	24.5 + 7.7 (22)
Sept	23.6 + 6.1 (23)	10.0 + 3.1 (10)	26.6 + 6.9 (25)
	24.0 + 5.9 (24)	16.5 + 4.7 (16)	36.0 + 11.7 (29)
Oct	26.0 + 7.6 (26)	18.5 + 6.6 (18)	26.3 + 7.4 (26)
	0.0 + 0.0 (20)	0.0 + 0.0 (16)	0.0 + 0.0 (23)

APPENDIX 1
Continued

	Grid G	Grid H	Grid I
1992			
June	0.0 + 0.0 (2)		0.0 + 0.0 (3)
July	5.0 + 2.2 (5)	6.0 + 0.0 (6)	17.0 + 3.9 (17)
Aug	10.0 + 2.6 (10)	9.5 + 3.8 (9)	18.9 + 8.4 (16)
	8.7 + 5.2 (8)	15.3 + 7.1 (13)	20.2 + 8.7 (17)
Sept	14.0 + 3.6 (14)	17.0 + 4.0 (17)	20.5 + 5.9 (20)
	19.0 + 4.2 (19)	12.0 + 4.6 (12)	29.6 + 6.0 (29)
Oct	15.5 + 8.4 (13)	22.0 + 15.1 (15)	25.0 + 7.4 (25)
	0.0 = 0.0 (17)	0.0 + 0.0 (16)	0.0 + 0.0 (19)

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PLANT VIRUS-INDUCED CHANGES IN APHID POPULATION DEVELOPMENT AND TEMPORAL FLUCTUATIONS IN PLANT NUTRIENTS

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Abstract—*Cucurbita pepo* plants were infected with zucchini yellow mosaic virus or maintained noninfected. *Aphis gossypii*, which transmits the virus, lived longer and produced more offspring on infected than on noninfected plants. On infected plants, the intrinsic rate of natural increase for *A. gossypii* increased with time after inoculation. In a similar experiment, concentrations of phloem sap nutrients, including free amino acids, total protein, and sugars from infected and noninfected plants were compared for 37 days after inoculation. Significant differences in levels of individual amino acids from phloem exudate between infected and noninfected plants were found, yet the concentration of total amino acids was not substantially different between infected and noninfected plants. Beginning four days after inoculation, the total protein content of phloem exudate generally was lower in infected plants than noninfected plants. Likewise, the total sugar content of phloem exudate from infected plants was lower than that of noninfected plants beginning nine days after inoculation. In contrast with the results from analyses of phloem exudate, foliage from infected plants had higher levels of almost all amino acids than noninfected foliage beginning nine days after inoculation. Concentrations of individual and total amino acids in infected foliage increased throughout the experimental period. Although no temporal effects were observed in the foliage sugar content for either individual or total sugars, starch content decreased

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with time in infected plants, while in noninfected plants, starch content remained level.

Key Words—*Aphis gossypii*, Homoptera, Aphididae, *Cucurbita pepo*, zucchini yellow mosaic virus, aphid population growth, amino acids, sugars, starch, phloem exudate.

INTRODUCTION

The effects of systemic viral infection of plants range from no discernible effects to severe morphological and metabolic alterations that lead to plant death. Even though viral symptomatology is well documented, the relationship, if any, between symptom formation and viral fitness is not clear. Cryptic viruses provide evidence that symptoms are not necessarily a by-product of virus replication (Antoniw et al., 1989). This has promoted much speculation concerning the role of virus-induced plant changes in virus fitness, but few conclusions have been drawn (Fraser, 1987). A fecund area of investigation in this regard concerns the effects of virus-induced changes in plant metabolism on insect vectors that interact with the same host plants, either at the level of population dynamics or at the level of behavior (Hammond and Hardy, 1988). The effects that viruses have on their insect vectors, either directly (in the case of circulative–propagative viruses) or indirectly via the physiological effects on a shared host (in the case of nonpersistent viruses) is especially important because of the dependency that most plant viruses have on vectors for spread.

Zucchini yellow mosaic virus (ZYMV) is a potyvirus that causes severe symptoms on *Cucurbita pepo* L. (squash) including leaf and fruit deformation, chlorosis, and stunting (Lisa and Lecoq, 1984). It is transmitted nonpersistently by at least 10 species of aphids (Adlerz, 1987; Castle et al., 1992; Lisa and Lecoq, 1984). *Aphis gossypii* Glover is one of the most efficient vectors of ZYMV (Castle et al., 1992) and is known to colonize many of the host species that ZYMV can infect (Blackman and Eastop, 1984).

Recent studies documenting the effects of ZYMV-infected zucchini on *A. gossypii* showed distinct changes in population growth, alatae formation, and behavior according to the stage of the infection process (Blua and Perring, 1992a,b). We believe that these changes could enhance virus spread (Blua and Perring, 1992b).

We hypothesize that infection with ZYMV induces changes in *C. pepo* phloem exudate and foliage nutrients that affect aphids. In the present study we have two objectives; the first is to determine if the intrinsic rate of natural increase (r_m) for *A. gossypii* is affected by infection of its host plant. The second objective is to document ZYMV-induced changes in nutrients that could account for our observations from the first experiment of this study, and from previous

experiments that documented changes in *A. gossypii* development and behavior on infected plants (Blua and Perring, 1992a,b). Nutrients analyzed from phloem sap, the primary source of food for aphids, include free amino acids, proteins, and sugars, all of which are important to aphid population growth (Srivastava, 1987; Weibull and Melin, 1990). In addition, dietary free amino acids are known to affect alatae production (Leckstein and Llewellyn, 1973) and, along with sugars, are believed to provide stimuli that allow aphids to locate phloem feeding sites and initiate nymphal deposition (Klingauf, 1987). Nutrients analyzed from foliage include free amino acids and sugars, which may indicate host suitability to aphids (Klingauf, 1987). Finally, starch content was analyzed to determine the effect of ZYMV-infection on photosynthate production and carbon translocation.

METHODS AND MATERIALS

A. gossypii *Development. C. pepo* plants (cv. Cheffini, Petoseed, Saticoy, California) were grown in a greenhouse (20–30°C) in 18-liter pots containing redwood humus and were irrigated daily with one-half strength Ward's (1973) solution. Treatments were noninoculated plants and plants inoculated with ZYMV at 0, 7, 14, and 21 days before infestation by *A. gossypii* newborn nymphs, as described previously (Blua and Perring, 1992a). Each treatment was replicated four times, for a total of 20 plants that were distributed at random. Nymphal development was monitored daily until each was reproductively mature, at which time newborn nymphs were removed with a camel's hair brush and counted every other day until nymph production ceased. Longevity of each original aphid was recorded. For each treatment, fecundity was recorded, and the intrinsic rate of natural increase (r_m) (Birch, 1948) was determined using an iterative computer program (Legner, personal communication).

ANOVA was used to compare days to reproductive maturity, longevity, and fecundity among treatments for the original aphid, after fecundity data were transformed (square root, SAS Institute, 1985). Linear contrasts were constructed within the ANOVA for ZYMV-infected treatments. Values of r_m for each inoculation day were analyzed by linear regression (SAS Institute, 1985).

Changes in Plant Nutrients. To access the effects of virus infection on changes in phloem sap and leaf tissue nutrients, an experiment was performed with *C. pepo* in the greenhouse under conditions described above. When plants had approximately 20 true leaves, they were assigned randomly to ZYMV-infected or noninfected treatments. The two oldest leaves of each plant were inoculated mechanically with extracts prepared from ZYMV-infected or healthy zucchini leaves (Blua and Perring, 1992b). ZYMV infection was confirmed by enzyme linked immunosorbent assay (Fereres et al., 1992). The completely

randomized experiment consisted of two treatments × 11 replicates (plants) per treatment.

Statistical analyses consisted of *t* tests comparing concentrations of phloem exudate or foliage components of ZYMV-infected plants to those of noninfected plants for each date that samples were collected (SAS Institute, 1985).

Phloem exudate was collected by making a small (ca. 1 mm deep × 5 mm long) transverse incision into the stem with a razor. A 30-μl aliquot of exudate, which in cucurbits flows for a short period under positive pressure, was removed with a pipet and immediately mixed with 500 μl of 80% ethanol (0°C) (Richardson et al., 1982). Although the cut stylet method of collecting phloem sap is often preferable, it requires long time periods (Fisher and Frame, 1984; Weibull et al., 1990), and our experience with *C. pepo* indicates that it is unreliable. The method of phloem sap collection we chose allowed us to gather many samples (22) within a short time period, thus minimizing variability in sap components due to diurnal fluctuations (Mitchell et al., 1992). Additionally, a study comparing the two techniques judged the stem exudate method favorably and found strong correlations in free amino acid composition between phloem sap samples collected from aphid stylets and stem exudates (Weibull et al., 1990).

Samples were taken between 1300 and 1400 hours (Pacific daylight time) from randomly selected internodes between 8 and 15 leaves from the terminal bud, an area that included only mature leaves exposed to the sun. Because we observed changes in *A. gossypii* alatae formation in the early stages of ZYMV infection (Blua and Perring, 1992a), phloem exudate was collected daily from days 2–9 after inoculation, and every fourth day thereafter to 37 days after inoculation. Extracts were stored at −70°C before chemical analyses.

Starting two days after inoculation, a paper punch was used to remove two 6-mm-diameter leaf disks from each of five randomly selected leaves of each plant. Samples were taken weekly for six weeks from the area of the first or second lobes on either side of the midvein from leaves between 8 and 15 nodes from the terminal bud of each plant. No more than one disk was ever taken from a lobe. Leaf disks were put on Dry Ice immediately and stored at −70°C before chemical analyses.

The ethanolic extracts of phloem exudate were spun in a microcentrifuge (14,000 rpm for 1 min) to pellet the precipitated proteins. Afterwards, 30 μl of supernatant were removed for amino acid analysis and 400 μl was removed for sugar analysis. The protein pellet was dried and weighed.

Individual amino acids were quantified by the methods of Yang and Sepulveda (1985) with the following exceptions. Each 30-μl ethanolic extract was mixed with 200 μl of drying buffer, and the solutions were vacuum centrifuged to dryness at room temperature. The extracts then were dissolved in 100 μl of PITC solution, and the mixtures were incubated at room temperature for 30 min

before drying. The derivatized, dried extracts were mixed with 500 μl resuspension buffer and filtered through 0.2-μm filters. A 20-μl aliquot was injected into a Beckman Ultrasphere ODS-C18 HPLC column (3.5 × 25 mm). Amino acid external standards were used for identification and quantification.

The 400-μl aliquots of exudate extracts saved for sugar analysis were vacuum centrifuged to dryness at room temperature. Dried extracts were resuspended in 60 μl HPLC grade water and filtered. Individual sugars were analyzed by HPLC (Madore et al., 1988). External standard sugar solutions were used to identify and quantify sample sugars.

Foliage disks were weighed fresh, lyophilized, and then reweighed. Dry tissues, in plastic 1.5-ml centrifuge tubes, were boiled briefly in 1 ml 80% ethanol before extracting for 20 min at 65°C. Disks were extracted three more times, and extracts from each sample were combined and saved for amino acid and sugar analyses. Starch was extracted from the leaf residue with 400 μl of 2 N potassium hydroxide heated in a 90°C water bath for 1 hr. The solution was then adjusted to pH 4.5 with acetic acid. One milliliter of amyloglucosidase solution (100 mg/ml from *Rhizopus*, Sigma Chemical Co.) was desalted on a 0.8-cm × 4-cm column of Sephadex G-25 and diluted to 25 ml with 0.1 N sodium acetate (pH 4.5). One milliliter of this diluted enzyme solution was added to each tube, before placing them in a 45°C water bath overnight (ca. 12 hr) to hydrolyze starch into glucose. Glucose in 10-μl aliquots of starch digest was determined with a spectrophotometric procedure using a glucose diagnostic kit [glucose (HK) 10, Sigma Chemical Co.].

Sugars and free amino acids in the ethanolic leaf tissue extracts were separated using coupled ion exchange columns described previously (Madore et al., 1988). Amino acids were derivatized and analyzed as described above, except final resuspension was in 4.0 ml of resuspension buffer. Neutral fractions were dried in a vacuum centrifuge, resuspended in 60 μl of water, and analyzed as described above.

RESULTS

A. gossypii *Development.* The number of days to reproductive maturity for the original aphids were not statistically different among treatments ($P = 0.183$). On noninfected plants, aphids matured in 6.3 days (SE = 0.2), and on infected plants, values ranged from 6.5 to 7.1 days (SE = 0.2, for each treatment). ANOVA demonstrated that longevity varied significantly among treatments ($P = 0.009$). Upon analyzing ZYMV-infected treatments separately from noninoculated controls, significant linear trends were revealed ($P < 0.001$). Aphids on noninfected plants lived an average of 13.0 days (SE = 0.8), the same as aphids on plants infected for seven days before inoculation (13.0 days, SE =

0.5), and very close to that of aphids on plants that were inoculated with ZYMV and infested with aphids on the same day (12.7 days, SE = 1.1). Aphids on plants inoculated 14 and 21 days before infestation lived an average of 15.2 (SE = 1.4) and 15.7 (SE = 0.8) days, respectively.

The pattern of aphid fecundity was similar to longevity in that there were significant differences among treatments ($P = 0.024$), with a significant linear trend for ZYMV-infected treatments ($P = 0.005$). Aphids on noninfected plants produced an average of 30.4 nymphs (SE = 3.5), close to the mean fecundity of aphids on plants infected for 0 days (32.3, SE = 7.4), and 7 days (32.8, SE = 4.9) before infestation. Aphids reared on plants inoculated 14 and 21 days before infestation had the highest fecundity, producing an average of 42.4 (SE = 5.2) and 44.4 (SE = 5.4) nymphs, respectively.

A significant and positive linear relationship existed between the intrinsic rate of natural increase for *A. gossypii* and the number of days between inoculation with ZYMV and aphid infestation ($r^2 = 0.91$, $P = 0.047$, Figure 1). The r_m for aphids on noninoculated plants was 0.398, a value close to those of plants infected for 14 days (Figure 1). Values of r_m determined in this experiment were within the estimates reported by other authors for *A. gossypii* (Kennedy and Kishaba, 1976; Wyatt and Brown, 1977).

Changes in Plant Nutrients. In phloem exudate collected from both virus-infected and noninfected plants there was a gradual increase in total amino acids through the first week after inoculation. During this period infected plants consistently had lower levels of total amino acids. At 21 days after inoculation the trend in total amino acids reversed, and phloem exudate from infected plants consistently had higher levels. However, statistical analysis indicated no sig-

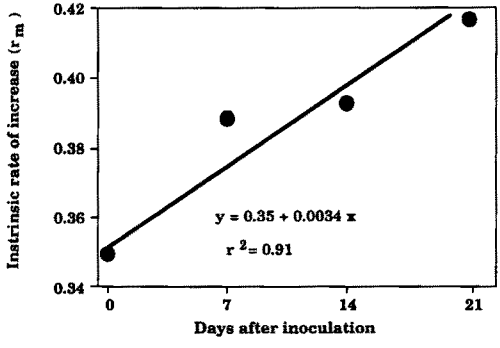

FIG. 1. Intrinsic rate of natural increase for *A. gossypii* as a function of the number of days between plant inoculation with ZYMV and aphid infestation. Fitted line is significantly different from 0 with $P = 0.047$.

nificant differences in total amino acid content between treatments ($P \geq 0.074$) (Figure 2).

Although highly significant differences in total amino acids did not exist, frequent significant ($P \leq 0.050$) differences in individual amino acids occurred that generally reflected the tendencies shown with respect to total amino acids (Table 1). Two days after inoculation, infected plants had higher levels of asparagine and glutamine than noninfected plants. From 3 to 13 days after inoculation, noninfected plants had higher levels of various amino acids. However, no clear pattern with respect to any individual amino acid emerged. Starting at 17 days after inoculation and continuing to the end of the experiment, differences between treatments in individual free amino acids were variable. For plants in both treatments the most predominant phloem exudate free amino acids throughout the experiment were glutamine (25%), glutamic acid (16%), arginine (15%), and homoserine (9%).

In both treatments there was an initial increase in phloem exudate protein concentration after the first collection. Thereafter, exudate from infected plants generally had a lower concentration of protein than healthy plants (Figure 3).

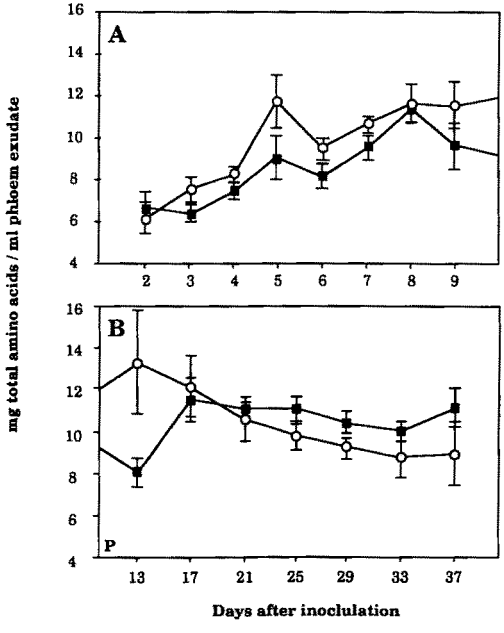

FIG. 2. Mean total free amino acid concentration (± 1 standard error, $N = 11$) in phloem exudate from ZYMV-infected (—■—) and noninfected (—○—) plants 2–9 days after inoculation (A) and 13–37 days after inoculation (B).

TABLE 1. MEAN CONCENTRATIONS OF PHLOEM EXUDATE AMINO ACIDS SHOWING STATISTICAL DIFFERENCES ($P \leq 0.05$, Student's t-test) BETWEEN HEALTHY AND ZYMV-INFECTED PLANTS THROUGH TIME[a]

Days after inoculation	Amino acid	Concentration (μg/ml)		P
		Healthy	ZYMV	
2	Asparagine	35.6	**55.6**	0.05
	Glutamine	594.3	**772.4**	0.05
3	Arginine	**1012.9**	768.3	0.02
	Valine	**124.8**	85.3	0.01
4	No sig. differences			
5	Arginine	**1649.6**	1000.5	0.03
6	Asparagine	**59.4**	32.4	0.03
	Citrulline	**344.1**	158.3	0.01
	Glutamic acid	**1098.7**	878.7	0.04
	Homoserine	**617.1**	280.1	0.01
	Tyrosine	**45.9**	5.2	0.01
7	Asparagine	**42.4**	15.1	0.05
	Citrulline	**332.0**	199.8	0.01
	Glutamic acid	**1024.5**	726.0	0.05
	Homoserine	**840.0**	547.5	0.01
	Phenylalanine	**41.2**	19.6	0.05
8	No. sig. differences			
9	Asparagine	**119.7**	82.5	0.05
13	Citrulline	**389.7**	106.1	0.05
	Glutamine	**3049.9**	1109.3	0.03
17	Tryptophan/ornithine	171.7	**353.1**	0.03
21	Alanine	68.4	**132.1**	0.02
25	Glutamic acid	**1652.4**	1160.9	0.05
	Glycine	**174.2**	96.5	0.04
	Homoserine	859.7	**1282.9**	0.03
	Isoleucine	178.2	**273.4**	0.05
	Leucine	**41.3**	9.6	0.02
29	Threonine	190.4	**425.7**	0.01
33	Alanine	315.6	**334.1**	0.05
	Valine	85.2	**151.3**	0.02
37	Aspartic acid	**253.1**	132.4	0.04
	Isoleucine	**238.5**	146.4	0.05
	Tryptophan/ornithine	109.7	**1143.4**	0.04

[a]Greatest values for each comparison are in bold type.

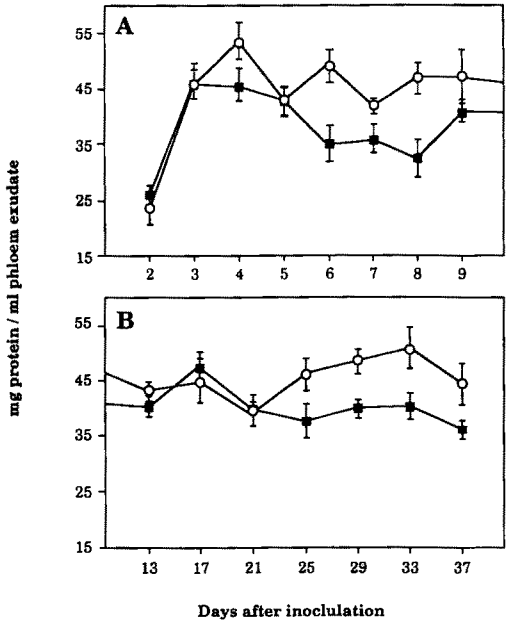

FIG. 3. Mean protein concentration (± 1 standard error, $N = 11$) in phloem exudate from ZYMV-infected (—■—) and noninfected (—○—) plants 2–9 days after inoculation (A) and 13–37 days after inoculation (B).

The mean protein content throughout the experimental period after the initial increase ranged from 32.4 to 47.3 mg/ml phloem exudate in infected plants, and from 38.8 to 53.5 mg/ml for noninfected plants. When significant ($P \leq 0.050$) differences occurred, noninfected plants had the higher concentration (Figure 3).

The predominant sugars in *C. pepo* phloem exudate for both infected and noninfected plants throughout the experimental period were stachyose, which accounted for an average of 45% and 58% of the total sugar content, respectively, and sucrose, which accounted for an average of 35% and 30% of the total sugar content, respectively. Glucose, galactose, fructose, myoinisitol, galactinol, and raffinose combined to make up an average of approximately 20% of the total sugar content for infected plants and 12% of the total for noninfected plants. There was no apparent trend with respect to treatments through time for any of these minor sugars. Due to the fact that cucurbits transport relatively low amounts of sugars compared to other plants (Ziegler, 1974) and to the limitations of our analytical procedures, we had to pool samples within treatments. This precluded statistical analysis and the calculation of standard errors. Yet, nine

days after inoculation, exudate from infected plants consistently had less than half the amount of total sugars than that of noninfected plants (Figure 4).

In infected plants, significantly greater values ($P \leq 0.040$) were consistently observed in tissue fresh weight per unit area, and the fresh weight to dry weight ratio, than in noninfected plants. These differences became apparent 16 days after inoculation and continued through the end of the experiment (Figure 5). Because there were no significant differences in dry weights per unit area between treatments, foliage components measured were expressed on a dry weight basis.

The most striking differences that occurred between infected and noninfected plants were those of leaf tissue free amino acid content. Infected plants had significantly ($P \leq 0.040$) higher concentrations of total amino acids beginning nine days after inoculation (Figure 6). The differences increased throughout the experimental period as amino acids continued to accumulate in infected tissue and remained relatively stable in noninfected tissue (Figure 6). Arginine + threonine (41%), glutamine (13%), and alanine (9%) were the most prevalent free amino acids in ZYMV-infected tissue, whereas arginine + threonine (43%),

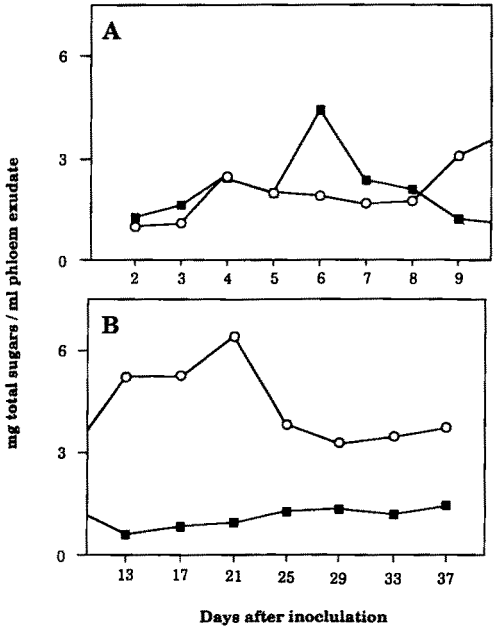

FIG. 4. Mean total sugar concentration in phloem exudate from ZYMV-infected (—■—) and noninfected (—○—) plants 2–9 days after inoculation (A) and 13–37 days after inoculation (B).

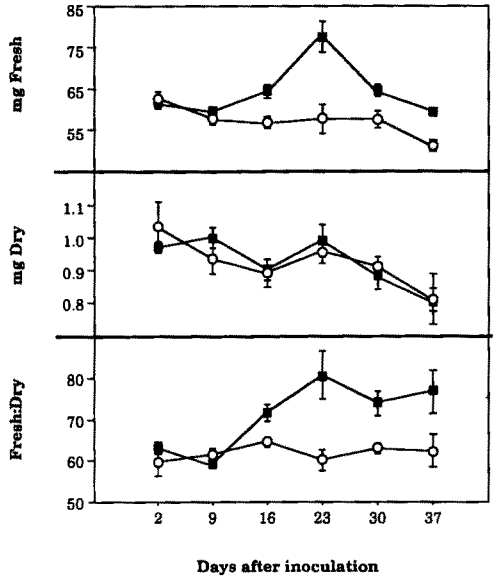

FIG. 5. Mean fresh weight and dry weight and ratio of fresh to dry weights of 283 mm^2 leaf samples ($\pm$ 1 standard error, $N = 11$) from ZYMV-infected (—■—) and noninfected (—○—) plants through time.

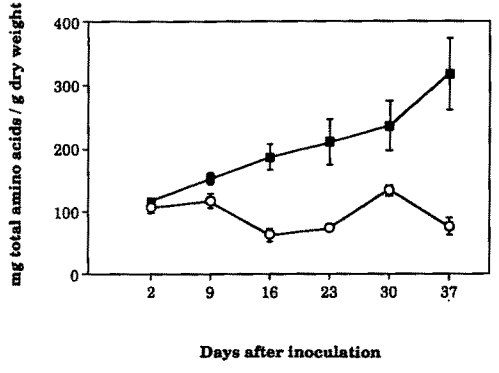

FIG. 6. Mean total free amino acid concentration ($\pm$ 1 standard error, $N = 11$) in leaf samples from ZYMV-infected (—■—) and noninfected (—○—) plants through time.

glutamic acid (11%), and alanine (10%) were the most predominant free amino acids in noninfected tissue throughout the experiment. It is interesting to note that infected foliage accumulated glutamine over glutamic acid, while noninfected foliage accumulated glutamic acid over glutamine. Together, glutamic acid and glutamine accounted for approximately the same proportion of total amino acids in infected plants as they did in noninfected plants.

Sugar content of both treatments was variable throughout the experiment, and no strong patterns could be discerned (Figure 7). However, a significantly ($P = 0.001$) greater concentration of glucose was found in noninfected plants

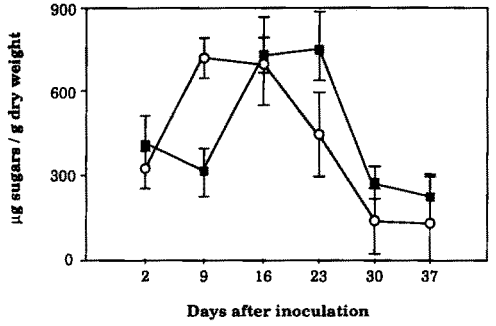

FIG. 7. Mean total sugar concentration (± 1 standard error, $N = 11$) in leaf samples from ZYMV-infected (—■—) and noninfected (—○—) plants through time.

TABLE 2. MEAN CONCENTRATIONS OF LEAF TISSUE SUGARS SHOWING STATISTICAL DIFFERENCES ($P \leq 0.05$, Student's t-test) BETWEEN HEALTHY AND ZYMV-INFECTED PLANTS THROUGH TIME[a]

Days after inoculation	Sugar	Concentration (mg/g dry wt.)		P
		Noninfected	Infected	
2	No sig. differences			
9	Glucose	**4.74**	2.93	<0.01
	Sucrose	**181.09**	104.47	<0.01
16	Fruct./myoin.	62.65	**132.80**	<0.01
	Sucrose	**297.06**	175.47	0.02
	Stachyose	103.57	**132.61**	0.04
23	No sig. differences			
30	Fruct./myoin.	45.90	**76.08**	0.05
37	No sig. differences			

[a]Greatest values for each comparison are in bold type.

than in infected plants nine days after inoculation (Table 2). Likewise, sucrose was more abundant in noninfected tissues nine days after inoculation (P = 0.001) (Table 2). Infected plants had greater concentrations of fructose + myoinisitol 16 and 30 days after inoculation (P = 0.045) and a greater concentration of stachyose 16 days after inoculation (P = 0.044) than did noninfected plants (Table 2). Concentration of total sugars was greater in noninfected foliage nine days after inoculation than in infected tissue (P = 0.002) (Figure 7). Throughout the experiment for both treatments, foliar sugars consisted of glucose (33%), sucrose (21%), stachyose (20%), fructose + myoinisitol (15%), raffanose (7%), and galactose (3%).

Starch content of leaves from infected plants decreased continually, while in noninfected plants, starch content was relatively stable after an initial decrease (Figure 8). Significant ($P \leq 0.043$) differences between treatments were observed in the last two weeks of the experiment.

DISCUSSION

Other investigators (e.g., Fraser, 1987; Goodman et al., 1986) have examined the effects of virus infection on plant tissue nutrients, including proteins, free amino acids, and sugars, that are important to aphids. Unfortunately, none of these studies have examined virus-induced changes of these nutrients in phloem sap—the primary site of aphid feeding (Srivastava, 1987). In the present study the effects of virus infection on the concentrations of nutrients in leaves and phloem exudate were examined through time after inoculation in order to understand the dynamics of the infection process that mediates plant growth and virus–vector interactions.

Nutritional components of leaf tissues and phloem sap are important in

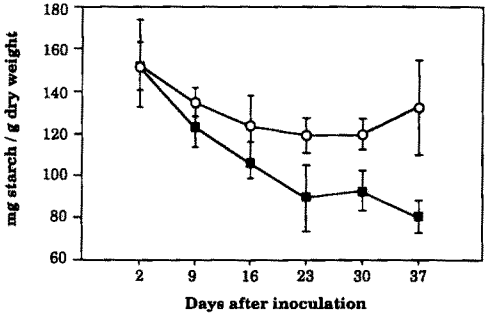

FIG. 8. Mean starch concentration (± 1 standard error, N = 11) in leaf samples from ZYMV-infected (—■—) and noninfected (—○—) plants through time.

several areas of virus–vector interactions, including aphid population development, alatae formation, and behavior. Nutrients such as sucrose and amino acids act as cues that stimulate aphids to locate feeding sites (Harris, 1977; Klingauf, 1987). Little is known of the stimulants important in the process of sieve cell location; however, aphids may guide their stylets to vascular bundles by following a sucrose gradient, and then locate sieve cells within the vascular bundles via a hit-or-miss approach (Harris, 1977).

Aphid population development depends on several aspects of nutrition. Amine nitrogen appears to be the most limiting nutrient controlling aphid population growth (Srivastava, 1987). Weibull (1988) found that growth of aphids on oats and barley was correlated closely with the free amino acid content of phloem exudate. In our study, there was an increase in the rate of population increase (r_m) for *A. gossypii* with time between ZYMV inoculation of zucchini and aphid infestation. This generally fits the pattern of changes in total amino acids even though total amino acid levels were not substantially different between infected and noninoculated plants. The effect of the accumulation of free amino acids over time to which aphids were exposed, however, may have been important to their longevity and fecundity. Aphids on plants inoculated and infested on the same day had the lowest r_m values (Figure 1), and were likely subjected to the lowest accumulated level of amino acids through their reproductive lifetime (Figure 2). Similarly, aphids on plants that were inoculated 7, 14, and 21 days before infestation had increasingly higher r_m values and likely were exposed to increasingly greater accumulated levels of amino acids. Finally, aphids on noninfected plants had an intermediate r_m value, which is predictable based on the level of amino acids accumulated over time. Phloem protein levels changed in opposition to the level of total amino acid (Figure 3). It is likely that these proteins are structural (Eschrich et al., 1971) and therefore may not be available as a nitrogen source for aphids. Although it is not known if *A. gossypii* can digest proteins, a related species, *Aphis acanti*, was shown not to digest proteins (Ehrhardt, 1962).

We do not know if the differences in free amino acids (Figure 2, Table 1) from phloem exudate between ZYMV-infected and noninfected plants could impact *A. gossypii* alatae formation as demonstrated in earlier studies (Blua and Perring, 1992a). Yet, alatae formation by various aphid species also has been linked to dietary free amino acids (Leckstein and Llewellyn, 1973).

Large differences between treatments were observed in concentrations of phloem sugars. Phloem exudate from infected plants had lower concentrations of sugars than exudate from noninfected plants throughout all but the first week of the experiment (Figure 3). If aphids locate vascular tissue by a sucrose gradient (Harris, 1977), this may explain the observation that *A. gossypii* alatae do not easily locate phloem feeding sites on plants that are infected with ZYMV for a long period of time (Blua and Perring, 1992a). This difficulty with locating

feeding sites is exacerbated by the relatively low amounts of sugars, particularly sucrose, in cucurbit phloem sap (Ziegler, 1975).

Factors not considered in this study that also may affect aphid development are differences between infected and noninfected plants in the levels of defensive compounds such as cucurbitacins (Tallamy, 1985). It also is possible that dissimilarities between nutrients in the phloem sap of the stem, where exudate was collected, and sieve elements fed upon by aphids were not consistent between infected and noninfected treatments.

It is interesting that differences in concentrations of individual (Table 1) and total amino acids (Figure 2) in the phloem sap of ZYMV-infected and noninfected plants did not reflect the large differences observed in the concentration of total amino acids in whole-leaf samples (Figure 6). The opposite situation occurred with sugars. Concentration of total sugars in leaves from infected and noninfected plants were statistically similar on four of the six dates examined (Figure 7), while large differences were observed for phloem exudate sugars, as noted above. These observations should serve as a warning to those who would make, or rely on, conclusions regarding the nutritive value of plants to phloem-feeding insects based on the nutritive value of whole-leaf samples. Such conclusions have been made in many studies done before technological advances allowed isolation of phloem sap and the analysis of picomole quantities of amino acids. These early studies, and more recent ones, have shown a direct relationship between aphid performance and the foliar content of free amino acids (Auclair et al., 1957; Carter and Cole, 1977; Havlícková, 1987; Jansson et al., 1987; Markkula and Laurema, 1964; Parry, 1977; Van Emden and Bashford, 1971). However, in recent studies that analyzed free amino acids in phloem sap, such relationships were not clear (Febvay et al., 1988; Weibull, 1988; Weibull and Melin, 1990; but see Weibull, 1987, for an exception).

The pattern in ZYMV-infected leaves of stunting, general chlorosis, a reduction in starch reserves, and an increase in free amino acids is consistent with a reduction in carbon fixation along with an increase in protein turnover. A similar increase in amino acids from phloem exudate was not observed, and phloem sugars were exceptionally low in infected plants. This suggests that translocation is reduced in ZYMV-infected plants. Studies with other plant viruses also indicate a reduction in photosynthesis (Fraser, 1987) and translocation (Jensen, 1972; Leal and Lastra, 1984), and increases in protein turnover (Fraser, 1987).

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PHTHALIDE-BASED HOST-PLANT RESISTANCE TO *Spodoptera exigua* AND *Trichoplusia ni* IN *Apium graveolens*

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Abstract—A chemical basis for the difference in suitability between two celeriac (*Apium graveolens* var. *rapaceum*) cultivars for the survival and growth of *Spodoptera exigua* (Hübner) and *Trichoplusia ni* (Hübner) was identified as sedanenolide (3-*n*-butyl-4,5-dihydro-isobenzofuranone). Sedanenolide was isolated using a bioassay-driven extraction and purification procedure and was identified using several spectrometric methods. Foliar concentrations of sedanenolide were negatively correlated with larval performance and were significantly higher in the cultivar less suitable for larval survival and growth. Sedanenolide and *Bacillus thuringiensis* Berliner acted additively in reducing larval growth when combined in artificial diets, a result that is consistent with previous studies in which the combined effect of host plant cultivar and *B. thuringiensis* on larval survival and growth were additive.

Key Words—Host-plant resistance, phthalide, sedanenolide, celeriac, *Bacillus thuringiensis*, tritrophic interaction, beet armyworm, cabbage looper, *Apium graveolens*.

INTRODUCTION

The importance of plant secondary metabolites in mediating host-plant suitability to herbivorous insects is widely recognized (e.g., Rosenthal and Berenbaum, 1991). It is therefore surprising that there are relatively few examples of intra-

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specific differences in host-plant suitability (i.e., host-plant resistance) based upon qualitative or quantitative variation in specific chemicals (Berenbaum and Zangerl, 1992).

The paucity of such examples can probably be attributed to two factors: (1) the difficulty in identifying potentially important chemicals and (2) the need to demonstrate that a specific chemical(s) can affect herbivore performance and that it exerts its effect in planta. The first problem is usually overcome with a bioassay-driven fractionation procedure in which a biologically active compound(s) is isolated. The second problem can be addressed by collecting two types of data: (1) direct evidence of the effect of a specific chemical(s) on herbivore performance, usually via bioassay of the isolated chemical(s) in an artificial diet; and (2) indirect evidence of the effect of a specific chemical on herbivore performance, usually by correlating the concentration of a specific chemical(s) found in the plant with herbivore performance. When this information is assembled, a compelling case for the role of a specific chemical in host-plant resistance can be made (reviewed by Berenbaum and Zangerl, 1992).

Secondary plant metabolites are also assumed to mediate interactions between herbivorous insects and their natural enemies. Studies that examine these tritrophic (plant–herbivore–natural enemy) interactions fall into two basic categories: (1) those that examine the interaction between host-plant genotypes and the herbivore's natural enemies where the herbivores are fed on plants (correlative) and (2) those that examine the interaction between specific allelochemicals and the herbivore's natural enemies where the herbivores are fed artificial diets (causative) (see Reichelderfer, 1991, and Hare, 1992, for recent reviews). Relatively few studies provide conclusive evidence for the role of specific chemicals mediating tritrophic effects both in the plant with correlative data and in isolation with causative data (but see Felton and Duffey, 1990).

Descriptions of the combined effects of host-plant resistance and natural enemies on herbivore mortality are often made from a pest manager's point of view with the terms "compatible" and "incompatible" being broadly applied (Bergman and Tingey, 1979). Hare (1992) points out, however, that the combined effects of these two mortality factors fall into three basic categories. Additive relationships between host-plant resistance and natural enemies occur when the combined mortality from the two factors can be predicted from the mortality of each considered alone (i.e., the mortality factors are independent). Synergistic relationships occur when the combined mortality from the two factors is greater than that predicted from the mortality of each factor considered alone. Antagonistic relationships occur when the combined mortality from the two factors is less than that predicted from the mortality of each considered alone. Host-plant resistance and natural enemies therefore are compatible pest management strategies when the relationship between them is additive or syn-

ergistic, and they are incompatible when the relationship is antagonistic (Hare, 1992).

Apium graveolens L. (celery, celeriac, and smallage) is an Old World herb that, like many members of the Umbelliferae, contains linear furanocoumarins among other allelochemicals (Scheel et al., 1963; Beier et al., 1983). The linear furanocoumarins present in *A. graveolens* are toxic to a number of herbivores, including two important pests of commercially grown celery (*A. graveolens* var. *dulce*) in California: the beet armyworm, *Spodoptera exigua* (Hübner) (Trumble et al., 1991), and the cabbage looper, *Trichoplusia ni* (Hübner) (Lee and Berenbaum, 1989) (Van Steenwyk and Toscano, 1981).

In previous work, we demonstrated that two celeriac (*A. graveolens* var. *rapaceum*) cultivars, USDA Plant Introduction Numbers 223333 and 357333 (hereinafter referred to as PI 223333 and PI 357333), differed in their suitability for the survival and growth of *S. exigua* and, to a lesser extent, those of *T. ni* (Meade and Hare, 1991). We also demonstrated that host-plant cultivar and *B. thuringiensis* acted additively on the survival and growth of *S. exigua* and *T. ni* (Meade and Hare, 1993, 1994).

Here, we describe the isolation and identification of a chemical responsible for differences in the suitability of the two plant genotypes for insect survival and growth and for the subsequent differences in susceptibility to *B. thuringiensis*. Specifically, we show that: (1) the difference between the cultivars is phytochemically mediated, (2) the difference is not related to linear furanocoumarin concentration, but to the concentration of a phthalide, sedanenolide, previously identified as an aroma and flavor constituent of *A. graveolens*, and (3) when incorporated into an artificial diet, sedanenolide causes an additive increase in the susceptibility of both insects to *B. thuringiensis*.

METHODS AND MATERIALS

Insects. S. exigua larvae were taken from a laboratory colony established in 1987 with adults collected on lettuce near Bakersfield, California. Larvae were reared on a baby lima bean diet (Patana, 1969), while adults received 25% honey in water. Larvae and adults were maintained under controlled environmental conditions (26 ± 1°C, 40–45% relative humidity, and 16:8 hr light–dark photoperiod).

T. ni larvae were taken from a laboratory colony established in 1965 from cabbage at the University of California's South Coast Field Station, Irvine. Larvae were maintained on a modified pinto bean diet (Shorey and Hale, 1965) while adults received 20% honey in water. Larvae and adults were maintained under controlled environmental conditions (24 ± 1°C, approx. 40% relative humidity, and 6:18 hr light–dark photoperiod).

Plants. Seeds of the two *A. graveolens* var. *rapaceum* cultivars were planted in steam-sterilized silica sand and allowed to germinate in a temperature-controlled room (26 ± 1°C). Seedlings were transplanted to steam-sterilized soil (University of California mix; Matkin and Chandler, 1957) and moved to a glasshouse. When the plants were ca. 15 cm tall, they were moved to a field plot at the University of California Agricultural Operation, Riverside, where they were maintained under conventional growing conditions (Sims et al., 1977). The leaf tissue used for the chemical isolation and identification came from plants of uniform chronological age, as did the tissue used in all bioassays.

Bioassay Conditions. Plant extracts and sedanenolide were bioassayed by applying them to 375 mg of cellulose (Alphacel, ICN Biochemicals), allowing the solvent to evaporate, and then incorporating the cellulose into a semisynthetic diet (Patana, 1969). The quantity of cellulose in the diets was equal to 5% of the dry weight of the diets, and all extracts were bioassayed at 0.1 g fresh mass equivalents/g fresh mass of diet (i.e., 1 g diet contained the equivalent of 0.1 g fresh host-plant foliage).

Hot (liquid) diet was poured into plastic Petri dishes (9 cm diam.) and, before gelling, a plastic grid was placed in the diet (Navon et al., 1990). Grids were cut from fluorescent light diffusers and consisted of a 4 × 4 array of 1.69 cm^2 cells (16 cells/Petri dish, 0.9 cm high). A single, newly eclosed (<6 hr), *S. exigua* first instar was placed in each cell, a 9-cm filter paper disk was placed over the cells, and a second plastic grid was placed over the first (Navon et al., 1990). The lid was then placed on the Petri dish and the edge of the dish was sealed with Parafilm (American National Can, Greenwich, Connecticut). Filter paper disks were changed every two days and larvae were weighed after 10 days. Each bioassay was repeated three times (16 larvae/replicate).

Extraction and Purification. Extracts and fractions were bioassayed as shown in Figure 1. Only those extracts and fractions showing differential activity between host-plant genotypes were separated further.

Two hundred grams (fresh weight) of leaf tissue from each genotype was extracted three times in boiling methanol. The three extracts were pooled and bioassayed, as was the solid residue from the extractions. The methanol extracts from each cultivar were reduced under vacuum, then dissolved in CH_2Cl_2. These mixtures were partitioned on the basis of polarity into four classes by extracting the CH_2Cl_2 solutions sequentially with aqueous acid (HCl, pH 2), aqueous weak base (0.1 M $NaHCO_3$, pH 8.3), and an aqueous strong base (NaOH, pH 11). This procedure produced extracts containing basic (e.g., alkaloids), strongly acidic (e.g., carboxylic acids), weakly acidic (e.g., phenolics), and neutral (e.g., furanocoumarins) compounds, respectively.

The concentrations of psoralen, bergapten, and xanthotoxin, the major linear furanocoumarins found in *A. graveolens* (Beier et al., 1983), were determined in the extracts containing neutral compounds using the method described

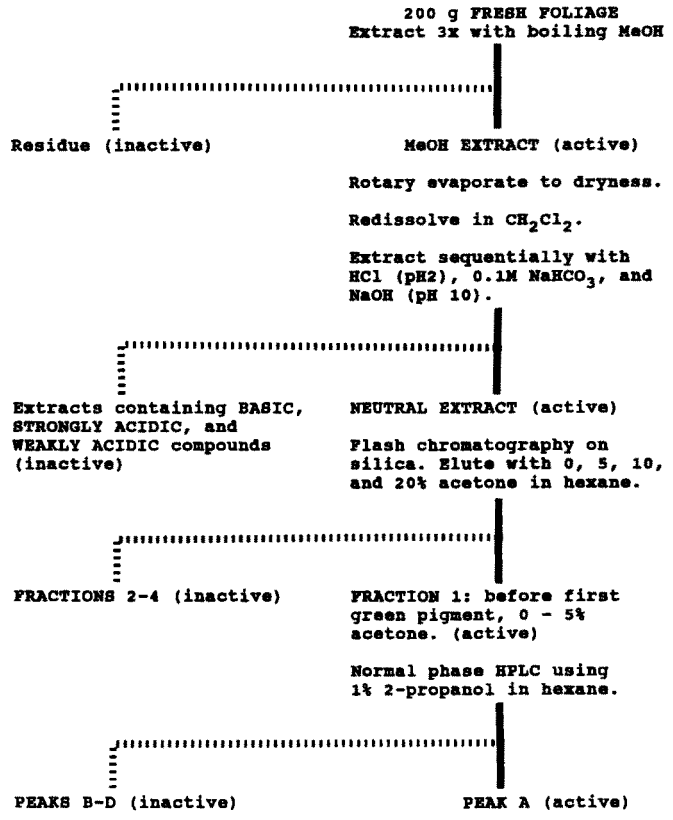

FIG. 1. Flow chart for the extraction and fractionation of sedanenolide from celeriac.

by Trumble et al. (1992). Two samples (500 mg fresh weight equivalent) from the extract from each host-plant genotype were analyzed for furanocoumarin content. Furanocoumarins were quantified using HPLC with UV detection. HPLC was performed on a Beckman model 332 (Beckman Instruments Inc., Fullerton, California) fitted with a silica analytical column (Ultrasphere, 250 × 4.6 mm, 5-μm particle size, Beckman Instruments Inc., San Ramon, California), a silica guard column (Ultramex 5, 50 × 4.6 mm, 5-μm particle size, Phenomenex, Torrance, California), and an Isco model V-4, variable wavelength absorbance detector (Isco Inc. Lincoln, Nebraska). Analytes were eluted isocratically with hexane–tetrahydrofuran (81 : 19) at a flow rate of 1.5 ml/min, detected at 290 nm, and quantified against an internal standard, 7-benzyloxycoumarin (Trumble et al., 1992).

The active fraction (containing neutral compounds) from the acid–base

extraction was further fractionated using flash chromatography (Still et al., 1978) on silica (230–400 mesh), eluting with a stepwise gradient (0, 5, 10, and 20% acetone in hexane). The active fraction from flash chromatography was further separated by HPLC using the instrument and column described previously. The analytes were eluted isocratically in hexane–2-propanol (99 : 1) at a flow rate of 2.0 ml/min and were detected by their absorbance at 220 nm.

Identification of Sedanenolide. Identification of the active component isolated by HPLC was accomplished by UV and IR spectrometry, EI and FAB mass spectrometry, and ^{1}H and ^{13}C NMR spectrometry. The UV spectrum was recorded in hexane and EtOH on a Spec-200 spectrophotometer (Bausch and Lomb Inc., Rochester, New York). The IR spectrum was recorded in CCl_4 on a 4020 Galaxy Series FTIR (Mattson Instruments, Madison, Wisconsin). The EI mass spectrum was recorded on a Hewlett-Packard 5970 mass selective detector (70 eV) coupled to a Hewlett-Packard 5890 gas chromatograph (Hewlett-Packard, Avondale, Pennsylvania). The gas chromatograph was fitted with a 20-m × 0.25-mm-ID × 0.25-μm film thickness Ultra 2 column (Hewlett-Packard) and was operated under the following conditions: splitless injection mode; temperature program 50°C for 2 min, then 10°C/min to 275°C for 10 min; head pressure 90 kPa, and injector and transfer line temperatures 250°C. The FAB mass spectrum was recorded on a VG ZAB-2FHF spectrometer (VG Instruments, Danvers, Massachusetts) using a thioglycerol sample matrix. The proton and carbon NMR spectra were recorded in $CDCl_3$ on a QE-300 NMR spectrometer (General Electric NMR Instruments, Fremont, California) at 300 MHz.

Sedanenolide Bioassays. The isolation and purification procedure originally employed was modified so that large quantities of sedanenolide could be isolated. Freeze-dried *A. graveolens* foliage was Soxhlet extracted in CH_2Cl_2 for 48 hr. The extracts were reduced under vacuum and the residue was dissolved in hexane. This extract was loaded onto a silica flash chromatography column and eluted sequentially with hexane, 5% acetone in hexane, and acetone (two column volumes of each). The 5% acetone in hexane fraction was reduced under vacuum and dissolved in hexane. Sedanenolide was isolated by HPLC using silica preparative (Econosil, 250 × 22.5 mm, 10-μm particle size) and guard columns (Econosil, 50 × 22.5 mm, 10-μm particle size, Alltech Assoc. Inc.). Samples were injected using a 500-μl injection loop with a flow rate of 10 ml/min. All other equipment and conditions were the same as those described previously. The purified sedanenolide (approximately 95% pure by HPLC analysis) was bioassayed against both *S. exigua* and *T. ni* using the previously described method at concentrations of 1, 10, 100, and 200 μg/g fresh weight of diet.

Sedanenolide Concentrations and Insect Performance on Intact Plants. In a previous study (Meade and Hare, 1994), we assessed the suitability of tissue from field-grown plants of each cultivar for *S. exigua* and *T. ni* larval growth.

These bioassays were conducted at approximately monthly intervals and plant tissue samples from each cultivar were taken at the beginning of the bioassays and stored at −20°C for chemical analyses. We used these samples to determine seasonal variation in sedanenolide concentration for both cultivars and correlated the previous bioassay data on larval performance with sedanenolide concentration.

We quantified sedanenolide in fresh tissue using a modification of the method described by Uhlig et al. (1987). Five hundred milligrams (fresh weight) of foliage were homogenized in 10 ml of HPLC-grade water with 5 μg of the synthesized internal standard, 7-benzyloxycoumarin using a tissue homogenizer (Tissumizer, Tekmar Co., Cincinnati, Ohio). The homogenate was vigorously extracted with 5 ml of CH_2Cl_2 and centrifuged at 10,000 *g* for 5 min to separate the aqueous and organic phases. The CH_2Cl_2 was removed with a Pasteur pipet and the extraction was repeated twice more for a total of three extractions. The CH_2Cl_2 was evaporated under a N_2 stream, and the dried extract was dissolved in 1 ml of hexane. The sample was loaded onto a solid phase extraction column containing 300 mg of silica (PrepSep, Fisher Scientific, Fair Lawn, New Jersey) which had been preconditioned with 2 ml of hexane. After rinsing with 3 × 1 ml hexane, the analytes were eluted with 4 × 1 ml hexane–2-propanol (19:1). The sample was again dried under a N_2 stream, dissolved in hexane, and stored at −10°C until HPLC analysis. HPLC analysis was performed as described previously. Analytes were eluted isocratically with hexane–2-propanol (99:1) at a flow rate of 2 ml/min and detected by their absorbance at 220 nm.

Combined Effect of Sedanenolide and B. thuringiensis. The combined effect of sedanenolide and *B. thuringiensis* on *S. exigua* and *T. ni* larval growth was determined by incorporating both sedanenolide and *B. thuringiensis* into semisynthetic diets. The bioassay procedure was that described previously. Sedanenolide was incorporated into diets at 0, 10, 50, and 75 μg/g fresh weight and *B. thuringiensis* was incorporated into the diets at 0, 22.5, 45.0, and 90 μg/g fresh weight for *S. exigua* and at 0, 0.1, 0.2, and 0.4 μg/g fresh weight for *T. ni* (4 sedanenolide concentrations ×4 *B. thuringiensis* concentrations = 16 treatment combinations). Each treatment combination contained 16 neonate larvae and the experiment was repeated four times. The *B. thuringiensis* preparation used in this experiment was a spore–crystal mixture derived from Javelin (Sandoz Crop Protection Corp., Des Plaines, Illinois), a commercial formulation of the NRD-12 isolate of *B. thuringiensis* subsp. *kurstaki* (Berliner) and whose preparation was described previously (Meade and Hare, 1993).

Statistics. Furanocoumarin concentrations were analyzed using a single-factor (host-plant cultivar) analysis of variance (ANOVA) (Sokal and Rohlf, 1981). The log-transformed larval weights of *S. exigua* reared on the diets containing extracts from each of the host-plant cultivars were analyzed using a single-factor (host-plant cultivar) ANOVA blocked by replicate (Steel and Tor-

rie, 1980; SAS Institute Inc., 1989). The log-transformed larval weights of *S. exigua* and *T. ni* from the bioassays of sedanenolide were analyzed using a single factor (sedanenolide concentration) ANOVA. Foliar concentrations of sedanenolide were analyzed using a two-factor (month × host-plant cultivar) ANOVA (SAS Institute Inc., 1989) and the hypothesis tests for a mixed-model were performed because month was considered a random effect (Sokal and Rohlf, 1981). Regression analysis (SAS Institute Inc., 1989) was used to test for correlations between host-plant sedanenolide concentrations (independent variable) and *S. exigua* and *T. ni* survival and log-transformed mass (dependent variables). Finally, a two-factor (sedanenolide concentration × *B. thuringiensis* concentration) ANOVA on the log-transformed masses of larvae was used to assess the interaction between sedanenolide and *B. thuringiensis* (SAS Institute Inc. 1989).

RESULTS

Extraction and Purification. The differential activity between the methanol extracts from the two host-plant cultivars (Table 1) mirrored the differences we observed in fresh tissue (Meade and Hare, 1991). Following acid–base extrac-

TABLE 1. BIOASSAYS OF EXTRACTS AND FRACTIONS FROM FIGURE 1[a]

	PI 223333	PI 357333	ANOVA[b]
Methanol extracts of fresh foliage			
Residue	30.2 (25.0–36.5)	28.7 (23.7–34.8)	$F = 0.00$; $P = 0.9973$
MeOH	34.3 (26.6–44.3)	9.6 (7.5–12.1)	$F = 36.62$; $P = 0.0262$
Acid–base extracts of the methanol extracts			
Strong acids	13.2 (9.6–18.1)	11.6 (9.1–14.8)	$F = 2.68$; $P = 0.2433$
Weak acids	10.4 (7.1–15.1)	11.7 (8.7–15.7)	$F = 0.09$; $P = 0.7958$
Neutrals	23.6 (19.7–28.3)	5.9 (4.7–7.5)	$F = 151.67$; $P = 0.0065$
Bases	21.8 (18.1–26.1)	25.6 (18.2–36.2)	$F = 13.75$; $P = 0.0656$
Flash chromatography of the neutrals extracts			
Fraction 1	41.6 (34.3–50.5)	14.1 (10.3–19.2)	$F = 28.10$; $P = 0.0338$
Fraction 2	41.1 (35.7–47.3)	41.3 (35.3–48.3)	$F = 0.04$; $P = 0.8602$
Fraction 3	34.1 (31.0–37.5)	34.6 (28.3–42.3)	$F = 0.02$; $P = 0.9023$
Fraction 4	46.7 (38.9–55.9)	57.0 (50.6–64.2)	$F = 0.51$; $P = 0.5510$
HPLC of flash chromatography fraction 1			
Peak A	48.7 (41.0–57.9)	26.3 (20.4–33.8)	$F = 21.48$; $P = 0.0428$
Peak B	47.3 (40.4–55.4)	39.9 (33.8–47.1)	$F = 0.01$; $P = 0.9449$
Peak C	59.2 (49.3–71.1)	58.3 (48.9–69.4)	$F = 0.39$; $P = 0.5964$
Peak D	48.4 (41.1–56.6)	52.4 (42.8–64.0)	$F = 2.15$; $P = 0.2801$

[a] Values are back-transformed means and 95% confidence limits of masses (mg) of *S. exigua* larvae from analyses of log-transformed data.
[b] $df = 1, 2$ for all tests.

tion, the difference in activity between extracts from the two cultivars was confined to the fraction containing neutral compounds (Figure 1, Table 1). Following flash chromatography, activity was restricted to the first fraction, which included compounds that eluted in 0–5% acetone in hexane (Figure 1, Table 1). Finally, following HPLC, the difference in activity between extracts from the two cultivars was confined to the first peak (Figure 1, Table 1).

Concentrations of the linear furanocoumarins bergapten and xanthotoxin were significantly greater in PI 223333, the cultivar more suitable for larval survival and growth, than in PI 357333, the less suitable cultivar (bergapten: $F = 15.32$; $df = 1, 3$; $P \leq 0.05$, xanthotoxin: $F = 1356.80$; $df = 1, 3$; $P \leq 0.0001$, Figure 2). Psoralen, the third furanocoumarin commonly found in celery, was present at trace levels, a result consistent with other published reports (Trumble et al., 1992).

Identification of Sedanenolide. The single component isolated from HPLC (peak A) was identified from the comparison of spectra with published values (Bjeldanes and Kim, 1977; Yamagishi and Kaneshima, 1977; Fischer and Gijbels, 1987) as sedanenolide (= senkyunolide) (Figure 3, Table 2). UV: λ_{max} (hexane) = 268 nm (ϵ3776), λ_{max} (EtOH) = 270 nm (ϵ1056). $[\alpha]_D^{26} = -100.3°$ (c = 2.87, in $CHCl_3$). FTIR: λ_{max} (cm^{-1}): 3050, 2982, 2963, 2936, 2874, 2836, 2685, 2305, 1767, 1661, 1460, 1430, 1263, 098, 1041, 1014, and 895. EI-MS, *m/z* (rel. abund.): 192 (15, M^+), 163 (2), 135 (4), 133 (5), 108 (9), 107 (100), 105 (6), 91 (5), 85 (10), 79 (30), 78 (12), 77 (32), 57 (8). FAB-MS: *m/z* 193.1222 (M + 1) (calculated for $C^{12}H^{17}O_2$ = 193.1229). NMR data are presented in Table 2.

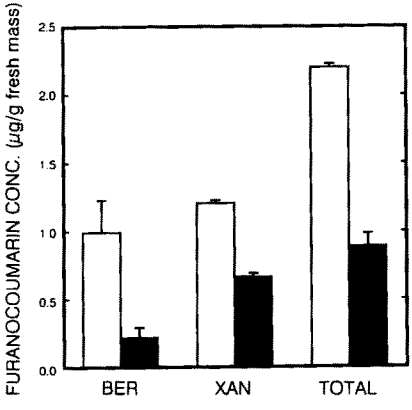

FIG. 2. Concentration of linear furanocoumarins in the neutrals containing extracts from PI 223333, the cultivar more suitable for *S. exigua* and *T. ni* growth (hollow bars), and PI 357333, the cultivar less suitable for larval growth (solid bars).

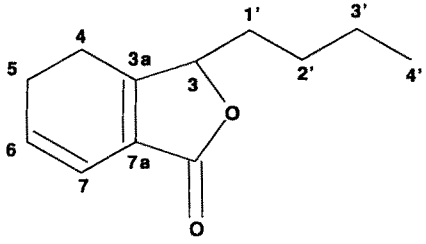

FIG. 3. Chemical structure of sedanenolide.

TABLE 2. ^{1}H AND ^{13}C NMR DATA OF SEDANENOLIDE

	This study			Fischer and Gijbels 1987		
H/C No.[a]	^{13}C	^{1}H	J_{H-H}	^{13}C	^{1}H	J_{H-H}
1	171.18			170.8		
3	82.44	4.92dddd	$J_{3-1'1}$ = 7.1 $J_{3-1'2}$ = 3.3 J_{3-4} = 0.4 J_{3-6} = 0.5	82.2	4.95	$J_{3-1'}$ = 6 + 6 J_{3-6} = 0.6 J_{3-7} = 1.0
3a	161.38			161.3		
4	22.32	2.47m		22.3	2 + 2.5	
5	20.73	2.47m		20.8	2.48	J_{5-6} = 2.5 + 2.5
6	128.27	5.91ddd	J_{6-3} = 0.5 J_{6-5} = 2.2 J_{6-7} = 9.7	128.1	5.93	J_{6-7} = 9.6
7	116.79	6.20dd	J_{7-5} = 1.5 J_{7-6} = 9.7	116.5	6.22	J_{7-5} = 1.5 + 1.5
7a	124.42			124.1		
$1'_1$	31.84	1.54m		31.9	1.54–1.90	
$1'_2$		1.86m				
2'	26.67	1.38m		26.7	1.7	
3'	22.38	1.41m		22.6	1.4	
4'	13.80	0.90t	$J_{4'-3'}$ = 7.0	13.8	0.95	$J_{4'-3'}$ = 6.0 + 6.0

[a]Hydrogen/carbon numbers refer to Figure 3.

The identification of sedanenolide was accomplished as follows. High resolution FAB-MS gave a M + 1 ion at 193.1222, corresponding to the formula $C_{12}H_{17}O_2$; the molecular formula was then $C_{12}H_{16}O_2$, corresponding to a molecule with five sites of unsaturation. The UV absorption maximum at 268 nm (hexane) suggested a molecule with at least some conjugation. The oxygen-containing functional group was identified as an ester by the strong IR bands at

1767 and 1263 cm^{-1} with the position of the band at 1767 cm^{-1}, suggesting a five-membered lactone (gamma-lactone). The ^{13}C and the APT (attached proton test) NMR spectra supported these data with a carbonyl carbon at δ 171.18 and a trisubstituted carbon with a C—O linkage at δ 82.44. Two, fully substituted, olefinic carbons at δ 161.38 (carbon 3a, Figure 3), and δ 124.42 (carbon 7a, Figure 3) in positions indicative of an α,β-unsaturated carbonyl system, suggested that the lactone carbonyl was conjugated to two fully substituted, potentially bridgehead carbons. In total, this information suggested an α,β-unsaturated lactone fragment, possibly fused to a second ring, with a substituent on the oxygen-bearing carbon.

The ^{13}C and APT spectra had other peaks indicative of two monosubstituted olefinic carbons (δ 128.27 and 116.79), five aliphatic or alicyclic methylenes (δ 20.73, 22.32, 22.38, 26.67, and 31.84), and a methyl group (δ 13.80). Further structural information was obtained from the ^1H and ^1H–^1H COSY spectra. The proton on the oxygen-bearing carbon of the lactone at δ 4.92 was coupled to a vicinal aliphatic methylene (δ 1.86 and 1.54), with two, small, long-range couplings. These two protons were in turn coupled to a four-proton multiplet at approximately δ 1.4. This multiplet was further coupled only to a methyl group at δ 0.90. This sequence of couplings identified the R-group on the oxygen-bearing carbon as a butyl group.

The remaining four carbons and six protons could be accommodated in a six-membered ring fused to the α,β-unsaturated, gamma-lactone. One of the monosubstituted, olefinic carbons (δ 128.37 and δ 116.79) had a strong coupling to an allylic methylene while the other did not. This information narrowed the possible structures to two: one with a double bond at carbons 4 and 5 and the other with a double bond at carbons 6 and 7 (Figure 3). At this point, a search of the literature revealed that our data matched the published data for sedanenolide, a compound known to occur in *A. graveolens* (Bjeldanes and Kim, 1977; Yamagishi and Kaneshima, 1977; Fischer and Gijbels, 1987).

Sedanenolide Bioassays. In semisynthetic diets, sedanenolide significantly reduced both *S. exigua* ($F = 18.65$; $df = 4, 8$; $P < 0.0001$; Figure 4) and *T. ni* ($F = 5.04$; $df = 4, 8$; $P = 0.0251$; Figure 4) larval growth. At a concentration of 100 μg/g fresh mass, the mean mass of *S. exigua* larvae reared on the sedanenolide-containing diet was reduced by 78% when compared to the control, while at 200 μg/g fresh mass, the reduction was 94% when compared to the control (Figure 4). *T. ni* were not affected as strongly by sedanenolide with a reduction in mean larval mass on the diet containing sedanenolide at 100 μg/g fresh mass of 49% when compared to the control and a reduction of 67% at 200 μg/g fresh mass (Figure 4).

Sedanenolide Concentrations and Insect Performance on Intact Plants. Concentrations of sedanenolide in the foliage of the more suitable cultivar, PI 223333, ranged from 868 to 1619 μg/g fresh mass, while in the less suitable

cultivar, PI 357333, they ranged from 1260 to 4154 μg/g fresh mass (Figure 5). Sedanenolide concentrations were significantly higher in the less suitable cultivar than in the more suitable cultivar ($F = 12.34$; $df = 1, 8$; $P = 0.0079$). Sedanenolide concentrations also differed significantly among months ($F = 29.03$; $df = 8, 18$; $P \leq 0.0001$) with the highest levels present in June, July, and August and the lowest levels present in September, December, January, and February (Figure 5). The difference in sedanenolide concentrations between cultivars varied over months (months × cultivar interaction: $F = 25.43$; $df =$

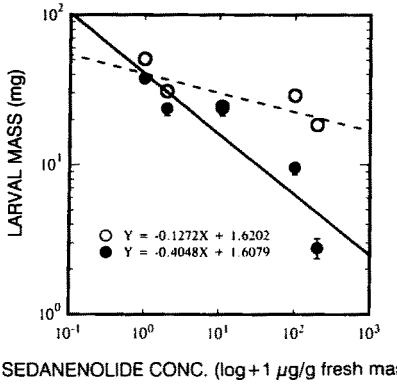

FIG. 4. Larval mass (mean and 95% confidence limits) of *S. exigua* (filled circles, solid line) and *T. ni* (hollow circles, dashed line) on artificial diets containing sedanenolide.

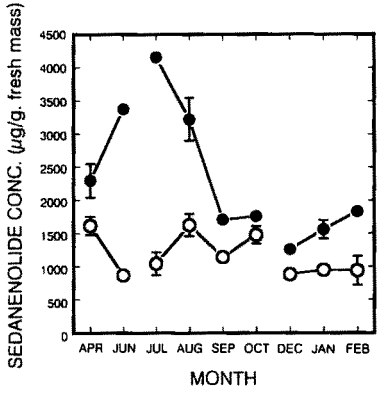

FIG. 5. Concentration of sedanenolide (mean ± SEM) in foliage of PI 223333, the cultivar more suitable for *S. exigua* and *T. ni* growth (hollow circles), and PI 357333, the cultivar less suitable for larval growth (solid circles) over time.

8, 18; $P \leq 0.0001$) with the smallest difference between cultivars occurring in October and the greatest difference occurring in July (Figure 5).

The survival and mass of *S. exigua* were significantly negatively correlated with sedanenolide concentration (survival: $r^2 = 0.5039$; $F = 34.53$; $df = 1, 34$; $P \leq 0.0001$, growth: $r^2 = 0.5913$; $F = 49.19$; $df = 1, 34$; $P \leq 0.0001$; Figure 6A and B). For *T. ni*, growth was significantly negatively correlated with sedanenolide concentration ($r^2 = 0.3161$; $F = 12.02$; $df = 1, 26$; $P = 0.0018$) but not survival ($r^2 = 0.0005$; $F = 0.01$; $df = 1, 26$; $P = 0.9080$) (Figure 6C and D).

Combined Effects of Sedanenolide and B. thuringiensis. Sedanenolide and *B. thuringiensis* both had a significant negative effect on *S. exigua* and *T. ni* mass (*S. exigua*, sedanenolide: $F = 4.63$; $df = 3, 48$; $P = 0.0063$; and *B. thuringiensis*: $F = 9.72$; $df = 3, 48$; $P \leq 0.0001$; Figure 7A. *T. ni*, sedanenolide: $F = 3.32$; $df = 3, 44$; $P = 0.0283$ and *B. thuringiensis*: $F = 187.78$; $df = 3, 44$; $P \leq 0.0001$; Figure 7B). There was no significant interaction between sedanenolide and *B. thuringiensis* for either insect species (*S. exigua*: $F = 0.22$; $df = 9, 48$; $P = 0.9901$; and *T. ni*: $F = 1.81$; $df = 9, 44$; $P = 0.0943$), indicating that the combined effect of the factors was additive.

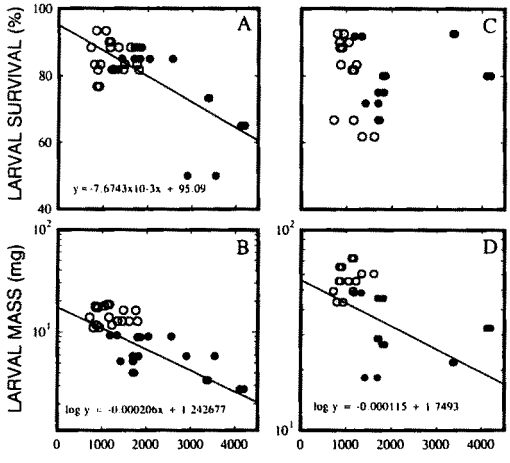

FIG. 6. Scatter plots of larval performance and the sedanenolide concentration of foliage fed to the larvae. *S. exigua* survival (A) and larval mass (B), *T. ni* (C) and larval mass (D). PI 223333 = hollow circles. PI 357333 = solid circles. Only statistically significant regressions are plotted.

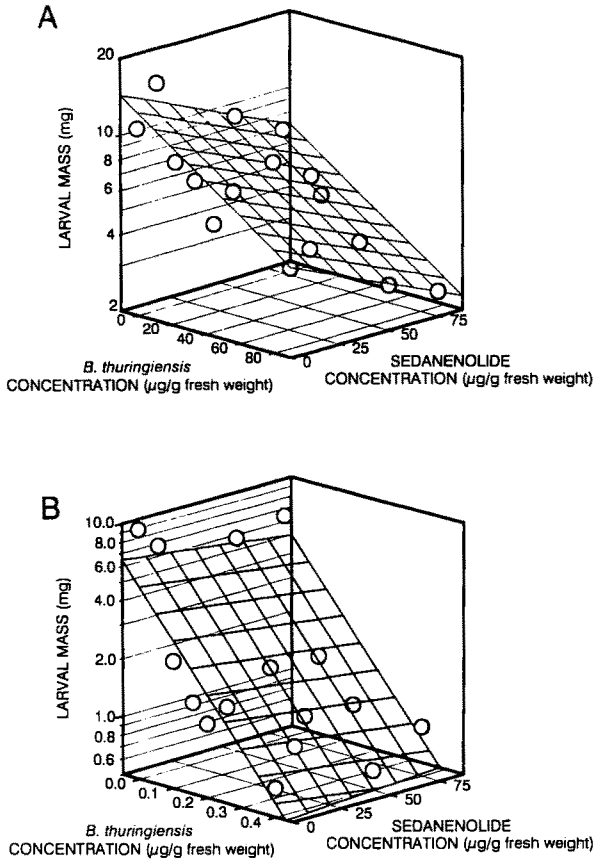

FIG. 7. *S. exigua* (A) and *T. ni* (B) larval mass (means) on artificial diets containing sedanenolide and *B. thuringiensis*. Grids in each graph represent the plane defined by the bivariate regression of sedanenolide and *B. thuringiensis* concentration on larval mass.

DISCUSSION

Most reports of sedanenolide in the literature focus on its role as a flavor and aroma component of *A. graveolens* (MacLeod and Ames, 1989; Uhlig et al., 1987), although it is reported to have a weak sedative effect in mice (Bjeldanes and Kim, 1978) and to act as a muscle relaxant in rats (Ozaki et al. 1989). Our results demonstrate for the first time that sedanenolide may have an ecological role as an allomone, protecting plants from herbivory.

Sedanenolide was first isolated by Ciamacian and Silber (1897) in their

studies of the essential oils of celery. They mistakenly believed they had isolated sedanonic anhydride, and it was only much later that the structural identity of sedanenolide was elucidated (Bjeldanes and Kim, 1977; Yamagishi and Kaneshima, 1977). Despite this confusion regarding its structural identity, there is sufficient spectral data in the literature (Bjeldanes and Kim, 1977; Yamagishi and Kaneshima, 1977; Fischer and Gijbels, 1987) to allow us to unequivocally identify the isolated compound as sedanenolide.

The correlative and causative data provide compelling evidence for the role of sedanenolide as the basis for the differences in suitability between the two host-plant genotypes. The fact that sedanenolide had a greater effect on *S. exigua* than on *T. ni* is consistent with the results from previous studies in which *T. ni* were less affected by the differences between cultivars than were *S. exigua* (Meade and Hare, 1991, 1994).

In the artificial diet studies, sedanenolide was effective in reducing larval growth at a concentration of 100 μg/g fresh weight diet; a level well below that found in the foliage of the more suitable cultivar (range 868–1619 μg/g fresh wt foliage). This result raises an important caveat with respect to the use of artificial diets in causative studies and underscores the importance of comparative data. All plant extracts and fractions were bioassayed at 0.1 gram leaf equivalents (GLE) because incorporation of the extracts and fractions at higher concentrations resulted in the suppression of larval growth and high mortality for both cultivars. When the foliar sedanenolide concentrations are adjusted to reflect the concentration at which plant extracts were bioassayed (0.1 GLE), the causative and correlative data are in close agreement.

Our data are also consistent with the hypothesis that the specific chemicals responsible for host-plant resistance mediate tritrophic interactions. In previous studies we demonstrated that the combined effect of host-plant cultivar and *B. thuringiensis* on herbivore survival and growth was additive (Meade and Hare 1993, 1994). Here, we show that the combined effect of a chemical responsible for the difference in suitability between cultivars and *B. thuringiensis* is additive, a result consistent with the aforementioned studies in planta.

The identification of a chemically based host-plant resistance to insect herbivores in *A. graveolens* has practical implications as there is an active interest in developing insect-resistant celery cultivars (Trumble and Quiros, 1988; Trumble et al., 1990). The linear furanocoumarins present in celery have been of concern because high concentrations of these compounds are potentially harmful to consumers (Ashwood-Smith et al., 1985). Although there are no reports of correlations between the linear furanocoumarin concentration of celery breeding lines and insect herbivore resistance, unsafe levels of linear furanocoumarins in celery breeding lines have been documented (Trumble et al., 1990). Our results demonstrate that the difference in suitability between the two celeriac cultivars

is not based on linear furanocoumarins because higher concentrations of these compounds were found in the more suitable cultivar.

The identification of sedanenolide as the chemical basis for host-plant resistance in this system may present a unique opportunity to plant breeders. Historical interest in sedanenolide has focused on its role as a flavor and aroma constituent, and plants with high concentrations of this compound may be more commercially desirable than plants with low concentrations. In the absence of any adverse toxicological effects, this may represent a case where breeding plants for increased resistance to insects is compatible with the goals of other breeding programs.

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CARIBBEAN FRUIT FLY, *Anastrepha suspensa* (Loew), ATTRACTION TO HOST FRUIT AND HOST KAIROMONES

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Abstract—Extracts of 22 fruits were tested for their attractancy to *Anastrepha suspensa* (Loew), the Caribbean fruit fly. Box-orange, calamondin, carambola, cattley guava, loquat, and Surinam-cherry were about equal in attractiveness to males and females. Nine synthetic chemicals, including four found

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in box-orange ripe seed, were attractive to females. Five synthetic chemicals, including two in box-orange ripe seed, were attractive to males. Farnesol, α-phellandrene, and 3-carene were highest in attractiveness to both males and females. Females were more attracted than males to 12 synthetic chemicals. These data suggest that host chemicals serve as attractants and that female and male specific attractants and traps could be developed from host kairomone data. These data also suggest that the volatilization of chemicals from water may play an important role in kairomone biology.

Key Words—Attractants, kairomone, Caribbean fruit fly, *Anastrepha suspensa*, host fruit, Diptera, Tephritidae.

INTRODUCTION

Anastrepha suspensa (Loew) was identified at Key West in 1931. *A. suspensa* was never reared from a field host, was not collected after the early 1930s, and may not have survived as a population. A new infestation was discovered in 1965 when more than 14,000 adult *A. suspensa* were trapped in Dade County (Miami). These were postulated to be a Puerto Rican strain that has many tropical and subtropical fruits as hosts (Weems, 1965). The economic importance of this fly has been reviewed (Greany and Riherd, 1993).

A. suspensa has at least 84 host fruits in 23 plant families in South Florida (Swanson and Baranowski, 1972). In an effort to attract and trap this economically important insect, research efforts have been directed at the male pheromone (Battiste et al., 1983; Chuman et al., 1988; Nation 1972, 1975, 1983, 1989, 1990; Robacker and Hart, 1987; Webb et al., 1983) and trap configurations, including color (Barros et al., 1991; Burditt, 1982; Davis et al., 1984; Greany et al., 1982; Perdomo et al., 1976; Sivinski, 1990; Witherell, 1982). An attractant program tested 1320 compounds for attraction to *A. suspensa* (Burditt and McGovern, 1979). Improved baits for traps were investigated (Sharp, 1987) and internal dye markers for trap field assessment have been developed (Sharp and Ashley, 1984).

No systematic search for kairomones that *A. suspensa* may use to orient to its hosts has been conducted. The objectives of this research were to determine the relative attractiveness of host fruit to *A. suspensa* and to isolate, identify, and rank host fruit kairomones.

METHODS AND MATERIALS

Laboratory Test Insects. A. suspensa pupae were supplied by the Florida Department of Agriculture and Consumer Services (FDACS/DPI), Division of Plant Industry, Gainesville, Florida, from the sterile male release rearing program. This colony was established in 1985 from the USDA colony (Miami,

Florida) which had been established from flies collected from a variety of wild hosts. Mass rearing began in 1987. The FDACS/DPI colony has been in culture for about 150 generations without the introduction of wild stock. Flies were shipped overnight to University of Florida, Lake Alfred, as 9-day-old pupae. Once adult emergence began about one week after arrival, pupae were transferred daily to obtain adult flies of known age.

Flies emerged in 30.54 × 30.54 × 30.54-cm (12 × 12 × 12-in.) cages with stocking access fronts (BioQuip, Inc., Gardenia, California 90248) and were sustained on a diet of sugar cubes and yeast hydrolysate (ICN Biomedicals, Costa Mesa, California 92626) and granular sugar mixture (1:3 w/w, with enough deionized water to form a paste). Glass-distilled, deionized water was supplied in the form of 1% w/v agar (Sigma Chemical Co., St. Louis, Missouri 63178). Food sources were placed on top of the cages and were covered with plastic wrap taped to the cage over the food. Flies fed ad libitum through the screen mesh. Cages were held at 23.3 ± 1.5°C and a 12:12 hr light–dark cycle.

Host Fruit Processing. Test fruits were collected by Florida Department of Agriculture and Consumer Services, Division of Plant Industry FDACS/DPI personnel. Fruit were sealed in plastic bags, tagged with date, location, and collector. Fruits were transported at ambient temperature within 48 hr to the FDACS/DPI Winter Haven, Florida, facility and were transferred within a few hours of arrival to the University of Florida, Lake Alfred, CREC. Fruit were classified as green, ripe, or rotten; assigned a number; immediately homogenized whole in a food processor; and strained through cheesecloth. Twenty milliliters of the filtrate were frozen in scintillation vials at −17°C for two to three days prior to bioassay. For bioassays, extracts were thawed, particulates were allowed to settle, and 200 μl were withdrawn from the center of the liquid with a 500-μl glass syringe.

Hexane, methanol, and water extracts for bioassays and chemical analyses were 1:10 w/v, fruit–solvent. Fruit were finely chopped on a cutting board and 1 g or more transferred into a 50-ml round-bottom glass centrifuge tube. After homogenization for 1 min in an ice water bath with a tissuemizer (Tekmar, Inc., Cincinnati, Ohio 45222), particulate matter was removed by centrifugation with a bench-top centrifuge. The supernates were removed with disposable glass pipets and were filtered through a 0.22-μm nylon 66 syringe filter attached to a 10-ml glass syringe. Extracts were stored at −17°C before analytical analyses or bioassays. No solvent was removed prior to GLC or GLC-MS analyses. Fruit extracts were tested by number. In the case of *Severinia buxifolia* (box-orange), no juice was obtained with the food processor, so initial bioassays were run by crushing the flesh and seed into a sugar cube by hand and removing the debris from the cube with a spatula. A minimum of three (usually four or more) bioassays were conducted per fruit extract.

Bioassay of Fruit Extracts. All bioassays were static air bioassays. To

remove flies for bioassay cage set up, dead flies were shaken to a rear corner of an emergence cage, and the cage was placed in a −17°C chest freezer for 6 min. Immobilized flies were transferred to 30.54 × 30.54 × 30.54-cm cages by grasping a wing with squeeze-to-close forceps. Each bioassay cage contained 50 females and 50 males. These flies were allowed to revive for 24 hr before bioassays began and were fed ad libitum before and between bioassays. Food and water were removed just prior to a bioassay and were replaced immediately afterward. Two sugar cubes to which 200 μl of test solution or water had been applied with a 500-μl syringe were placed wet side down 14 cm apart on the cage screen top. Fly visits were recorded by sex over 10 min. Fly visits included flies landing, but not feeding, plus flies landing and feeding, plus flies that walked away from a cube and returned to feed, plus flies landing near a cube and then walking to a cube. This total for each cube–lure combination was used with the totals for other replications (three or more) to calculate mean fly visits ± standard deviation (SD). Very attractive materials may have 15–20 flies feeding for the entire 10 min. Flies used for these bioassays were 1–7 days old. A maximum of two flies per sex died over the few days each cage was used for bioassays. Dead flies were not replaced with live flies nor were these deaths used in statistical analyses. Oviposition in these flies can begin at age 5 days, but is not heavy until day 8 (H.N.N., L.L.M., S.E.S., and J.A.A., unpublished).

The same two observers conducted all bioassays: one observer from approximately 8:15–9:30 AM and the second from approximately 1:15–2:30 PM. An individual cage was bioassayed only once during these periods or in some cases only once per day. Up to four fruit extracts were bioassayed during each testing period. The same four extracts were analyzed over several days to provide replication over several fly ages. Extracts were refrozen between bioassay periods.

Bioassay of Individual Chemicals. Chemical bioassays were conducted with 200 μl of a solution or an emulsion made from 10 ml glass-distilled deionized water and 10 μl of liquid chemicals or several crystals of solid chemicals. Flasks were shaken vigorously and allowed to stand for 15 min before test aliquots were removed with a 500-μl glass syringe from the center of the volumetric. Compounds bioassayed here were chosen based on a positive identification from box-orange seed or for similarity in structure to an identified compound in box-orange seed or in the cases of D-lyxose and acetic acid from probable GLC-MS identifications. Aziridines were presumptively identified in box-orange seed. Aziridines, however, are unstable and may be explosive. They do contain a nitrogen in a three-membered ring and pyridine was chosen as a test substance with a ring nitrogen. Only positively identified compounds were included in Table 4 below.

Chemical Sources. The following chemicals were obtained for attractive-

ness testing: α-pinene (99%, Aldrich, Milwaukee, Wisconsin); β-pinene (99%, Aldrich); *d*-limonene (97%, Florida Chemical Co., Lake Alfred, Florida); α-phellandrene (Aldrich, FCC); *trans*-caryophyllene (pfs Sigma Chemical Co., St. Louis, Missouri); farnesene (Bedoukian Res. Inc., Danbury, Connecticut; no purity listed); bisabolene (Givaudan, Clifton, New Jersey; no purity listed); farnesol (Aldrich, 95% isomeric mixture); (+)-cedrol (Lancaster Synthesis, Windham, New Jersey); isopimpinellin, xanthotoxin, bergapten, and psoralen (Dr. Ross Beier, USDA, College Station, Texas; 100% by HPLC); α-terpinene (Aldrich, 85%); γ-terpinene (Aldrich, 97%); 3-carene (Aldrich, 95%); glacial acetic acid (Fisher Chem. Co., Orlando, Florida); D-xylose (Sigma, pfs); 6-methyl coumarin (Aldrich, 99%); 7-methyl coumarin (Aldrich, 98%); pyridine (Sigma, 99%); scoparone (Aldrich, 98%); and scopoletin (Aldrich, 95%). Solvents were HPLC grade (Fisher Chem. Co.).

Gas–Liquid Chromatography–Mass Spectroscopy. To identify potential host-fruit kairomones, gas–liquid chromatography (GLC) was performed on a Tracor 540 gas chromatograph equipped with a 30-m × 0.53-mm-ID megabore DB-1 column (J & W Scientific, Folsom, California 95630), film thickness 1.5 μm, and a flame ionization detector. Operating conditions were: temperature program from 50 to 270°C at 3°C/min with a 5-min final hold; injector 225°C, and detector 250°C.

GLC-MS was performed on a Finnigan 4500 quadrapole GLC-MS system equipped with a 30-m × 0.25-mm DB-1 column, film thickness 0.25 μm (J & W Scientific), 50–270°C at 3°C/min, carrier He ~1.5 ml/min, 70 eV EI, injection 200°C.

GLC-MS was also performed with a Kratos MS-25 magnetic sector GLC-MS equipped with a Tecknivent Data System (NIST MS library), and a 30-m × 0.25-mm, 0.5-μm film RTX-5 column (Restek Inc., Bellefonte, Pennsylvania 16823). Operating conditions were: temperature program 40°C, 3 min hold; 7°C/min to 230°C; 5 min final hold; injector 220°C, source 220°C, GLC-MS interface 200°C, H_2 carrier 1 ml/min; and 70 eV EI, 2 kV accelerating voltage.

GLC-MS was also performed on a Perkin-Elmer 8420 capillary GLC attached to a Finnigan ion trap model 6210. The column was DB-1, 30 m × 0.25 mm, 0.25-μm film (J & W Scientific), He carrier, linear flow 25 cm/sec, injection port 180°C, 60–120°C at 10°/min, to 150°C at 6°/min; scan range 181–1800.

Statistical Analyses. The GLM procedure (SAS Institute, Inc., 1989) was used to indicate differences in attractancy. This procedure is similar to an ANOVA, but corrects for tests where every treatment is not presented at each test. An $\alpha = 0.05$ was used to indicate differences in attractancy. The LSD (least significant difference) test was used to test for differences by using comparisonwise error (the risk of making a statistical error was 5% for each com-

parison). Tukey's studentized range (HSD) test was useful in making comparisons by using experimentwise error (the risk of making a statistical error was 5% for all comparisons). The Waller-Duncan *K*-ratio *T* test was chosen over Duncan's multiple range test because Waller-Duncan includes a correction factor that minimizes making type-I errors (rejecting the null hypothesis when it is true).

RESULTS AND DISCUSSION

The fruit tested are listed in Table 1. For females, there were no significant statistical differences between responses to ripe and rotten cattley guava and ripe box-orange (water extract), ripe box-orange (crushed into cube), carambola, loquat, Surinam-cherry, plum, and green Surinam-cherry (Table 2). Ripe box-orange extract was very attractive to male flies, but there was no statistical

TABLE 1. FRUIT TESTED FOR ATTRACTANCY TO *Anastrepha suspensa*

Common name	Scientific name
1. Balsam-apple	*Momordica balsamina* L.
2. Box-orange	*Severinia buxifolia* (Poiret) Ten.
3. Carambola	*Averrhoa carambola* L.
4. Calamondin	*Citrofortunella microcarpa* (Bunge) Wijnands (= *Citrofortunella mitis*)
5. Elderberry	*Sambucus canadensis* L.
6. Fig	*Ficus carica* L.
7. Guava	*Psidium guajava* L.
8. Guava, cattley	*Psidium littorale* Raddi var. *longipes* (O. Berg.) Fosb.
9. Guava, strawberry	*Psidium littorale* Raddi var. *littorale*
10. Kumquat, oval	*Fortunella margarita* (Lour.) Swingle
11. Kumquat, sweet	either *Fortunella crassifolia* Swingle (meiwa kumquat) or *F. japonica* (Thunb.) Swingle
12. Kumquat, Robinson	?
13. Loquat	*Eriobotrya japonica* (Thunb.) Lindley
14. Mandarine, Cleopatra	*Citrus reticulata* Blanco Cleopatra
15. Minneolaquat	*Fortunella* hybrid
16. Papaya	*Carica papaya* L.
17. Peach	*Prunus persica* L.
18. Persimmon, American	*Diospyruos virginiana* L.
19. Plum	*Prunus* spp.
20. Surinam-cherry	*Eugenia uniflora* L.
21. Strawberry	*Fragaria* × *ananassa* Duchesne
22. Tangelo, Minneola	*Citrus* × *tangelo* J. Ingram and H.E. Moore

TABLE 2. FRUIT EXTRACT ATTRACTANCY TO FEMALE TO FEMALE *Anastrepha suspensa*

Fruit	*N*	Fly visits (mean ± SD)	Footnote
Cattley guava (rotten)	4	17.75 ± 2.50	[a,b]
Cattley guava (ripe)	4	17.75 ± 2.50	[a,b]
Box-orange (ripe) water extract[c]	4	16.25 ± 4.11	[a]
Carambola (ripe)[d]	4	16.00 ± 5.72	[a]
Loquat	4	14.25 ± 4.50	[a]
Box-orange (ripe) (crushed into cube)	4	14.00 ± 10.08	[a]
Surinam-cherry (green)	4	13.75 ± 6.55	[a]
Surinam-cherry (ripe)	4	13.75 ± 5.50	[a]
Plum (ripe)	4	13.25 ± 3.20	[a]
Minneolaquat (ripe)	4	12.00 ± 7.70	[a,e]
Calamondin (rotten)	8	12.00 ± 6.46	[a,e]
Box-orange (ripe) Methanol extract	4	11.75 ± 8.69	[a,e]
Fig (ripe)[d]	4	11.50 ± 4.20	[e]
Kumquat (ripe)[d]	8	11.44 ± 5.78	[a,e]
Carambola (rotten)	4	11.00 ± 2.71	[e]
Minneola (ripe)	4	11.00 ± 4.69	[e]
Peach (ripe)[d]	4	10.75 ± 5.68	[e]
Surinam-cherry (rotten)	8	10.50 ± 6.12	[a,e]
Guava (ripe)[d]	4	10.50 ± 2.38	[e]
Sweet kumquat (ripe)	8	10.00 ± 4.66	[a,e]
Box-orange (ripe) Hexane extract	4	9.75 ± 2.22	[e]
Elderberry (ripe)	6	9.50 ± 5.05	[e]
Strawberry cattley guava (ripe)	7	9.43 ± 4.39	[e]
Balsam-apple (ripe)[d]	7	9.43 ± 4.39	[e]
Strawberry (ripe)	4	8.75 ± 4.99	[e]
Persimmon (ripe)[c]	4	8.50 ± 3.32	[e]
Robinson kumquat (ripe)	4	8.25 ± 3.77	[e]
Calamondin (ripe)[d]	4	7.00 ± 4.24	[e,f]
Cattley guava (green)[d]	4	6.25 ± 1.26	[e,f]
Cleo orange (ripe)	10	5.60 ± 5.27	[e,f]
Papaya (green)[d]	4	3.50 ± 2.38	[e,f]
Water	156	5.58 ± 3.94	[e,f]

[a] Significantly more attractive than water using Dunnett's *T* tests ($\alpha = 0.05$).

[b] Significantly more attractive than all other fruit and water except ripe box-orange water extract, carambola (ripe), loquat, box-orange (ripe), Surinam-cherry (green), Surinam-cherry (ripe), and plum (ripe) by Waller-Duncan *K* ratio *T* tests ($\alpha = 0.05$).

[c] Males significantly different than females.

[d] Listed as a host by Swanson and Baranowski (1972).

[e] Significantly less attractive than cattley guava (rotten) by LSD (least significant difference) *T* tests ($\alpha = 0.05$, comparisonwise error).

[f] Significantly less attractive than cattley guava (rotten) by Tukey's studentized range (HSD) test ($\alpha = 0.05$, experimentwise error).

difference between this extract and ripe and rotten cattley guava, loquat and ripe carambola (Table 3).

Box-orange, considered a minor host, probably because of the small size of its large-seeded fruit, was very attractive to males and to females when crushed into the sugar cube (data were presented) and as a water extract, and it was moderately attractive as a methanol extract (Tables 2 and 3). Therefore, chemical analyses concentrated on the ripe seed of box-orange with GLC and GLC-MS, and the 19 compounds listed in Table 4 were identified. GLC comparison of methanol extracts of unattractive early box-orange seeds with methanol extracts of attractive ripe seeds revealed the absence of compounds from the injection point to a retention time of approximately 37 min in green, unattractive seeds. Green seed did contain compounds 15–19 (Table 4). GLC comparison of methanol extracts of box-orange seed versus methanol extracts of flesh and skin revealed a very different profile for the moderately attractive flesh and skin (data not presented). Further chemical analyses of the unattractive flesh and skin were not undertaken.

Of the eight more attractive chemicals, bisabolene, 3-carene, farnesol, α-phellandrene, and β-pinene were attractive to males and females. However, females were more attracted than males to 12 chemicals, including scopoletin (Table 5). Psoralen was moderately attractive to males (Tables 5 and 6). Psoralen and other furanocoumarins may be found on the leaf surface of plants exposed to UV radiation (Zobel and Brown, 1988, 1993). (+)-Cedrol, 6-methyl coumarin, and 7-methyl coumarin were comparatively attractive to females but not to males (Tables 5 and 6).

In our study, farnesene was a deterrent and proved to be a mixture of at least four isomers by GLC. β-Bisabolene appears to be a main component of male *A. suspensa* and also *A. ludens* pheromones (Nation, 1990) and was identified in box-orange ripe seed (Table 4). However, bisabolene, obtained from Givaudan Corp. (Clifton, New Jersey 07014; CAS #495-62-5), was a mixture of at least 20 compounds (our GLC-MS analysis), none of which seemed to be β-bisabolene. Among the 20 compounds in the mixture were (*E*,*E*)-α-farnesene, α-bergamotene, and possibly valencene. Each of the compounds in the Givaudan mixture had a mass spectrum characteristic of a sesquiterpene. This commercial mixture was attractive to males and females (Tables 5 and 6).

General methods for kairomone research have been adopted from pheromone research (Heath and Manukian, 1992). Pheromones are commonly trapped on an absorbent, desorbed with a solvent or occasionally thermally desorbed, and analyzed, identified, and bioassayed (Heath et al., 1992). Absorbent trapping tends to collect compounds of high volatility; that is, low boiling compounds, and airflow and collection time are important factors for reliability of data (Surburg et al., 1993). The vacuum headspace method appears to be reproducible and produces essences closer to nature than absorbent trapping (Surburg

TABLE 3. FRUIT EXTRACT ATTRACTANCY TO MALE *Anastrepha suspensa*

Fruit	N	Fly visits Mean ± SD	Footnote
Box-orange (ripe) water extract	4	19.50 ± 3.32	a,b
Cattley guava (rotten)	4	18.75 ± 5.85	a,b
Cattley guava (ripe)	4	18.75 ± 5.85	a,b
Loquat	4	16.75 ± 5.56	a
Carambola (ripe)	4	15.50 ± 7.94	a
Carambola (rotten)	4	13.25 ± 1.26	a,c
Calamondin (ripe)	4	13.00 ± 10.49	a,c
Calamondin (rotten)	4	12.75 ± 4.50	a,c
Strawberry cattley guava (ripe)	7	12.71 ± 3.86	a,c
Surinam-cherry (rotten)	4	12.38 ± 2.26	a,c
Surinam-cherry (green)	4	12.25 ± 5.57	a,c
Box-orange (ripe) methanol extract	4	11.75 ± 6.02	a,c
Sweet kumquat (ripe)	8	11.38 ± 3.74	a,c
Elderberry (ripe)	6	11.17 ± 7.28	a,c
Minneola (ripe)	3	7.75 ± 6.02	c
Kumquat (ripe)	6	7.50 ± 5.63	c,d
Robinson kumquat (ripe)	4	9.75 ± 5.50	c,d
Fig (ripe)	4	9.75 ± 3.95	c,d
Plum (ripe)	3	7.25 ± 4.92	c
Box-orange (ripe) (crushed into cube)	4	9.33 ± 5.16	c,d
Balsam-apple	7	9.29 ± 4.79	c,d
Minneolaquat (ripe)	4	9.25 ± 4.43	c,d
Strawberry (ripe)	4	8.50 ± 7.55	c,d
Peach (ripe)	4	8.50 ± 3.70	c,d
Guava (ripe)	3	6.25 ± 5.62	c,d
Surinam-cherry (ripe)	4	7.75 ± 2.06	c,d
Box-orange (ripe) hexane extract	4	7.50 ± 4.80	c,d
Cattley guava (green)	4	7.50 ± 4.80	c,d
Cleo orange (ripe)	10	7.40 ± 5.64	c,d
Persimmon (ripe)	4	6.25 ± 3.30	c,d
Papaya (green)	4	3.50 ± 1.91	c,d
Water	151	5.46 ± 4.22	c,d

[a] Significantly more attractive than water using Dunnett's T tests ($\alpha = 0.05$).
[b] Significantly more attractive than all other fruit and water except loquat and carambola (ripe) by Waller-Duncan K-ratio T tests ($\alpha = 0.05$).
[c] Significantly less attractive than box-orange (ripe) water extract by LSD (least significant difference) T tests ($\alpha = 0.05$, comparisonwise error).
[d] Significantly less attractive than cattley guava (rotten) by Tukey's Studentized Range (HSD) test ($\alpha = 0.05$, experimentwise error).

TABLE 4. COMPOUNDS IDENTIFIED FROM BOX-ORANGE RIPE SEED METHANOL EXTRACT, IN GLC–MS ELUTION ORDER

Compound	Mol wt	R_t (min)	Relative %[a]	CAS No.
1. α-Pinene	136	6:37	0.80	80-56-8
2. Linalool acetate	196	7:51	9.10	115-95-7
3. D-Limonene	136	9:42	5.78	138-86-3(dl)
4. α-Phellandrene	136	10:49	1.06	99-83-2
5. Caryophyllene	204	27:24	13.58	87-44-5
6. Longicyclene	204	27:40	25.91	1137-12-8
7. (*Z*, *E*)α-Farnesene	204	28:17	1.61	26560-14-5
8. α-Caryophyllene	204	28:45	4.02	6753-98-6
9. (*Z*)-β-Farnesene	204	29:18	7.7	28973-97-9
10. Elixene	204	30:35	3.55	3242-08-8
11. β-Bisabolene	204	31:23	2.49	495-61-4
12. (+)-Cedrol	222	32:07	11.20	77-53-2
13. Bicyclo[3.3.1]nonan-9-ol	140	36:58	11.05	15598-80-8
14. (*Z*, *E*)-Farnesol	222	37:34	2.15	3790-71-4
15. Seselin	228	48:34	[b]	523-59-1
16. Isobergapten	216	52:01	[b]	482-48-4
17. Isopimpinellin	246	57:00	[b]	482-27-9
18. 7H-furo[3,2-G][1] benzopyran-7-one,9-[(4-hydroxy-3-methyl-2-butenyl)ol]	286	60:01	[b]	65853-14-7
19. Aurapten	298	68:01	[b]	495-02-5

[a]Based on GLC–FID total area % of identified compounds.
[b]Not used in relative % calculation because in unattractive area of chromatogram.

et al., 1993; Joulain, 1993). Organic solvent extraction often requires the removal of solvent prior to bioassay, and systems for solvent removal, such as nitrogen and rotary evaporators, do not discriminate so as to avoid loss of some compounds, especially very volatile ones. Any method for extracting or collecting attractant compounds may produce artifacts, including the methods we used here (Surburg et al., 1993). Pheromonal compounds are usually lipids or lipophilic substances, while some host kairomonal compounds are likely to be much more polar and water soluble. Hence, we chose juice and pulp processing to make extracts that would include polar compounds.

Water delivery of a kairomone is supported by physical data and by *A. suspensa* field and laboratory behavioral data. Dew falls in Florida every night except during occasional cold fronts and rare dry periods. For example, from June 16, 1992, to September 30, 1992, dew hours at the University of Florida, Lake Alfred, averaged 8.7 hr. During this period, 56 days had dew for >8 hr. There were no days without dew. The longest dew day was 19.6 hr and the

TABLE 5. CHEMICAL WATER SOLUTION ATTRACTANCY TO FEMALE *Anastrepha suspensa*

Farnesol[a,b]	6	40.50 ± 15.41	c,d
α-Phellandrene[a,b]	6	37.67 ± 9.54	c,d
3-Carene[b]	6	31.50 ± 9.22	c,e
Bisabolene[a,b]	6	29.33 ± 10.39	c,e
(+)-Cedrol[a,b]	6	25.67 ± 8.24	c,e,f
7-Methyl coumarin[b]	6	24.83 ± 3.66	c,e,f
α-Terpinene[b]	6	23.50 ± 13.07	c,e,f
6-Methyl coumarin[b]	6	23.00 ± 4.10	c,e,f
β-Pinene[b]	6	21.67 ± 14.76	c,e,f
α-Pinene[a,b]	6	19.17 ± 3.13	e,f
D-Limonene[a,b]	6	18.83 ± 5.91	e,f
Scopoletin[b]	6	16.67 ± 5.13	e,f
Glacial acetic acid	3	12.67 ± 4.04	e,f
Isopimpinellin[a]	6	12.67 ± 12.91	e,f
Psoralen	12	11.75 ± 4.67	e,f
Xanthotoxin	6	11.00 ± 2.53	e,f
Scoparone	6	7.83 ± 5.98	e,f
Bergapten	6	6.00 ± 3.58	e,f
Farnesene[a]	3	4.67 ± 1.53	e,f
D-Lyxose	3	3.00 ± 2.00	e,f
γ-Terpinene	6	2.50 ± 2.95	e,f,g
Pyridine	3	1.33 ± 1.53	e,f,g
Water[b]	126	12.35 ± 9.59	e,f

[a] Identified from box-orange.
[b] Females significantly different from males (Table 6).
[c] Significantly more attractive than water by Dunnett's *T* tests ($\alpha = 0.05$).
[d] Significantly more attractive than all other compounds and water by Waller-Duncan *K*-ratio *T* tests ($\alpha = 0.05$).
[e] Significantly less attractive than farnesol by LSD (least significant difference) *T* tests ($\alpha = 0.05$, comparisonwise error).
[f] Significantly less attractive than farnesol by Tukey's studentized range (HSD) test ($\alpha = 0.05$, experimentwise error).
[g] Significantly less attractive than water by Dunnett's *T* tests ($\alpha = 0.05$).

shortest 0.3 hr. From October 1, 1992, to June 15, 1993, dew hours averaged 7.6. There were 127 days with >8 hr dew. The longest dew day was 24 hr and the shortest 0 hr (Robert Sorrell, University of Florida, Lake Alfred, NOAA weather data, personal communication). Since dew falls every night around 2:00 AM, leaves and fruit are wet until about 10:00 AM for most days of the year in Florida.

Burk (1983) made field observations on the behavioral ecology of *A. suspensa* on guava (*Psidium guajava* L) in July through October and Surinam-cherry (*Eugenia uniflora* L) in May and June in Florida. Observations were

TABLE 6. CHEMICAL WATER SOLUTION ATTRACTANCY TO MALE *Anastrepha suspensa*

Compound	*N*	Fly visits (mean ± SD)	Footnote
Farnesol[a]	6	17.50 ± 7.23	b,c
α-Phellandrene[a]	6	15.50 ± 3.62	b,c
3-Carene	6	11.67 ± 5.24	b,d
α-Terpinene	6	11.33 ± 6.28	b,d
Bisabolene[a]	6	10.50 ± 5.21	d
Glacial acetic acid	3	10.33 ± 2.52	d
Psoralen	12	10.17 ± 3.30	b,d,e
β-Pinene	6	9.83 ± 5.81	d
Isopimpinellin[a]	6	9.67 ± 5.99	d
Scoparone	6	9.67 ± 7.61	d
Xanthotoxin	6	8.83 ± 5.85	d,e
(+)-Cedrol[a]	6	8.67 ± 3.14	d,e
D-Limonene[a]	6	7.83 ± 3.49	d,e
Bergapten	6	7.00 ± 4.43	d,e
6-Methyl coumarin	6	7.00 ± 3.64	d,e
α-Pinene[a]	6	5.50 ± 1.76	d,e
Farnesene[a]	3	5.33 ± 3.21	d,e
7-Methyl coumarin	6	5.17 ± 4.67	d,e
Scopoletin	6	4.67 ± 2.80	d,e
D-Lyxose	3	3.67 ± 2.08	d,e
γ-Terpinene	3	3.00 ± 2.00	d,e
Pyridine	3	1.33 ± 1.53	d,e
Water	122	6.21 ± 3.82	d,e

[a] Identified from box-orange.
[b] Significantly more attractive than water by Dunnett's *T* tests ($\alpha = 0.05$).
[c] Significantly more attractive than all other compounds and water except 3-carene and α-terpinene by Waller-Duncan *K*-ratio *T* tests ($\alpha = 0.05$).
[d] Significantly less attractive than farnesol by LSD (least significant difference) *T* tests ($\alpha = 0.05$, comparisonwise error).
[e] Significantly less attractive than farnesol by Tukey's studentized range (HSD) test ($\alpha = 0.05$, experimentwise error).

made every hour from 7:00 AM to 7:00 PM EDT. Data on the two hosts were indistinguishable and were combined. Males (feeding and courting) and females (feeding and ovipositing) were active on fruit until about 10:00 AM when they shifted to leaves. This shift was delayed until 11:00 AM or 12:00 PM on cool or cloudy mornings. After 10:00 AM, most flies were on the underside of leaves as single flies. At 3:00 PM, males became active with mating behavior and most mating pairs were observed after 4:00 PM. Male puffing and calling reached a peak between 5:00 PM and 6:00 PM. In Burk's observations, only two attempted copulations were observed on fruit (Burk, 1983). Burk correlated these

behaviors with increasing temperature and sunlight levels. Temperature and sunlight are also confounded with the presence or absence of dew. The shift of *A. suspensa* from fruit to leaves in the field occurs about 10:00 AM when the dew disappears. Thus, the feeding behavior of *A. suspensa* is possibly determined by chemicals evaporating from and solubilized in water.

Water is important in the volatilization of DDT and other pesticides from soil (Woodwell et al., 1971; Spencer and Cliath, 1973), gas scavenging for phenol in air (Leuenberger et al., 1985), and pesticides may be enriched in fog droplets (Glotfelty et al., 1987). The rate of volatilization of chemicals "insoluble" and compounds "soluble" in water can be rapid from water, even for compounds with a vapor pressure of less than 10^{-5} mbar at 25°C (Jordan et al., 1970; Mackay and Wolkoff, 1973; Mackay and Leinonen, 1975; Mackay et al., 1979; Kilzer et al., 1979). The rates of volatilization and half-lives of compounds evaporating from water are not temperature-dependent even for compounds with a fairly high vapor pressure such as benzene (95.2 mm Hg at 25°C; compare with DDT at 1×10^{-7} mm Hg at 25°C) (Mackay and Leinonen, 1975). Compounds not normally considered water soluble, such as monoterpenes, may be biologically active at levels that do indeed exist in aqueous extracts (Weidenhamer et al., 1993). Under Florida's dew conditions, *A. suspensa* must face kairomone mixtures evaporating from water, mixtures that relate most probably to water solubility, nonideal solutions, and diffusion processes (Mackay and Leinonen, 1975; Glotfelty et al., 1979) and odor plume behavior (Murlis et al., 1992).

Important factors in the search for kairomones from plants are hour of the day for collection, climate, environment (sun, shade, dew, etc.), physiologic status: "intact vs. picked," "living vs. dead" (Joulain, 1993), and insect field behavior. The collection of volatiles from dry fruit (or other plant parts) may miss attractive compounds that volatilize from water. In addition, the release rates and relative gaseous concentrations are likely to be different for wet fruit and leaves. Consequently, it seems reasonable that testing compounds in water "solutions" would provide gaseous kairomone concentrations closer to biological reality, and the limitation of gaseous concentration by water solubility has the potential of reducing the complexity and number of concentrations tested. In at least one report, male pheromone response was affected by humidity (Royer and McNeil, 1993). We suggest that dew plays an important role in kairomone response for animals active in the morning and perhaps also for animals that feed at night, such as moths and bats (Fleming, 1993).

Bioassays used in this study did not discriminate between an arrestant and an attractant. Flies that flew to the sugar cube/test chemical were attracted. Flies that stayed and fed were arrested. We only used arrival data here, i.e., attractant data. In any case, the definition of kairomone includes attractants, arrestants, and excitants (Metcalf and Metcalf, 1992). Furthermore, tephritid flies may form

leks and are attracted to other flies of the same species. Once a fly or several flies arrive at an attractant, that substance may appear more attractive to other flies than another choice. Our experimental design did not eliminate this possibility. Mating and pheromone release by males may also skew results. Males at 7 days of age are capable of producing substantial quantities of pheromone (Nation, 1990). Mating was observed occasionally in our bioassays. Typically, a male would land on a feeding female, fall to the bottom of the cage with the female, and attempt to mate. However, the behavior of these flies at our food sources was aggressive, particularly with the better attractants. Flies forced their way to the cube by landing on other flies or landed, walked into feeding flies, and forced one or two flies off the food source.

A similar approach to kairomone discovery has been taken by Robacker et al. (1990a,b, 1992, 1993) for Mexican fruit fly, *A. ludens*, and a similar host kairomone approach has been suggested by Heath et al. (1993) for lure development for *A. suspensa*. Our experiments differ from those of Robacker in that flies in our work were fed until just before bioassay while Robacker starved his flies for 48 hr before bioassay.

Our data do suggest that a female/male lure could best be developed from compounds found in cattley guava, loquat, box-orange, and carambola. Male-specific lures might be developed from compounds found in box-orange. Synergism or potentiation between the individual compounds we tested here will probably be important in this development (Byers, 1992, and references therein). Our data generally support the supposition that host chemicals serve as kairomones and attractants for *A. suspensa*. The concept of water delivery has important implications for kairomone research and for the formulation of lures and baits.

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Journal of Chemical Ecology, Vol. 20, No. 3, 1994

FEMALE SEX PHEROMONE COMPONENTS OF JASMINE MOTH *Palpita unionalis* (LEPIDOPTERA: PYRALIDAE)

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Abstract—(*E*)-11-Hexadecenal and (*E*)-11-hexadecen-1-yl acetate were found in abdomen tip extracts from females of the jasmine moth *Palpita unionalis* (Hübn). The identification was based on capillary GC analyses, mass spectrometry, and laboratory and field tests. In laboratory bioassays, both components elicited a low level of upwind flight by males. The two components when tested separately in the field were inactive, but the blend of the two components at a ratio of (3:7) was highly attractive to males. Traps baited with 1 mg of the two-component blend were competitive to traps baited with five virgin females. The addition of *Z* isomers components reduced male capture.

Key Words—Sex pheromone, (*E*)-11-hexadecenal, (*E*)-11-hexadecen-1-yl acetate, *Palpita unionalis*, Lepidoptera, Pyralidae, Pyrustinae, *Anisodes* sp., Geometridae, jasmine moth.

INTRODUCTION

The jasmine moth, *Palpita unionalis* (Hübn) (Lepidoptera: Pyralidae), is present throughout the Mediterranean region, Asia minor, and North Africa. It is an important pest of *Jasminun* sp., *Ligustrum* sp., *Olea europea*, and *Philirea media*. Larvae of this moth first attack young shoots and leaves. In heavy infestations, larvae attack the olive fruit, especially of table varieties, making them unacceptable to the commercial market (Balashowsky, 1972).

Relatively little information is available on the biology, ecology, and

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behavior of this pest. Preliminary studies in our laboratory indicated that the virgin females produce a sex attractant to attract males.

We report here the chemical identification of two components of the *P. unionalis* female-produced sex pheromone and provide data on behavior and field attractiveness.

METHODS AND MATERIALS

Insects. The insects used in this study were collected as larvae from infested olive trees and reared in the laboratory on *Ligustrum ovalifolium* (L) leaves at 25 ± 2°C, 60 ± 5% relative humidity, and 16:8 hr light–dark (Vasilaina-Alexopoulou and Santorini, 1973).

Moths were sexed three to four days after pupation. Emerged moths were transferred daily to 20-cm^3 Plexiglas cages in separate chambers and kept under the conditions mentioned above. Adults were provided with a 10% sucrose solution.

Pheromone Collection, Purification. The pheromone was collected from 3 to 4-day-old virgin females exhibiting calling behavior. The peak of calling occurred 6 hr after lights off. The abdomen tips with the pheromone gland from 800 females were excised and extracted in methylene chloride for 30 min. The concentrated crude extract was fractionated by preparative GC, on a 5% OV-101 column, on Chromosorb G/HP 80–100 mesh, using the same instrumentation and gas flows described by Mazomenos (1989).

The active fractions collected from the OV-101 column were analyzed on a capillary gas chromatograph. The fractions were injected on DB-5 30-m × 0.32-mm-ID and DB-WAX 30-m × 0.32-mm-ID fused silica columns (J & W Scientific, Rancho Cordoba, California 95630). The column temperature program for DB-5 was 50–180°C at 15°C/min, held for 10 min, and then programmed up to 250°C at 25°C/min; for DB-Wax the temperature program was 50–190°C at 8°C/min. The injector temperature was set at 200°C and the detector temperature at 260°C. Helium was the carrier gas; the column flow rate was adjusted at 2 ml/min, and the make up flow rate at 30 ml/min. Sample aliquots (2 μl) of each active fraction were injected, and the retention times of the components were compared to those of authentic standards on the two columns.

GC-MS Analysis. The mass-spectral analyses of the active components were performed using a GC-MS VG-Trio-1000 (Manchester M23 9BE, U.K.) spectrometer system in the electron impact (EI) mode equipped with a DB-5 30-m × 0.32-mm-ID column (J & W, Scientific), held at 50°C for 1 min, then programmed at 25°/min to 250°C.

Chemicals. Synthetic (*Z*)-11-hexadecen-1-yl [(*Z*)-11-16:Ac], (*Z*)-11-

hexadecenal [(*Z*)-11-16:Ald], hexadecanyl acetate (16:Ac), and hexadecanal (16:Ald), were obtained from Sigma Chemical Co. (St. Louis, Missouri); (*E*)-11-Hexadecen-1-yl acetate [(*E*)-11-16:Ac], (*E*)-11-hexadecenal [(*E*)-11-16:Ald], and (*E*)-11-hexadecenol [(*E*)-11-16:OH] were kindly provided by Dr. J.H. Tumlinson, (Insect Attractants Behavior and Basic Biology Laboratory, Gainesville, Florida). The chemicals were found to be 96–98% pure when analyzed by capillary gas chromatography (DB-5 and DB Wax 30-m × 0.32-mm-ID columns).

Laboratory Bioassays. Bioassays were performed in a screen cage (Mazomenos, 1989); 50–80, 2 to 5-day-old males were present in the cage during the bioassay. The bioassays commenced 6 hr after lights off under a uniform low level of red light. Samples of the crude extract, the fractions obtained during the purification procedure, and synthetic components were tested. Males exhibiting upwind flight and landing on the filter paper was the response criterion.

Field Tests. Preliminary field experiments indicated that Delta traps (AgriSence-BCS Ltd Treforest, Pontypridd CF37 5SU, U.K.) and funnel-type traps (Phytophyl, Averof 16, Athens, Greece) baited with 1 mg of the two pheromone components were equally attractive to *P. unionalis* males. Two field experiments were conducted during 1991 in two olive groves located at Papagou (I) and Markopoulo, Attikis (II) near Athens. For experiment I in the olive grove at Papagou, Delta traps were used. The traps were baited either with rubber septa (A.H. Thomas Co., Philadelphia, catalog No. J^{12} 1780) loaded with 1 mg of each pheromone component and blends of the two components and their geometric isomers, or with five virgin females 1–3 days old, placed in a small screen cage 4 × 2.5 cm. Females were provided with 10% sucrose solution and were renewed every seven days. Three traps per treatment were hung from July 3 to July 30, 1991, as a complete randomized block design. The traps were suspended 50 m apart, on olive trees outside the tree canopy, approximately 2.5 m from the ground.

For experiment II, conducted in the olive grove at Markopoulo, Attikis during July 25–September 5, 1991, funnel traps were used. The traps were baited with different doses of the two pheromone components blend. Three replicates were used for each treatment in a complete randomized block design. A slow-release formulation of DVP (Vapona) was used as killing agent.

Traps were serviced once a week. Weekly trap captures were subjected to log $(x + 1)$ transformation, and differences between mean capture were tested by Duncan's multiple-range test.

RESULTS AND DISCUSSION

Pheromone Chemistry. Laboratory bioassays indicated that the activity was found in the fractions that eluted between 16 and 20 min (fraction 5) and 24 and 28 min (fraction 7) after injection. The capillary GC analyses showed that

in both polar and nonpolar columns the retention times of the major peaks corresponded to those of synthetic monounsaturated 16-carbon aldehydes and acetates, respectively. The ratio of the two components in the crude extract was found to be 3:7.

The mass spectra of the major components of fraction 5 and fraction 7 indicated a C_{16} monounsaturated aldehyde with diagnostic peaks at *m/z* 238 (relative intensity: 5%), *m/z* 220 (8.1%), and *m/z* 55 (100%) and a C_{16} monounsaturated acetate with characteristic peaks of *m/z* 282 (relative intensity 3%), *m/z* 222 (6%), and *m/z* 43 (100%), respectively. The double-bond geometry and position could not be deduced from the mass spectra, but the spectra were identical to those of synthetic (*E*)-11-16:Ald and (*E*)-11-16:Ac. These assignments were supported by laboratory assays and field trials.

Laboratory Bioassays. Ovipositor extracts elicited to males the highest response (39.8%). The response elicited by the two active fractions collected from the preparative GC was significantly lower compared to that elicited by the ovipositor extracts (21.2% for fraction 5, and 11.4% for fraction 7). The activity increased when the two fractions were combined (32.4%). The same type of response was observed when the synthetic (*E*)-11-16:Ald and (*E*)-11-16:Ac were tested individually. The synthetic blend with a component ratio of 3:7 (*E*)-11-16:Ald to (*E*)-11-16:Ac, elicited to males the same pattern (upwind flight and landing on the filter paper) and level of response as that of 3 FE ovipositor extracts (39.2%).

Field Tests. The results of the field trapping experiments, the effects of each pheromone component and the effect of their blend used on male captures are presented in Table 1. Traps baited either with 1 mg of (*E*)-11-16:Ald or with 1 mg of (*E*)-11-16:Ac did not catch males. Males were captured in traps, when the two components were combined at the ratio of 3:7. These results indicated that the two pheromone components act synergistically for male attraction under field conditions. The number of males captured in the traps baited with two-component blend was not significantly different than the number of males captured in the traps baited with the live females.

Blends of the two pheromone components with each of the *Z* isomers reduced male captures, while combinations of each component with its *Z* isomer proved to be inactive. Reduction of males approaching the pheromone traps with the addition of the geometric isomers is very common for many lepidopteran species (Tumlinson, 1990). The addition of 100 μg (*E*)-11-16:OH or 16:Ald to the two-component blend reduced its attraction (Table 1). The addition of 100 μg of 16:Ac did not affect the male captures.

The dose–response relationship of males to the pheromone traps revealed that traps baited with higher concentration of the two-component blend proved to be more effective in male captures than traps baited with lower concentrations (Figure 1).

TABLE 1. CAPTURES OF *P. unionalis* MALES IN TRAPS BAITED WITH FIVE 1- TO 3-DAY-OLD VIRGIN FEMALES AND DIFFERENT COMBINATIONS OF SYNTHETIC SEX PHEROMONE COMPONENTS, GEOMETRIC ISOMERS, AND RELATED CHEMICALS; PAPAGOU, ATTIKIS GREECE, JULY 5–30, 1991

Components (μg)							Males captured
*E*11-16:Ac	*E*11-16:Ald	*E*11-16:OH	*Z*11-16:Ac	*Z*11-16:Ald	16:Ac	16:Ald	(Means[a]/replicate)
1000							0
	1000						0
700	300						14.1a
700	300	100					4.6b
700	300		100				1.4c
700	300			100			1.7c
1000			1000				0
	1000			1000			0
700	300				100		12.5a
700	300					100	6.4b
5 virgin females							15.7a
Blank							0

[a] Means followed with the same letter are not significantly different according to Duncan's multiple-range test.

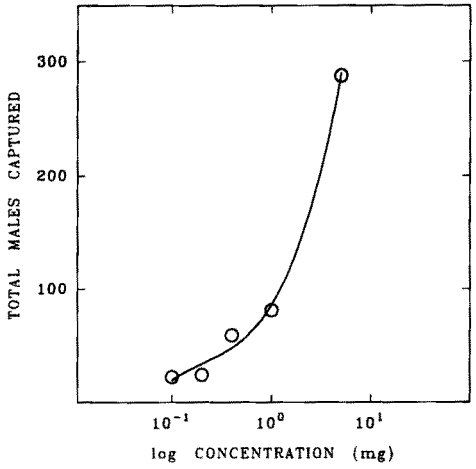

FIG. 1. Total number of *P. unionalis* males captured in traps baited with different concentrations of the female sex pheromone blend. Curve drawn by polynomial interpolation.

(Z)-11-16:Ald and (Z)-11-16:Ac have been frequently identified as pheromone components for many Lepidopteran species (Inscoe et al., 1990). However, very few species have been reported to use (*E*)-11-16:Ald and (*E*)-11-16:Ac. In the subfamily of Pyraustinae, there are several species presently known to use either (*E*)-11-16:Ald or (*E*)-11-16:Ac isomers. *Sceliodes cordalis* (Doubleday), *Mnesistema flavidalis* (Clearwater et al., 1986), *Leucinodes orbonalis* (Guenne) (Zhu, 1987), and *Diasemia litterata* (Ando, 1977) are attracted to (*E*)-11-16:Ac. The closely related species *Diaphania hyalinata* (Linne) and *D. nitidalis* (Stoll) use a blend of C_{16} aldehydes and alcohols in which (*E*)-11-16:Ald is an essential component for male attraction (Raina et al., 1986; Klun et al., 1986).

Traps baited with the *P. unionalis* sex pheromone also captured low numbers of *Anisodes* sp. Geometridae males. Although *Anisodes* sp. males were captured in traps placed in the olive orchards this species is not considered as an olive pest.

The results presented here support the identification of (*E*)-11-16:Ald and (*E*)-11-16:Ac as the main sex pheromone components for *P. unionalis*. When these chemicals are properly formulated, they will be useful for monitoring, as a component in an integrated system for the control of this pest.

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Journal of Chemical Ecology, Vol. 20, No. 3, 1994

TASTING GREEN LEAF VOLATILES BY LARVAE AND ADULTS OF COLORADO POTATO BEETLE, *Leptinotarsa decemlineata*

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Abstract—Larvae and adults of the Colorado potato beetle, *Leptinotarsa decemlineata* (Say), are shown to have galeal gustatory cells that are highly sensitive to distillate of potato leaf extracts, (*E*)-2-hexen-1-ol, (*E*)-2-hexenal, and other saturated and unsaturated six-carbon alcohols. In larvae and adults, the sensory response patterns elicited by leaf homogenate, leaf distillate and a mixture of these two extracts differ in subtle ways. Beetle larvae feed most readily on Millipore disks treated with leaf homogenate and the mixture, but they did not feed on disks treated with leaf distillate. The differences in behavioral response and sensory input are used to derive a potential gustatory code that may stimulate different levels of feeding. This code may be disrupted by compounds present in nonhost leaves, thus leading to reduced feeding. Possible interactions of sapid leaf volatiles, amino acids, sugars, and potentially deterrent plant compounds are discussed.

Key Words—Contact chemoreception, electrophysiology, taste, leaf alcohols, galeal sensilla, feeding behavior, host selection, gustatory coding, Colorado potato beetle, *Leptinotarsa decemlineata*, Coleoptera, Chrysomelidae.

INTRODUCTION

Mouthpart sensilla on the galea of both the larva and adult stages of the Colorado potato beetle (CPB), *Leptinotarsa decemlineata* (Say), respond to leaf homogenates of various plants, as well as to L-alanine, gamma-amino butyric acid (GABA) and sucrose (see references in Mitchell, 1988, 1993). All but one of

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the 12 galeal sensilla of adult CPB have four chemosensitive cells (Sen and Mitchell, 1987). The response of each adult galeal sensillum to potato (*Solanum tuberosum*) leaf homogenate is primarily due to the activity of a single sensory cell, despite the complexity of the stimulus (Mitchell et al., 1990). Homogenates of host plants other than potato also stimulated a simple, primarily single-cell response in *L. decemlineata* and in *L. texana* (Haley Sperling and Mitchell, 1991). Responses of this system to leaf homogenates of nonhost plants showed that these complex mixtures stimulated several cells in each sensillum of *L. decemlineata* (Mitchell et al., 1990). These authors argued that the single-cell galeal response to host leaf homogenate represents a chemosensory labeled line associated with initiation of feeding and perhaps host-recognition in these beetles. The multicell, highly variable responses obtained by stimulating with nonhost leaf homogenates were thought to lead to less feeding and ultimately to plant rejection (see also Haley Sperling and Mitchell, 1991). Although additional sensilla are undoubtedly involved in the regulation of feeding and plant recognition in these beetles, the above correlation of plant selection behavior and galeal sensory input encouraged further study.

The electrophysiological response of the galeal sensilla to potato leaf homogenate is highly reproducible and easily obtained. It can thus be used as a sensory bioassay to study the effects of extracts of leaf homogenates. Unpublished studies by us using organic solvents of different polarity to obtain extracts of potato leaf homogenate did not yield any fractions with activity similar to the original homogenate. Apparently, the drying steps necessary in this type of extraction procedure destroyed most of the activity. Freshly prepared and subsequently dried leaf homogenate, reconstituted with distilled water, also showed substantially reduced activity. These observations led to the present study, wherein vacuum distillates of leaf homogenates were found to be highly stimulatory to the galeal gustatory cells. The basis of this response to the vacuum distillate and the effectiveness of leaf distillate as a feeding stimulus are explored.

METHODS AND MATERIALS

Insects and Plants. L. decemlineata larvae and adults were obtained from a continuous culture of animals collected near Edmonton and reared in the laboratory on *S. tuberosum* plants grown in a greenhouse. Insects were maintained at 23°C on a 16:8 hr light–dark photoperiod using a combination of cool white and wide-spectrum Grolux fluorescent bulbs (GTE Sylvania Canada Ltd.). Further details on rearing procedures can be found in Haley Sperling and Mitchell (1991).

Potato shoots (*S. tuberosum* cv. Norland) were used for rearing insects and for preparing homogenates to examine feeding and electrophysiological

responses. Plants were grown from tubers in soil in the greenhouse under ambient light and humidity. High-pressure sodium lamps were used to maintain a 16-hr photoperiod during the winter. Temperature ranged from 18 to 28°C throughout the year and a general purpose fertilizer (20:20:20 NPK) was applied weekly. Insect pests were controlled by spraying with a pesticide soap (Safer Agro-Chem Ltd., Scarborough, Ontario) as required.

Preparation of Leaf Homogenate and Distillate. Freshly cut potato leaves (10 g) were rinsed in distilled water and sliced into 2-mm-wide strips. The slices were ground in a chilled mortar with silica sand and 5 ml of 50 mmol/liter KCl (4°C). The slurry was transferred with rinses (2 × 2 ml of 50 mmol/liter KCl) to a Potter Elvehjem ground glass tissue homogenizer to complete the disruption. Samples (200 μl) of the crude homogenate were taken for chlorophyll determination (Bruinsma, 1963). The crude homogenate was centrifuged (2000*g*) for 2 min in a clinical centrifuge, and the supernatant was stored on ice. The chlorophyll concentration of the supernatant was adjusted to 700 mg/liter using 50 mmol/liter KCl.

For distillation, 10 ml of the homogenate was placed in a 100-ml round-bottom flask connected by a still head and vacuum take off to a 250-ml round-bottom flask that served as a receiver. The receiver was immersed in a Dry Ice–ethanol mixture and the sample was warmed to 40°C under vacuum (690 mm Hg) and taken to dryness (about 20 min). The distillate was thawed and KCl (1 mol/liter) was added to increase the conductivity to equal that of the homogenate (about 630 mS/m at 23°C). The dried residue was dissolved in 10 ml of distilled water. Samples were stored at 0–4°C.

Working concentrations for electrophysiology and feeding behavior were prepared by adding 2 ml of sample (homogenate, distillate or residue) to 2 ml of 50 mmol/liter KCl. The mixture was prepared by combining 2 ml of distillate and 2 ml of residue. The effective chlorophyll concentration of all four working solutions was approximately 350 mg/liter.

The alcohols to be tested electrophysiologically were prepared by dissolving each in a small amount of ethanol and diluting with KCl solution (50 mmol/liter). The final ethanol concentration was less than 2% (w/v) and the test alcohol concentration was 100 mg/liter. Reagent-grade primary saturated alcohols containing three to nine carbons and (*E*)-2-hexenal were obtained from Sigma Chemical Company (St. Louis, Missouri) and Aldrich Chemical Corp. (Milwaukee, Wisconsin). Isomers of 1-hexanol [(*Z*)-2-hexen-1-ol, (*E*)-2-hexen-1-ol, (*Z*)-3-hexen-1-ol, and (*E*)-3-hexen-1-ol] were purchased from Carl Roth GmbH and Co., Karlsruhe, Germany. The purity of all compounds was determined by gas chromatography.

Distillate Extraction and Gas Chromatography. Volatile components of the distillate were extracted with a mixture of pesticide-grade diethyl ether–hexane (2:1, v/v) in a Mixxor Separatory Cylinder (Xydex Corporation, Bedford, Mas-

sachusetts). The distillate was extracted six times with 5 ml of the solvent mixture. The combined extracts were dried over anhydrous sodium sulfate (20% w/v) and injected into the gas chromatograph.

A Hewlett-Packard HP5890 gas chromatograph with a 3392A integrator and a flame ionization detector was equipped with an HP-20M (Carbowax 20 M) fused silica capillary column (50 m × 0.2 mm ID × 0.2-μm film thickness). Carrier gas (helium) flow rate was 0.6 ml/min (linear velocity 39 cm/sec) and 1 μl of sample was injected in splitless mode (splitless injection liner, purge delay 60 sec). The injector and detector temperatures were 100 and 200°C and the oven temperature program was 40°C for 1 min, then increased, at 3°C/min, to 80°C and then 5°C/min to 150°C. To help confirm the identity of some of the peaks present in the distillate, a second column (HP-5, crosslinked 5% phenyl methyl silicone, 25 m × 0.2 mm ID × 0.11-μm film thickness) was used at a linear velocity of 20 cm/sec in splitless mode. The temperature program was 40°C for 1 min, then increased, at 1°C/min, to 48°C and again increased, at 5°C/min, to 150°C. Samples and standards were also analyzed by gas chromatography–mass spectrometry on a Varian Vista 6000 gas chromatograph (using the HP20M column and program) connected to a VG Analytical VG 7070E mass spectrometer. Compounds were identified by comparing their retention times and mass spectra with authentic standards and published spectra.

Standard solutions of the alcohols used for electrophysiological recordings were prepared in hexane containing 1-octanol as an internal standard to compensate for injection variation. Almost all the compounds were baseline resolved on the Carbowax column. A test mixture of the major alcohols and aldehyde found in potato leaf distillate [(*E*)-2-hexenal, 1-hexanol, (*Z*)-3-hexen-1-ol and (*E*)-2-hexen-1-ol each at 20 mg/liter] was prepared in water and used to assess the recovery during the distillation, extraction and drying process.

Electrophysiology and Feeding Behavior. The tip-recording method (Hodgson et al., 1955) was used throughout. Animal preparations for tip-recording of galeal sensilla of both adults and larvae were described previously (Haley Sperling and Mitchell, 1991; Mitchell et al., 1990). Intact adults were used but larvae were decapitated and their heads mounted on a glass micropipete containing 50 mmol/liter NaCl. Parts of the larval mandibles were excised to expose the galea. The four working solutions were used as stimuli (homogenate, distillate, residue and mixture) along with 50 mmol/liter KCl as a control. Data were analyzed as described by Haley Sperling and Mitchell (1991), using 1 sec of the recording starting 100 msec after contact with the sensillum. The first-stage clamping preamplifier used was especially designed for this recording method (George Johnson, Baltimore, Maryland, see Frazier and Hanson, 1986). Other recording equipment was standard.

Tape-recorded data were digitized and analyzed via computer with SAPID

Tools (Smith et al., 1990). With this software, multicell records can be analyzed by first separating individual waveforms into classes. Waveforms from each of the cells in the sensilla studied can be assigned to a particular cell type and compared across preparations. Details of using the programs are available in Smith et al. (1990), Mitchell et al. (1990), and Haley Sperling and Mitchell (1991). A handbook describing analysis procedures and individual programs is also available from B.K.M. Basically, the software allows one to separate waveforms representing cellular responses on the basis of spike height and overall shape. Waveforms resulting from simultaneous or near-simultaneous firing of two or more cells can be identified by the user and excluded for the purposes of calculating an average waveform for each cell in the sensillum. Such average waveforms are first compared for cells within each sensillum for all recordings from that sensillum. Then, waveforms extracted from all recordings with the same stimulus (across sensilla) can be compared. Finally, across-sensilla and across-stimuli records can be compared, if required by the analysis. The across-sensillum but within-stimulus comparison is a powerful tool to determine which cells are typically responding and the relative shapes of the waveforms obtained from that sensillum type with that particular stimulus. It is on the basis of such a comparison that we can be confident that the large and most frequent waveform obtained with leaf distillate, for example, is from a homologous cell in each sensillum and that responses from other cells are indeed infrequent (Figure 2 below). A similar comparison across sensilla and across stimuli [e.g., distillate, homogenate, (*E*)-2-hexen-1-ol and residue] allows direct comparison of waveforms across the entire data set. Thus we can conclude that the residue, for example, stimulates very little activity in the cell with the large waveform that responds so well to distillate, homogenate and mixture (Figure 2 below). Example traces such as those given in Figure 2 (below) give only an idea of a typical record. In all cases, multiple responses must be analyzed to form a reasonable data set. Detailed comparison of individual traces has severe limitations; thus, the traces shown in Figure 2 (below) should be regarded as mainly illustrative.

Feeding experiments were conducted by placing individual larvae under Petri dishes (5.5 cm diameter) over six sheets of moistened filter paper mounted on a Styrofoam base. A single disk (9.3 mm diameter) cut from Millipore filter (type RA, 1.2 μm) with a cork borer was mounted on a pin in the center of the Petri dish and the disk was wetted with 20 μl of a working solution (homogenate, distillate, residue, or mixture). Fourth-instar larvae [average fresh weight 103.9 $\pm$ 2.4 mg (mean $\pm$ SE; N = 204)] were randomly selected and placed under the dish and allowed to feed at 24°C for 16 hr (6:8:2 light–dark–light). Disk consumption was measured by area determination after scanning with a digital image analysis system (Tracor Northern, Series II, X-Ray Microanalysis System).

RESULTS

Basic Response Pattern. Leaf homogenate from potato leaves stimulated a high level of activity from primarily a single cell in the lateral galeal sensilla of larval beetles. Figure 1 illustrates a typical response. In addition to their largely single-cell nature, such responses were very long-lived. The sensillum also recovered from prolonged stimulation within 30 sec, to give a response near the initial maximum (unpublished observation). The same response pattern was seen in adult galeal sensilla, where it was shown to be dose dependent (Mitchell et al., 1990). This response is highly reproducible in both larvae and adults.

Sensory Effects of Distillate, Residue, and Mixture. Compared with homogenate and distillate, residue of potato leaf homogenate produced a very different

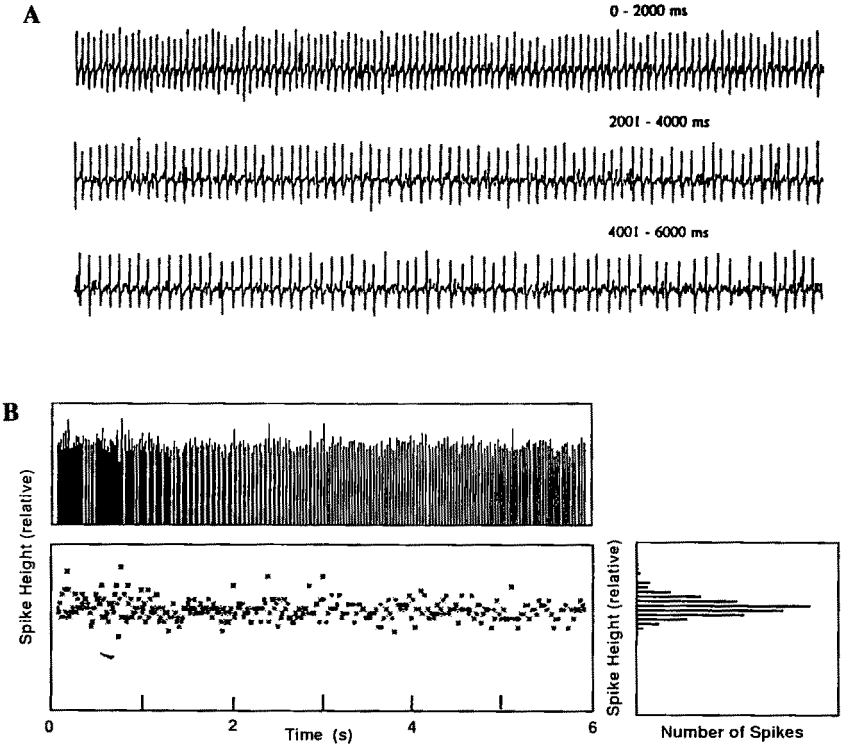

FIG. 1. (A) Typical response from a larval galeal sensillum to potato leaf homogenate. Records represent continuous activity from the cell during a 6-sec stimulation. (B) The same recording analyzed by SAPID Tools software showing the intensity of the response and its single-cell nature (spike height/frequency histogram).

sensory response pattern in both larvae and adults. This is seen both in the sample records (Figure 2) and in the histograms of mean sensory cell response (Figures 2 and 3). Responses were determined by computer analysis to comprise the activity of up to three cells (cell numbers 1, 2 and 3 in Figures 2 and 3), depending on the stimulus. Cell 1, the same physiological cell type as shown in Figure 1, was highly responsive to homogenate and distillate, while responding significantly less, on average, to residue (in the sample response to residue in figure 2, there was no activity from cell 1). Cells 2 and 3 were moderately sensitive to homogenate and residue, and cell 2 was significantly more active with residue stimulation than with homogenate. Distillate stimulated very little activity in cell 2 or 3.

To test for possible effects of the distillation process, distillate and residue were recombined (mixture) and used to stimulate the system. Comparison of the responses to mixture and homogenate in Figure 2 shows that the original sensory activity was substantially recovered by recombining. Cell 1 was significantly less sensitive to the mixture, relative to homogenate, but its activity was still substantial enough to dominate the response and significantly higher than in the response to residue. Activity from cell 2 and cell 3 to stimulation with mixture was equivalent to stimulation with homogenate.

In summary, when only the distillable components of the homogenate were used as stimuli, only cell 1 remained active. Removal of the distillable components, as in the residue, reduced but did not eliminate activity in cell 1 and led to increased activity from cells 2 and 3. Recombining distillate and residue largely restored an homogenate-like response pattern. The 50 mmol/liter KCl control stimulus was practically inactive. Essentially the same sensory patterns prevailed in galeal sensilla of adult beetles (Figure 3).

An important aspect of sensory response not revealed in Figures 2 and 3 is variability. Table 1 gives the coefficients of variation (CV) for the responses of larval and adult sensilla to the four plant extracts and to KCl. These coefficients were calculated from the data illustrated in Figures 2 and 3. The response of cell 1 was highly consistent when homogenate, distillate, and mixture were the stimuli. For larvae and adults combined, the mean CV for these three stimuli combined was 28%. By comparison, the mean CV for the response of cell 1 to residue in adults was 83% and in larvae 132%. Compared with the 28% CV for cell 1, the next most consistent response was that of cell 2 in adults to residue (CV = 59%). All other responses were highly variable with CVs ranging from 77% to 413% with a mean of 161% (for CVs greater than 60%).

Feeding Behavior. When the four stimuli (homogenate, distillate, residue, and mixture) were applied to Millipore filter disks and offered to fourth-instar larvae in no-choice tests, substantial feeding occurred only on the homogenate treated disk. Feeding on these disks was significantly greater than on any of the other three treatments (ANOVA, $F = 15.7$; $df = 3$, $N = 51$) (Table 2). The

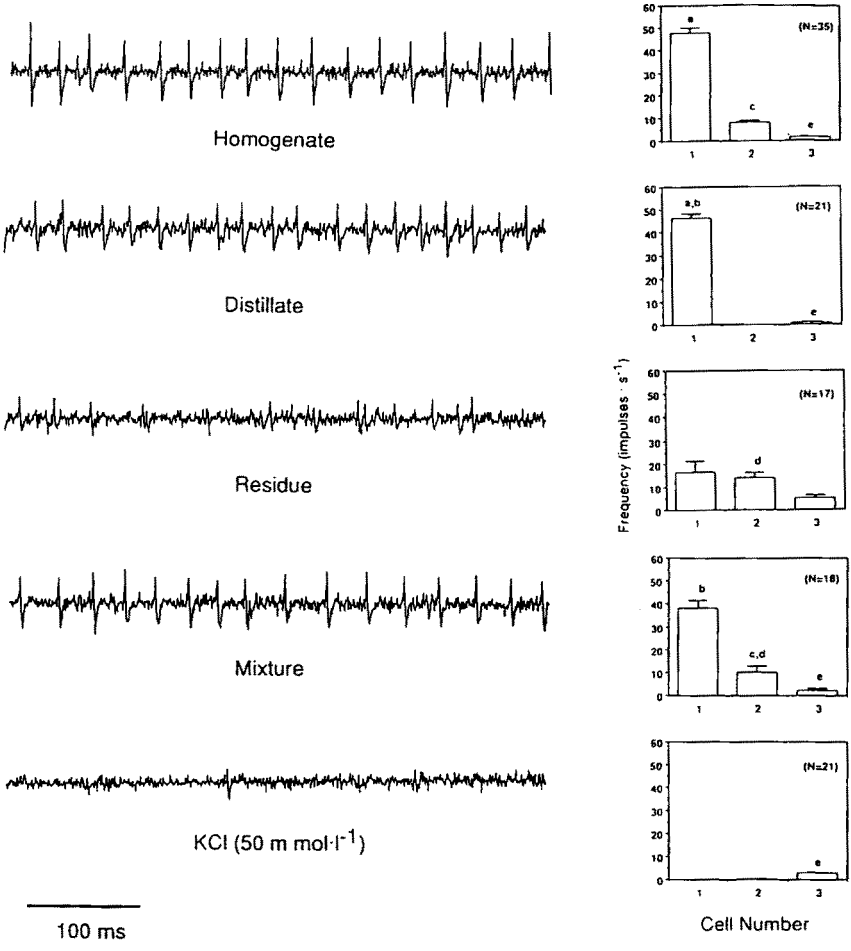

FIG. 2. Electrophysiological responses from larval galeal sensilla to homogenate, distillate, residue, mixture, and 50 mmol/liter KCl. The five recorded traces were from a single sensillum and show the response 350 msec after contact. Mean data from 17 to 35 stimulations (on nine larvae) are summarized in the histograms beside each trace. Activity could be attributed to as many as three cells in some records (1, 2, and 3 on the abscissa). Error bars are $\pm$SE. Columns, representing responses from one cell (e.g., cell 1) to each of the four stimuli, that have the same letter, are not significantly different ($P < 0.05$, Duncan's multiple-range test).

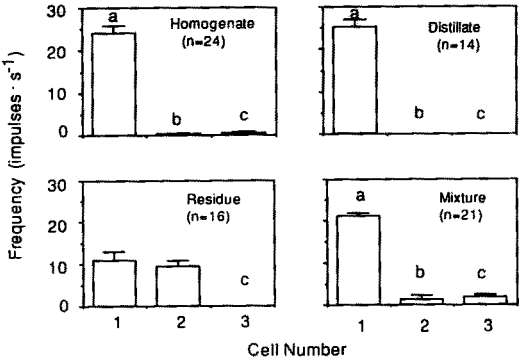

FIG. 3. Summaries of mean electrophysiological responses from 14 to 24 contacts on adult galeal sensilla (two animals, total of 10 sensilla) to homogenate, distillate, residue, and mixture. Error bars are ±SE. Columns representing responses from one cell (e.g., cell 1) to each of the four stimuli that have the same letter are not significantly different ($P < 0.05$, Duncan's multiple range test).

TABLE 1. COEFFICIENTS OF VARIATION (%) FOR CELLULAR RESPONSE DATA IN FIGURES 2 AND 3[a]

	Larvae cell number			Adults cell number		
Stimulus	1	2	3	1	2	3
Homogenate	32	101	235	38	289	413
Distillate	21		251	25		
Residue	132	82	103	83	59	
Mixture	38	114	168	17	363	197
KCl		261	84			200

[a]Missing data indicates no response from that cell.

mixture- and residue-treated disks stimulated similar feeding activity and distillate-treated disks were barely touched.

Compounds Responsible for Cell 1 Activity. Some of the volatile components in potato foliage were identified by Visser et al. (1979). We chose to study the leaf alcohols, since they are widely distributed, and it is known that olfactory cells in *L. decemlineata* respond to them (Ma and Visser, 1978). Indeed, cell 1 in both larval and adult galeal sensilla responded to (*E*)-2-hexen-1-ol and electrophysiological traces of responses to this alcohol were indistinguishable from responses to distillate of potato leaf homogenate.

Straight-chain saturated alcohols with three to nine carbons were tested at

100 mg/liter on the adult (nine different hairs on 3 animals) galea. Ethanol, up to 2% (v/v) final concentration, was used to facilitate dissolution of the longer alcohols but alone did not stimulate any cell activity compared to the control (50 mmol/liter KCl, data not shown). Only 1-hexanol showed substantial activity at 100 mg/liter and the frequency and waveform appeared identical to that resulting from leaf homogenate or distillate. In two of the three adults (three of the nine hairs), 1-heptanol showed some activity but both the frequency and impulse amplitude were half of the hexanol response. None of the other alcohols was stimulatory.

Dose–response data were obtained for (*E*)-2-hexen-1-ol on larval and adult sensilla and for 1-hexanol on adults only. Figure 4 shows the dose–response curve for (*E*)-2-hexen-1-ol on larvae. Table 3 summarizes dose–response parameters as determined using a Lineweaver-Burk plot.

In addition to these saturated alcohols, four *cis* and *trans* isomers with a single double bond were also tested at 100 mg/liter [viz. (*E*)-2-hexen-1-ol, (*E*)-

TABLE 2. CONSUMPTION BY FOURTH-INSTAR LARVAE OF MILLIPORE DISKS TREATED WITH ONE OF FOUR POTATO LEAF EXTRACTS[a]

Treatment	Consumption (mm^2)	Significance
Homogenate	15.2 ± 2.3	a
Distillate	1.2 ± 0.5	b
Residue	5.1 ± 1.2	b,c
Mixture	8.7 ± 1.5	c

[a] Data expressed as mean ±SE. Values with the same letter do not differ significantly ($P < 0.05$).

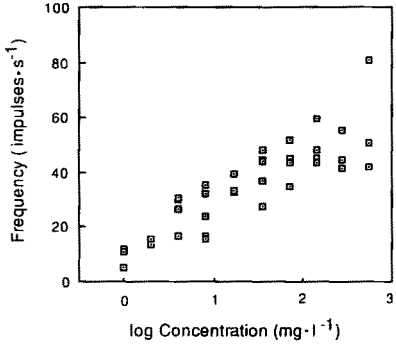

FIG. 4. Dose–response curve for larval galeal cell 1 response to (*E*)-2-hexen-1-ol ($N = 2$).

TABLE 3. PARAMETERS OF DOSE–RESPONSE RELATIONSHIP FOR TWO LEAF ALCOHOLS[a]

Alcohol	V_{max}	K_h	r^2	N
1-hexanol	35 ± 2	28 ± 8	0.77 ± 0.06	5
(*E*)-2-Hexen-1-ol	42 ± 5	37 ± 9	0.82 ± 0.05	5
(*E*)-2-Hexen-1-ol[b]	49 ± 7	5 ± 3[c]	0.78 ± 0.09	3

[a] V_{max} and K_h were determined by fitting a straight line double-reciprocal plot of individual dose-response curves. The first two experiments were on adult sensilla while the third experiment [second (*E*)-2-hexen-1-ol] was on larval sensilla. K_h is the concentration at which the response was half-maximal, and V_{max} is the maximum response. V_{max} = impulses/sec; K_h = mg/liter; r^2 = regression coefficient of double-reciprocal dose-response plot; average ±SE; N = number of senilla tested.
[b] Data from larval sensillum.
[c] Significantly different from other K_h values. t test, $P < 0.05$.

3-hexen-1-ol, (*Z*)-2-hexen-1-ol and (*Z*)-3-hexen-1-ol]. Some of these unsaturated alcohols were detected in potato leaf steam distillate (Visser et al., 1979) and are involved in plant lipid metabolism. The galeal response to these four alcohols appeared to be the same as that to 1-hexanol and the impulse frequency [44.7 ± 2.2 (SE) impulses/sec] was not significantly different among these five treatments ($P < 0.05$).

(*E*)-2-Hexenal was also found in potato leaf steam distillate (Visser et al., 1979) and was preliminarily tested here on another adult (five sensilla) along with 1-hexanol and (*E*)-2-hexen-1-ol at 100 mg/liter. The waveforms from the aldehyde were indistinguishable from those of 1-hexanol and (*E*)-2-hexen-1-ol, and there was no significant difference in the frequency [29.0 ± 1.4 (SE) impulses/sec, $N = 21$] among these stimuli (Duncan's multiple range test, $P < 0.05$).

The purity of all of the compounds tested for electrophysiological activity was assessed on the gas chromatograph and averaged 97.4 ± 0.6% (SE).

Identification of Potato Volatiles. The chromatogram of potato leaf distillate on a Carbowax capillary column is shown in Figure 5. The predominant peak was identified as (*E*)-2-hexenal and contained 81% of the total peak area. Other peaks were (*Z*)-3-hexen-1-ol, (*E*)-2-hexen-1-ol, and 1-hexanol, and together these four peaks accounted for 93% of the total area. Only trace amounts of (*E*)-3-hexen-1-ol, (*Z*)-2-hexen-1-ol, and linalool were noted. The total concentration of the four main components in potato leaves was 165 mg/kg fresh weight [with (*E*)-2-hexenal at 149 mg/kg] and the leaf chlorophyll content was 2.17 g/kg fresh weight. Experiments with a mixture of the four main components found in potato leaf distillate showed that after distillation, extraction and drying, recovery averaged 99.1 ± 1.8% (SE, $N = 5$) for all four compounds.

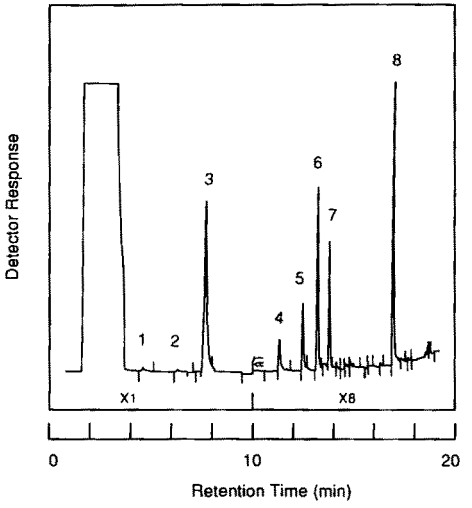

FIG. 5. GLC-FID of volatiles from potato leaf distillate on a Carbowax 20 M capillary column. Signal response was increased eight-fold at 10 min. Peaks 1, 2, and 4 are unknown, peak 3: (*E*)-2-hexenal, peak 5: 1-hexanol, peak 6: (*Z*)-3-hexen-1-ol, peak 7: (*E*)-2-hexen-1-ol, peak 8: 1-octanol (internal standard; 8.23 ng/μl).

DISCUSSION

Gustatory Response to Volatiles. Gustatory responses to volatiles dissolved in water have rarely been shown in insects. The anesthetic halothane stimulates the water-sensitive cell in the blowfly (Dethier and Goldrich Rachman, 1976) and leaf volatiles presented in air stimulate cells in the galeal styloconica of *Manduca sexta* (Staedler and Hanson, 1975). Sensitivity of the olfactory system of plant-feeding insects to green leaf volatiles has been well established (Dickens, 1989; Light and Jang, 1987; Honda et al., 1986, and reviewed by Visser, 1986), and specific antennal sensilla of *L. decemlineata* have been shown to be sensitive to (*E*)-2-hexen-1-ol and related compounds (Ma and Visser, 1978).

We detected the same compounds identified in potato shoot steam distillate by Visser et al. (1979), although the proportions were quite different. They found the largest peak to be (*E*)-2-hexen-1-ol, and (*E*)-2-hexenal was only a minor component. This variance in composition may reflect differences in plant variety, growth conditions, or methods of sample preparation. For example, Visser and colleagues extracted aboveground plant parts including stems, fruits, and flowers, while we used only leaves. Due to the high sensitivity of the capillary chromatography used here, sample preparation was relatively simple and recoveries of test mixtures were shown to be virtually quantitative. While

we do not know if the composition of volatiles in the homogenate is identical to that which the animals experience during leaf feeding, we believe that the concentrations found here are the same as in the homogenate used for our electrophysiology and feeding behavior experiments with the Colorado potato beetle.

Of the primary saturated alcohols tested here, the galeal sensilla of both adult and larval Colorado potato beetle responded only to hexanol and heptanol. Antennal receptors on a number of phytophagous insects are known to respond to individual alcohols and aldehydes commonly found in "green leaf volatiles" and the responses showed chain-length optima of five, six, or seven carbons (e.g., Light and Jang, 1987; Honda et al., 1986). Thus, antennae have relatively broad optima in terms of response to carbon chain length (C4–C8) whereas the galeal response reported here was virtually specific for six or seven carbons. In addition, the response to heptanol was easily distinguished from that to hexanol in terms of spike height, suggesting that different cells were involved. The galea also responded to other components found among the green leaf volatile mixture such as the monounsaturated *cis* and *trans* isomers of hexenol and a six-carbon aldehyde shown to stimulate antennal receptors in weevils (Dickens, 1984) and Colorado potato beetles (Visser, 1979). Such compounds have also been shown to influence orientation in the Colorado potato beetle (Visser and Ave, 1978) and to enhance aggregation of boll weevils when mixed with pheromones (Dickens, 1989).

The dose–response data for (*E*)-2-hexen-1-ol (Figure 4, Table 3) show that this compound is effective on the gustatory system at levels comparable to those found in potato leaves (range 3–149 mg/kg fresh weight). The responses of cell 1 (primary cell) to leaf distillate, to (*E*)-2-hexen-1-ol, and other six-carbon alcohols were remarkably similar. Responses of secondary cells to (*E*)-2-hexen-1-ol and the other effective alcohols were practically zero (data not shown but determined from dose–response and structure–activity studies reported above) as they also were for leaf distillate. We conclude that six-carbon leaf alcohols/aldehydes are an important stimulus component for the cell 1 (primary) response of the galeal taste system in larval and adult *L. decemlineata*, when this system is stimulated with potato leaf homogenate, distillate, or mixture. It remains to be seen if distillates of nonhosts such as tomato are effective sensory stimuli. Responses to tomato homogenate are multicellular, highly variable, and quite unlike responses to potato homogenate (Haley Sperling and Mitchell, 1991).

Predicting Feeding Response from Sensory Activity. From the electrophysiological data shown in Figure 2, one might expect homogenate, mixture, and distillate to stimulate similar levels of feeding. Homogenate and mixture were fairly effective feeding stimuli, and the significantly lower feeding on the mixture (Table 2) might be attributed to the distillation/recombination procedure having altered some component(s). Indeed, the activity of cell 1 to mixture, although

strong, was significantly less than to homogenate (Figure 2). Cell 1 responses to mixture were also slightly more variable (CV for homogenate, 32% vs. for mixture, 38%) (Table 1). Secondary cell (cells 2 and 3) activity was the same for homogenate and mixture, suggesting that the decrease in activity from cell 1 was at least partly the cause of reduced feeding on residue treated disks compared with homogenate treated disks. Distillate treated disks were barely touched by larvae, despite the high activity that distillate stimulated in cell 1, which is so like that stimulated by homogenate and mixture. Responses to distillate were practically unicellular, with cell 1 active, by contrast to responses to homogenate and mixture where secondary cell activity was evident. It is likely that compounds that remained in the residue account for the difference in activity of cells 2 and 3 when responses to distillate and mixture or homogenate are compared. These compounds include sugars, amino acids, salts, and other nonvolatile components of leaf homogenate, several of which are known to stimulate cells in this system (see Mitchell, 1988, 1993) and to stimulate feeding by larvae (Hsiao and Fraenkel, 1968; Mitchell, 1974).

This brings us to the interesting case of the residue. Residue-treated disks stimulated moderate feeding by larvae, at a level similar to the mixture (Table 2), yet, the sensory profiles of residue and mixture were markedly different (Figure 2). Recovery of residue included a drying step, which, coupled with the distillation processes itself, should have removed most of the volatiles. Nevertheless, residue stimulated some activity in cell 1, although this was significantly reduced compared to mixture, homogenate, or distillate. Furthermore, the response of cell 1 to residue was highly variable (CV = 132%, Table 1) compared with the same cell's responses to homogenate, distillate, and mixture (mean CV = 30%). Assuming insufficient volatiles remained in the residue to stimulate cell 1, the activity may have been due to nonvolatiles such as amino acids or sugars. Some amino acids stimulate a large-impulse cell in larval sensilla (Mitchell and Schoonhoven, 1974). This is also the case for adult galeal sensilla, where there is evidence that GABA, L-alanine, and sucrose stimulate the same cell (Mitchell and Harrison, 1984). Furthermore, the characteristic large, long-time-course waveform obtained with the amino acids and sugars is similar to that obtained with homogenate, distillate, mixture, and leaf alcohols (compare Figure 6 in Mitchell and Harrison, 1984, with Figures 1 and 2). We suggest that L-alanine, GABA, sucrose, and leaf alcohols all stimulate the same cell, cell 1, which forms the primary response of these galeal sensilla when the animal bites into a leaf. If this is true, it may well have been amino acids and sugars in the residue that stimulated the low level and variable activity from cell 1. The response to residue also includes activity from cell 2 and cell 3; in fact, cell 2 was almost as active as cell 1. This is consistent with what is known about responses of the adult system to amino acids and sucrose where secondary cell activity is also present (Mitchell and Harrison, 1984).

It is possible that both a moderate level of activity in cells 2 and 3 and high activity in cell 1 are necessary to stimulate high levels of feeding. Thus, normal high activity from cell 1 (primary response), in the absence of activity in cells 2 and 3 (secondary response), as obtained with distillate, would be insufficient to stimulate any feeding. A lower than normal primary response coupled with normal or slightly higher than normal secondary response, as with residue and mixture, will stimulate some feeding. If this interpretation is correct, the prominent activity from cell 1, so characteristic of responses to homogenates of host leaf sap, is necessary but not sufficient for plant acceptance in *L. decemlineata*. Cells 2 and 3 together comprise the secondary response analyzed by Mitchell et al. (1990) in the adult system. In adults, this response was highly variable compared to the primary response from cell 1, and, because of this variability, the secondary response was considered to convey little information useful for host-plant recognition. However, activity from these cells may signal the presence of primary plant compounds such as sugars, amino acids, and salts. Even where this activity is highly variable, these sporadic sensory inputs may be an important component driving feeding behavior.

In adult sensilla, when homogenates of nonhost plants are used as stimuli, activity from cell 1 is highly variable, considerably reduced, and sometimes completely absent, (Mitchell et al., 1990; Haley Sperling and Mitchell, 1991). It may be that compounds in nonhost plants inhibit cell 1, implying that this cell would respond to volatile components, sugars, and amino acids of nonhosts if it were not inhibited. If high and consistent activity in cell 1 is a necessary part of the code signaling host plant, its inhibition and/or an increase in response variability could lead to plant rejection. The response of cell 1 in adult sensilla to sucrose is inhibited by quinine and papaverine and that to GABA by tomatine, solanine, papaverine, and sparteine (Mitchell, 1987). There is no information on inhibitors for the strong response to leaf alcohols. Work to test the hypotheses that: (1) amino acids, sucrose, and leaf alcohols stimulate the same cell and (2) nonhost leaves contain compounds that interfere with these responses is in progress.

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USE OF CONIFER VOLATILES TO REDUCE INJURY CAUSED BY CARROT PSYLLID, *Trioza apicalis*, FÖRSTER (HOMOPTERA, PSYLLOIDEA)

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Abstract—The feeding and oviposition of the carrot psyllid, *Trioza apicalis*, were reduced by the application of fresh spruce and pine sawdust along the seedling rows in carrot fields. Turpentine and separate monoterpene hydrocarbons, mixed into old sawdust and/or placed in polyethylene tubes, were also effective. At a dose of 0.5 liter/m, fresh sawdust reduced the damage to 18% of the plants, compared to 100% damage in untreated plants. The sawdust materials were spread on the soil surface at four- or seven-day intervals during the oviposition period. The tubes were placed along the carrot rows before the oviposition started. Turpentine and separate monoterpene hydrocarbons afforded a protective effect of the same order of magnitude as that obtained from fresh sawdust. The volatile profiles of the spruce and pine sawdust as well as of the turpentine used were determined.

Key Words—Homoptera, Psylloidea, Triozidae, *Trioza apicalis*, *Daucus carota*, repellent, dispenser, sawdust, monoterpene, enantiomeric composition, TMP-turpentine.

INTRODUCTION

The carrot psyllid, *Trioza apicalis* Förster, can cause serious damage in carrot crops. Young carrot seedlings are attacked shortly after emerging. While feed-

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ing, the insects inject a substance that causes the leaves to curl and reduces growth, especially of the roots. Adult female psyllids are more injurious than adult males and immature individuals (Markkula et al., 1976). The adults pass the winter in diapause on coniferous trees, mostly Norway spruce, *Picea abies* (L.) Karst., (Rygg, 1977). After leaving their hibernation sites in spring, they seek their summer hosts, carrots, where mating and egg-laying take place. The eggs are laid one by one on the edges of the carrot leaves and one female can lay up to 900 eggs (Láska, 1964). The oviposition period, during which the plants are injured, lasts for about three to six weeks. There is one generation per year, and the adults die shortly after egg-laying. The eggs hatch after 9–14 days, and the nymphs remain sedentary on the underside of the leaves, passing through five instars before they become adults. The new adults emerge during August (with geographical variation) and leave the carrots two to three days after eclosion (Láska, 1976). They seek their winter hosts during autumn, and after hibernation they migrate back to the carrot seedings in spring.

This pest has been reported from all of Europe as well as from temperate Asia including Japan (Burckhardt, 1986). It is of great economic importance in carrot-producing areas located near conifer forests, where up to 100% of the carrots may be damaged.

In Sweden, carrot psyllids are commonly controlled by spraying at 10-day intervals with pesticides such as dimethoate, deltametrin, acephate, or fenitrothion (Kemiska bekämpningsmedel, 1991). Up to eight sprayings may be needed. A desirable alternative control strategy would be to manipulate the behavior of the insect.

An old gardener's trick, mentioned in the amateur-gardener literature, is to use sawdust to reduce injury by the carrot psyllid (Arman, 1977). Apsits (1931), who used sawdust as a soil mulch to control weeds in carrot fields, noted that the treatment also reduced the level of damage by the psyllid. This effect was verified by Rämert and Nehlin (1989).

Various products containing volatile conifer constituents have been used as deterrents against other insects. For example, pine oil, a by-product from certain pulp mills, has been shown to deter attacks by bark beetles, *Dendroctonus* spp. (Nijholt et al., 1981, Richmond, 1985), pine weevils, *Pissodes strobi* (Alfaro et al., 1984), and the onion maggot, *Delia antiqua* (Javer et al., 1987). Another product from the pulp industry, a terpene distillation residue with the commercial name XPQ, has also been used as a repellent against several forest insects (Eidmann and Nordenhem, 1984).

Our aim was to explore further the possibility of using sawdust and its volatile constituents, separate monoterpene enantiomers, as protectants against *T. apicalis*.

METHODS AND MATERIALS

Experimental Field Procedures; Evaluation Methods

Four randomized-block design experiments were performed during 1990–1992 in commercial carrot fields (in Karlstad and Sala, Sweden). Each experiment comprised four blocks of experimental plots. Each plot consisted of four rows of carrots. The lengths of the plots differed, depending on the space available in the field and on the type of experiment to be carried out. The carrots, cv. Duke, were sown in mid-May.

The protectants to be tested were either sawdust—with or without added chemicals—or chemical products enclosed in polyethylene tubes. The first application of the protectants was made along the carrot rows when the first seedlings were emerging. The sawdust was applied over the seedlings and additional applications were made at fixed intervals. The tubes were nailed with metal wires to the soil surface along the rows.

For estimation of the protective effect, two middle stripes of seedlings with the length of 1 m each were randomly chosen in each plot and the numbers of damaged and undamaged plants were counted. In total about 400 plants per treatment were examined. The psyllid injury was defined as the proportion of plants with curled, injured leaves. The effect of the treatment was evaluated using the method of analysis of variance after arc-sine transformation. The mean values found were compared by means of the least significant range (LSR) method (Sokal and Rohlf, 1969).

Experiments

Experiment 1 was a dose–response study using an undefined mixture of fresh sawdust from Norway spruce, *Picea abies* and Scot's pine, *Pinus sylvestris* L. The conifer logs were sawn at local sawmills about one to two months after being cut. The sawdust, which contained a small proportion of bark material, was collected immediately and then stored at $-18°C$ until its application to the carrot field. Six applications of sawdust in doses of 0.10, 0.25, and 0.50 liter/m were made at one-week intervals throughout the oviposition period. Each experimental plot was 4 m long in this experiment.

In experiment 2, the protective effects of spruce and pine sawdust were tested separately. The activity of an old sawdust preparation (see below) was tested for comparison. This old sawdust, used in experiments 2 and 3, was of the same origin as the fresh sawdust used in experiment 1 (a mixture of spruce and pine). After production, it had been stored outdoors, exposed to the weather during January–May. The volatiles present in the three sawdust preparations were analyzed using capillary GC (see below).

The application dose of the sawdust was 0.2 liter/m and the application started when about half of the seedlings had emerged. It was repeated every four days until the egg-laying of the carrot psyllids had ceased. Four applications were made before the oviposition period, which in 1991 was very short, lasting for just about two weeks. Four applications were made during this period (total of eight applications). The damage was recorded four days after the last application. Each of the experimental plots was 3 m.

In experiment 3, individual monoterpene hydrocarbons were assessed as protectants. α-Pinene, β-pinene, and limonene, major components of the volatile fractions of the sawdust and turpentine, were soaked up in old sawdust. The chemicals were mixed with the sawdust in plastic bags (Pingvin polyester oven bags) and kept at $-18°C$ before use. The concentration of the monoterpenes on the sawdust was 1 : 500 (4 g of a compound in 200 ml of pentane was mixed with 2 kg of old sawdust). We tested both the (+) and the (−) enantiomer of α-pinene and limonene but only the (−) form of β-pinene. [The (+) form was not commercially available.] The origins and purities of the chemicals used were as follows: (+)-α-pinene [Koch-Light pract., 75% (+) enantiomer], (−)-α-pinene [Firmenich 95%, 91% (−) enantiomer], (−)-β-pinene [Firmenich 95%, 97% (−) enantiomer], (+)-limonene [Aldrich 97%, 97% (+) enantiomer], (−)-limonene [Fluka 97%, 97% (−) enantiomer].

A sawdust dose of 0.2 liter/m was applied every four days during the oviposition period in 1991 (total of three applications and damage recorded four days after the last application). The plots used in this experiment were 2 m in length.

In Experiment 4, separate monoterpene hydrocarbons and turpentine, containing a mixture of monoterpenes, were used as protectants. In this experiment polyethylene tubes were used as dispensers instead of sawdust. The volatile fraction of the turpentine was analyzed using capillary GC, as was done with the sawdust volatiles in experiment 2.

Preliminary studies of the release rate of a monoterpene from the tubes had been made (see below).

The chemicals used were: (+)-α-pinene, (−)-α-pinene, (−)-β-pinene, (+)-limonene, (−)-limonene, (+)-3-carene, and turpentine. The origin and purity of the chemicals were as described in experiment 3, (+)-3-carene [Fluka 95%, 99% (+) enantiomer]. The turpentine originated from thermomechanical pulping (TMP) of spruce wood (SCA, Sundsvall, Sweden).

The polyethylene tubes (Kaltoplast AB), with an outer diameter of 5 mm and an inner diameter of 4 mm, were cut in 2-m lengths. The chemicals were filled into separate sets of tubes (four pieces of 2-m tubes per plot). The ends of the tubes were sealed by first heating them and then pressing them with a pair of pliers.

The carrot seedlings were covered by insect nets (Agronest) until ovipo-

sition was observed on carrots nearby and the test started. The net was removed and the tubes were put close to the stem base of the carrot seedlings along the carrot rows. The plots were 2 m long. The damage was recorded two and seven days after the tubes had been placed in the carrot field.

Collection and Analyses of Sawdust Volatiles

Trapping of Volatiles from Sawdust. The volatiles emitted by samples of fresh sawdust from spruce and pine and of the old sawdust used in experiments 2 and 3 were analyzed separately. The sawdust (3 g) was placed on a glass Petri dish enclosed in a glass flask with two openings. During 4 hr, filtered air was passed at room temperature at the rate of 100 ml/min first through the flask and then through a plug filled with Porapak Q (100 mg, 80–120 mesh). The volatile compounds adsorbed in the plug were then eluted with pentane (0.5 ml), and the pentane solution was submitted to GC and GC-MS analyses, using the same instruments and techniques as were used for gaseous samples (see below).

Headspace Analysis Technique. The analyses of the volatiles thus collected from fresh spruce and pine sawdust and from old sawdust (see experiment 2) were made in order to estimate the proportions of the volatiles emitted by these three sawdust materials. A method of external calibration of the procedure was employed, using calibration samples of the old sawdust, containing adsorbed turpentine in different concentrations.

Preparation of a Calibration Sample. Old sawdust, 7 g, was placed in a 50-ml Erlenmeyer flask with a septum-equipped cap, and a 2-ml sample of a pentane solution of turpentine was added. The flask was shaken for 30 min and then placed in a freezer. The concentrations of turpentine in the four calibration samples prepared were 1 : 100 (70 mg), 1 : 1000 (7 mg), 1 : 10,000 (0.7 mg) and 1 : 100,000 (0.07 mg).

After 24 hr, a flask with one of the calibration samples was taken from the freezer. The flask had been sealed with a septum cap, which was now removed, and a piece of aluminum foil was placed over the opening. The septum cap was then replaced, and the flask was kept at room temperature for 20 min before the cap was removed again. The needle of a gas-tight syringe was inserted through the aluminum foil, and 20 μl of the air above the sawdust was taken and injected into a fused silica capillary DB-WAX column (30 m $\times$ 0.25 mm). A Varian 3400 gas chromatograph equipped with a splitless injector and a flame-ionization detector was used. The temperature program was: 40°C for 1 min, followed by 50°C/min to 65°C and then 8°C/min to 130°C.

Two-dimensional gas chromatography (Borg-Karlson et al., 1993) was used for the chiral analyses. Two Varian 3400 gas chromatographs were coupled in series. The first one was equipped with a DB-WAX column and the second one with two chiral columns. a Cyclodex B column (30 m $\times$ 0.25 mm) was used

to separate the enantiometers of α- and β-pinene, camphene, limonene, and β-phellandrene. The sabinene and 3-carene enantiomers were separated on a Lipodex E column (30 m × 0.25 mm). The temperature during the chiral separations was kept at 30°C for 30 min and was then increased to 75°C at the rate of 3°C/min.

Studies of Release through Polyethylene Tubing Walls. Preliminary studies of the rate of release of a monoterpene from the polyethylene tubes (4 mm ID, 1 mm wall thickness) were made under laboratory conditions (25°C). A piece of tube (length 30 cm) was filled with α-pinene and closed at both ends. The tube was weighed at 24-hr intervals during a period of three weeks.

RESULTS

Experiment 1. Two weeks after the start of the oviposition period, 91% of the plants in the untreated plots were heavily attacked by the psyllids (Figure 1, June 14). One week later, all of the plants were injured. The lowest dose of sawdust, 0.1 liter/m, protected the carrot seedlings to some degree, but at the

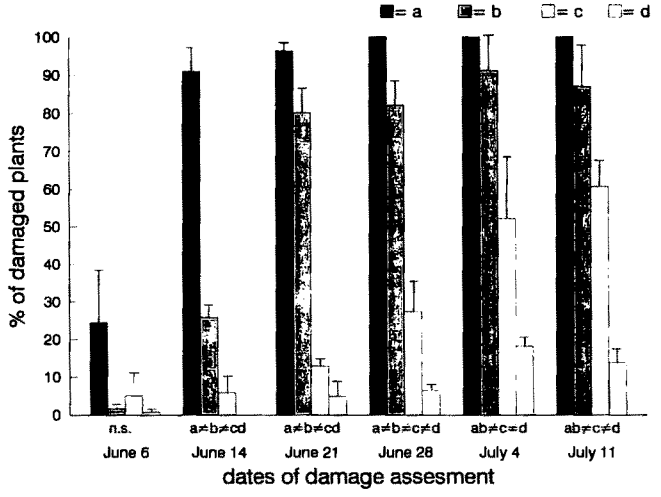

FIG. 1. Experiment 1: temporal variation in the frequency of psyllid-injured carrot plants during the oviposition period (mean values). a = untreated plants; b = sawdust 0.10 liter/m; c = sawdust 0.25 liter/m; d = sawdust 0.50 liter/m. An analysis of variance was made for each date, using arc-sine transformed values, and the results of the treatments were compared using the LSR method. (Significant differences at each date are marked below the bars in the diagram). LSD 5% for untransformed values were: 17.3, 10.1, 9.2, 10.5, 18.7, and 17.9, respectively.

end of the third week, the injury levels were high, 80%. By the end of the oviposition period, there was no significant difference in injury levels between the low-dose and control plots (Figure 1, July 4). In the 0.25 liter/m plots, the injury levels remained low for two weeks and then rose steadily to 61% at the end of the oviposition period. At the highest dose of sawdust (0.5 liter/m) the injury levels never rose above 18%. The final injury levels found in the medium- and high-dose treatments were significantly different ($P > 0.05$) from each other and from those found in the lowest dose treatment and the control plots.

The sawdust was effective only at very close range. Plants growing further than 10 cm away from the edge of the fresh sawdust were damaged. At the end of July, a slight yellowing of the carrot foliage could be seen in the sawdust-treated plots, indicating a deficit of nitrogen.

Experiment 2. by the end of the psyllid oviposition period, 85% of the untreated plants were injured (Table 1). The injury levels in the plots treated with old sawdust were somewhat lower, while fresh pine and spruce sawdust afforded the best protection (10–17% damaged plants). There was no statistically significant difference between the fresh sawdust of spruce and pine ($P < 0.05$), despite the quantitative differences in their head-space monoterpene composition (Table 4 below).

Experiment 3. Of the carrot plants in the control plots, 98% were injured (Table 2). The plants in the monoterpene-treated plots were significantly less damaged, with attack rates ranging between 12 and 38%. (−)-Limonene, with 12% damaged plants, showed the best protection and (−)-β-pinene the worst, 38% damage. There was no significant difference between the enantiomers.

Experiment 4. The weather in 1992 was warm and sunny during the pre-oviposition period. This led to a rapid invasion of a high field population of carrot psyllids. The psyllid damage was recorded two and seven days after the tubes had been applied to the field. Differences in effect between the protectants were seen only at the first recordings, two days after application (Table 3).

TABLE 1. EXPERIMENT 2: MEAN FREQUENCIES OF CARROT PLANTS INJURED BY CARROT PSYLLID (*Trioza apicalis*) IN PLOTS SUBJECTED TO VARIOUS TREATMENTS[a]

Treatment	Mean value (%)	SD
Untreated	84.6 a	8.3
Old sawdust	48.1 b	19.9
Spruce sawdust	17.3 c	5.3
Pine sawdust	9.7 c	3.8

[a]SD = standard deviation. LSD 5%: 18.9. All figures represent values before arc-sine transformation. Mean values in a column followed by different letters are significantly different (analysis of variance, LSR test, arc-sine transformation).

TABLE 2. EXPERIMENT 3: MEAN FREQUENCIES OF CARROT PLANTS INJURED BY CARROT PSYLLID (*Trioza apicalis*) IN PLOTS TREATED WITH OLD SAWDUST PLUS DIFFERENT SEPARATE MONOTERPENE HYDROCARBONS[a]

Treatment	Mean value (%)	SD
Untreated	97.8 a	0.4
(+)-α-Pinene	25.1 bc	9.5
(−)-α-Pinene	18.1 bc	10.6
(−)-β-Pinene	37.7 b	16.2
(+)-Limonene	21.4 bc	13.0
(−)-Limonene	11.6 c	4.5

[a]Figures represent values before arc-sine transformation. SD = standard deviation. LSD 5%: 15.8. Mean values in a column followed by different letters are significantly different (analysis of variance, LSR test, arc-sine transformation).

TABLE 3. EXPERIMENT 4: MEAN FREQUENCIES OF CARROT PLANTS INJURED WITHIN 2 DAYS BY CARROT PSYLLID (*Trioza apicalis*) IN PLOTS WHERE SEPARATE MONOTERPENE HYDROCARBONS WERE APPLIED IN POLYETHYLENE TUBES IN A CARROT FIELD[a]

Treatment	Mean value (%)	SD
Untreated	76.5 a	16.8
(+)-α-Pinene	64.3 a	12.5
(−)-α-Pinene	62.8 a	25.9
(−)-β-Pinene	68.5 a	18.0
(+)-Limonene	26.8 bc	10.2
(−)-Limonene	35.0 b	20.7
(+)-3-Carene	35.3 b	18.1
Turpentine	16.8 c	6.8

[a]Figures represent values before arc-sine transformation. SD = standard deviation. LSD 5%: 18.5. Mean values in a column followed by different letters are significantly different (analysis of variance, LSR test, arc-sine transformation).

When the damage was recorded after seven days, 100% of the plants in all treatments were damaged. Two days after the application of the tubes, 77% of the untreated plants were injured, whereas (+)- and (−)-limonene, 3-carene, and turpentine have reduced the injury to 17–35% damaged plants. Among these protectants, turpentine and (+)-limonene were the best according to the statistical analysis. In contrast to the low and stable release rates in the laboratory, the evaporation of the tested compounds in the field was quite fast due to the hot weather during the testing period.

Chemical Compositions. The volatile-constituent profile of the turpentine

used was similar to the profile of the volatile fraction emitted by the spruce sawdust: α-pinene, β-pinene, 3-carene, and limonene were the main components (Table 4). In pine sawdust, 3-carene, α-pinene, limonene, and terpinolene were the main components. The enantiomeric composition of the monoterpenes, with the exception of 3-carene, varied between the samples. Pure (+)-3-carene was found in all three samples. As regards β-phellandrene, the (−) enantiomer was strongly prevalent in all samples. The spruce and pine sawdust used in our experiments differed most notably in their emissions of α-pinene, β-pinene, and 3-carene.

The old sawdust emitted less than 1 g of α-pinene per 100 kg of sawdust (according to GC analysis). The total monoterpene concentration in fresh sawdust was estimated at 1 : 1000–1 : 100 according to the calibration curve of the standard turpentine–sawdust samples.

Rates of Release. The polyethylene dispenser showed a stable release rate under laboratory conditions. During the first three days, the release was very low due to slow diffusion through the tubing walls. After three days, the release became stable, reaching about 2.4%/24 hr period; 0.02% of the compound had been released after the first 24 hr, 1.1% after 3 days, 22% after 12 days, and 43% after 21 days.

DISCUSSION

Our results show that the application of fresh sawdust from coniferous trees on the soil in carrot fields can reduce injury caused by the carrot psyllid. The good effect shown also by single monoterpenes as well as by turpentine indicated that the protective effect of fresh sawdust was due to the volatile monoterpenes emitted by it. The old sawdust showed a much lower capacity of reducing psyllid attack.

The most likely mode of action of the substances applied in our experiments is that they act as close-range repellents (Dethier et al., 1960), i.e., "a chemical which causes an insect to make oriented movements away from its source." Repellents can interfere at different stages of the host selection. Our repellents seem to work directly on the insects at very close range, yet before the insects settle on the carrot plants for sucking and egg-laying.

An explanation of repellency might be that the volatiles from sawdust interact with attractive chemical stimuli from the carrot plants. The reception of the stimuli by the insect neurons might then be blocked, and the insects might lose their orientation towards the hosts (Davis, 1985).

Although the psyllids hibernate in conifers and are probably attracted by conifer odors during autumn, they might be repelled by these odors in nature during their spring migration into the carrot fields. The concentration of the

TABLE 4. RELATIVE AMOUNTS AND ENANTIOMERIC COMPOSITIONS OF MONOTERPENE HYDROCARBONS IN VOLATILE FRACTIONS FROM SPRUCE (*Picea abies*) AND PINE (*Pinus sylvestris*) SAWDUST AND IN TMP TURPENTINE

Compound	% monoterpene fraction (+/− enantiomer)		
	Spruce	Pine	TMP turpentine
α-Pinene	44.7 (44/56)	33.2 (70/30)	57.4 (63/37)
Camphene	0.7 (22/78)	0.4 (50/50)	1.1 (40/60)
β-Pinene	23.6 (4/96)	1.2 (25/75)	23.7 (2/98)
Sabinene	0.5 (72/28)	1.3 (2/98)	0.3 (76/24)
3-Carene	13.5 (>99.5/<0.5)[a]	48.2 (>99.5/<0.5)[a]	6.8 (>99.5/<0.5)[a]
Myrcene	1.7 nonchiral	2.1 nonchiral	1.0 nonchiral
α-Terpinene	0.1 nonchiral	0.1 nonchiral	0.0
Limonene	4.7 (26/74)	5.0 (9/91)	6.5 (17/83)
β-Phellandrene	5.7 (1/99)	2.4 (0.1/99.9)	2.0 (7/93)
γ-Terpinene	0.3 nonchiral	0.4 nonchiral	0.0
p-Cymene	0.6 nonchiral	0.4 nonchiral	0.0
Terpinolene	1.9 nonchiral	4.7 nonchiral	0.4 nonchiral

[a]Corresponds to the detection limit of the Lipodex E column.

conifer volatiles from the protectants might be too high. It is known from other insects that substances normally attracting an insect may, if present in higher concentration, instead repel the same insect (Davis, 1985).

The similarity of the protective effects of spruce sawdust and turpentine was probably due to their similar contents of volatiles. The similar effects of different kinds of sawdust, turpentine, and monoterpene dispensers show that the presence of an individual active compound may not be crucial for the repelling effect. However, limonene showed a high protective effect in experiments 3 and 4 and will, together with turpentine, be of special interest in further investigations. (+)-Limonene has already been tested as a protectant against several insects (Karr and Coats, 1988). In their experiments, (+)-limonene was found to be both slightly toxic and repellent to some insects. Both α- and β-pinene showed different effects in our two tests, being active on sawdust (Table 2), but having little or no repelling effect when applied with the tubes (Table 3).

Except for limonene in the tube experiment, the enantiomers did not show any significant difference in effect, although this would have been expected due to several literature reports. Thus, (+)-α-pinene elicited an oviposition response from the female spruce budworm, *Choristoneura fumiferana*, but the (−) enantiomer did not (Städler, 1974). In EAG testing, *Ips typographus*, a bark beetle feeding an spruce, was more sensitive to (−)-α-pinene than to the (+) enan-

tiomer (Dickens, 1978). This was found for both males and females. Thus, their choice of a breeding place will be the most suitable tree, since only (−)-α-pinene is transformed into the aggregation pheromone, (*S*)-*cis*-verbenol during feeding (Lindström et al., 1989). Monoterpenes from termite soldiers, acting as defense substances, showed different toxicities of their two enantiomers for other insects. (−)-α-Pinene was more toxic than the (+) enantiomer for *Formica rufa* ants, *Dysdercus congulatus* bugs, and *Tenebrio molitor* beetles (Everaerts et al., 1988). Samples of (+)-limonene showed a higher toxicity than the (−) enantiomer for *Monomorium pharaonis* ants (Valterová et al., 1988). Further examples of the activities of enantiomers of monoterpene derivatives were given by Silverstein (1979).

The continuing decrease observed in the protective effects of all treatments might have been due to loss of active material because of rapid evaporation at high field temperatures. There might also have been an overriding effect of psyllid population pressure, forcing psyllids to oviposit on plants that they had earlier considered unacceptable (Miller and Strickler, 1984).

Old sawdust, which contained only trace amounts of monoterpenes, still afforded some degree of protection. This indicated that the sawdust might have affected the visual perception of the host plant by the insect.

The effective high dose of fresh sawdust in experiment 1 (6 times 0.5 liter/m) is equivalent to a total dose of 15 liter/m^2 or 150 m^3/ha. On a commercial scale it would not be feasible to use such high application rates. The spreading is time-consuming when it must be repeated every four to seven days. Moreover, high doses of sawdust might have adverse effects on the crop; thus, being essentially organic, the sawdust might contribute to the immobilization of nitrogen during the mineralization, or it might release some phytotoxic substances. Therefore, applications of fresh sawdust cannot be recommended for large-scale use.

Our future research will focus on developing a dispenser with a constant terpene release rate. The carrier material should preferably be biodegradable and easy for the grower to handle and apply in practice. The repellent used could be turpentine or limonene. (+)-Limonene is a natural product of low toxicity. Turpentine is a complex by-product of the pulping industry and could be used as a cheap insect repellent if it were possible to spread it satisfactorily. However, before use, its possible plant toxicity must be investigated.

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RESPONSES OF FIG WASPS TO HOST PLANT VOLATILE CUES

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Abstract—Fig wasps (Chalcidoidea; Agaonidae) are intimately associated with the 750 or so species of fig trees (*Ficus*, Moraceae). Each tree species is usually pollinated by a single species of wasp belonging to the subfamily Agaoninae, while other wasps of the family are parasitoids or seed predators. Previous experiments have shown that the wasps are attracted to the trees by volatiles emanating from the figs. Using fig-bearing trees and arrays of sticky traps baited with figs, we investigated the specificity of wasp attraction and its timing. The pollinators of two closely related *Ficus* species were specifically attracted to figs of their host species and only at the time when figs were ready to be pollinated. Some nonpollinating fig wasps appear to respond to the same volatile cues.

Key Words—Hymenoptera, Agaonidae, evolution, fig wasps, host finding, volatile attractants, *Ficus*, Moraceae.

INTRODUCTION

Many insects show restricted host-plant preferences and consequently limit their feeding to a single (or a few closely related) plant species. Although visual components may be important (Rausher, 1978; Owens and Prokopy, 1986), long-distance identification of preferred host plants is usually olfactory (Miller and Strickler, 1984). Where the host plant is patchily distributed, the searching insect must be able to recognize its particular host signals from the myriad of

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other naturally occurring plant volatiles concurrently present in the atmosphere. Plant odors are complex mixtures of compounds of which only a few are typically used by the insect as cues to locate its potential host (Visser, 1986; Bergström, 1987). The nondirected dispersal flight of an insect is converted into active host search behavior once specific host volatile information is perceived (Cardé, 1984). Many plants are only suitable for the insects at a particular stage of their development, i.e., when bearing flowers or fruit. In these cases the insect must be able to recognize its host plant and to differentiate between its stages of development. Stage-specific changes in the volatile cues of the plants might involve the production of additional plant volatile compounds or may result from differences in the relative concentration of already existing compounds.

Where foraging insects play a role in pollination, the differences in floral fragrances of closely related plant species may act as isolating mechanisms. Bees have been shown to associate a particular plant odor with nectar reward (von Frisch, 1967) while *Elleschodes* beetles (Coleoptera; Curculionidae), which are the exclusive pollinators of *Eupomatis* (Eupomatiaceae), are attracted by floral fragrances (Bergström et al., 1991). Many interfertile orchid species (Orchidaceae) are ecologically isolated as they selectively attract a single species of bee (Hills et al., 1972; Borg-Karlson et al., 1985; Paulus and Gack, 1990).

Some plants offer larval feeding sites as a reward to pollinators. Yucca moths (Lepidoptera; Incurvariidae) pollinate the flowers of the yucca plant (Agavaceae), which in turn provides sites for larval development. In at least two cases this interaction is species-specific (Davis, 1967). Fig wasps species (Chalcidoidea, Agaonidea) have a similar intimate association with fig trees (*Ficus* spp., Moraceae) (Bouček, 1988). Each of the 750 or so *Ficus* species (Berg, 1988) is generally pollinated by a specific species of pollinating wasp belonging to the subfamily Agaoninae (Wiebes, 1979; Wiebes and Compton, 1990). The fig trees are totally dependent on the wasps for pollination and in return provide sites for their larval development inside the fruits—the figs. The high degree of specificity shown by the pollinating wasp species to their particular *Ficus* species has led to the assumption that the trees attract their specific pollinators through the release of volatile chemicals when the figs are ready to be pollinated (Hill, 1967; Galil, 1977; Janzen, 1979). Circumstantial evidence for the existence of such volatile attractants was first provided by Bronstein (1987) and this was later confirmed by van Noort et al. (1989).

In addition to the pollinators, there are many species of nonpollinating fig wasps whose larvae also develop inside the figs. These belong mainly to subfamilies of the Agaonidae other than Agaoninae, but include representatives of other chalcid families (Bouček, 1988). Some of the species gall the fig ovules, while others parasitize the gall formers. A few nonpollinating wasp species are like

the pollinators and enter the center of the fig, the lumen, through a narrow bract-protected entrance, the ostiole, prior to oviposition (Compton and van Noort, 1992), but the majority reach the ovules from the outside, penetrating the wall of the figs with their long ovipositors. Although the host relationships of most nonpollinating species are unknown, some of them, like the pollinating wasps, appear to be exclusively associated with a single *Ficus* species (Ulenberg, 1985; van Noort, 1992).

In most *Ficus* species, the development of fig crops tends to be synchronized within any one tree, but is not synchronized between trees (Wharton et al., 1980; Bronstein, 1987, 1992; Bronstein et al., 1990; Bronstein and Patel, 1992; Compton, 1993). Adult females of pollinating fig wasps are short-lived, surviving at most a few days (Kjellberg et al., 1988), while the longevity of some female nonpollinating wasps can extend to one or two months (Joseph, 1958; Compton et al., 1994). The gaps between fig crops on each tree may be months or even years (Bronstein, 1989; Windsor et al., 1989). The combination of within-tree fruiting synchrony and the short life-spans of the wasps means that both the pollinating and the nonpollinating female wasps must usually leave their natal trees in order to find figs that are suitable for oviposition (Bronstein, 1987, 1992).

Van Noort et al. (1989) showed that the pollinating wasp *Elisabethiella baijnathi* Wiebes located the figs of its host tree, *Ficus burtt-davyi* Hutch., using volatiles released by the figs when they were ready to be pollinated (receptive or female phase figs: Galil, 1977) but did not react to receptive figs of conspecific species. Figs at other stages of development were not attractive to the pollinators, nor were figs which had their ostioles covered, suggesting that the attractants emanated from within the figs during this short period in their development (van Noort et al., 1989). The responses of nonpollinators were not investigated, but those species that oviposit at the same stage of fig development as the pollinators could potentially make use of the same volatiles, whereas wasps that oviposit into figs at a later stage of development might be expected to utilize alternate cues.

In this paper we expand on the findings of Bronstein (1987) and van Noort et al. (1989) by simultaneously investigating the specificity of attraction of two conspecific species of *Ficus* to their pollinating fig wasps under natural conditions. Using arrays of sticky traps baited with figs of different developmental stages, we determined when wasps are attracted to the figs of *F. thonningii* Bl. and compared the specificity of the volatile attractants produced by this tree and *F. burtt-davyi*. We also experimentally prolonged the period when figs remained attractive to their pollinators, in order to determine the length of time figs would "wait" for their pollinators.

METHODS AND MATERIALS

The study was conducted in the 1820 Settlers Botanical Gardens situated at Grahamstown, in the eastern Cape Province of South Africa. Three local eastern Cape *Ficus* species grow in the gardens. Two, *F. burtt-davyi* and *F. thonningii*, are closely related and are placed in the section Galoglychia of the subgenus Urostigma, while the third, *F. sur* Forssk., belongs to the subgenus Sycomorus. *Ficus burtt-davyi* has a variable growth form but in the gardens grows as a shrub among the rocks and seldom exceeds 2 m in height. The larger *F. thonningii*, which naturally occurs as a strangler of other trees, has been planted among indigenous and exotic trees and obtains a height of 15 m while *F. sur* can grow to a height of 12 m. The locations of the fig trees in the gardens are indicated in Figure 1, and the fig wasps associated with these species locally are listed in Table 1.

The approximately 110 naturally occurring *F. burtt-davyi* in the botanical gardens grow mainly on the rocks of the north and northeast facing hill slopes and are usually surrounded by bush up to 2 m in height. Most of the 57 *F. thonningii* are planted trees and have been established between other tall, mature indigenous and exotic trees. The average distance between the *F. thonningii* and the *F. burtt-davyi* census trees and a fig-bearing heterospecific was, in both cases, approximately 150 m. The distances between *F. thonningii* census trees and their nearest fig-bearing conspecifics averaged 15 m while that between *F. burtt-davyi* census trees and their conspecifics was 10 m (Figure 1).

Sticky traps, each consisting of a cylinder (10 cm radius; 30 cm length) covered with cellulose and sprayed with pruning sealant (Frank Fehr, Durban), were used to investigate the attraction of fig wasps to figs at different stages of fig development. Poles, bearing the sticky traps placed at a height of 1.2 m, were placed in a 3 × 3 array about 40 m from the nearest fig tree. Each pole was positioned 5 m from its nearest neighbor. Twenty-five receptive-phase *F. thonningii* figs, determined by noting that the ostiole that provides entry of the pollinators had opened, were placed in each of the three cotton bags (treatment A) and 25 postpollinated figs in each of another three bags (treatment B). The final three empty bags acted as controls (treatment C). The bags were attached to the poles immediately above the sticky traps and placed in position (oriented ABC:BCA:CAB) at 0700 hr. The sticky traps were removed for analysis 6 hr later. The experiment was conducted twice in December 1989.

We then investigated how long unpollinated figs could potentially remain attractive to fig wasps. *F. burtt-davyi* was chosen for these experiments because it is a smaller tree species than *F. thonningii*, and all its figs are within reach from the ground. We selected two *F. burtt-davyi* trees growing about 100 m apart that were of comparable size, each bearing approximately 5000 figs at the same stage of development. Approximately half of the figs on one of the trees

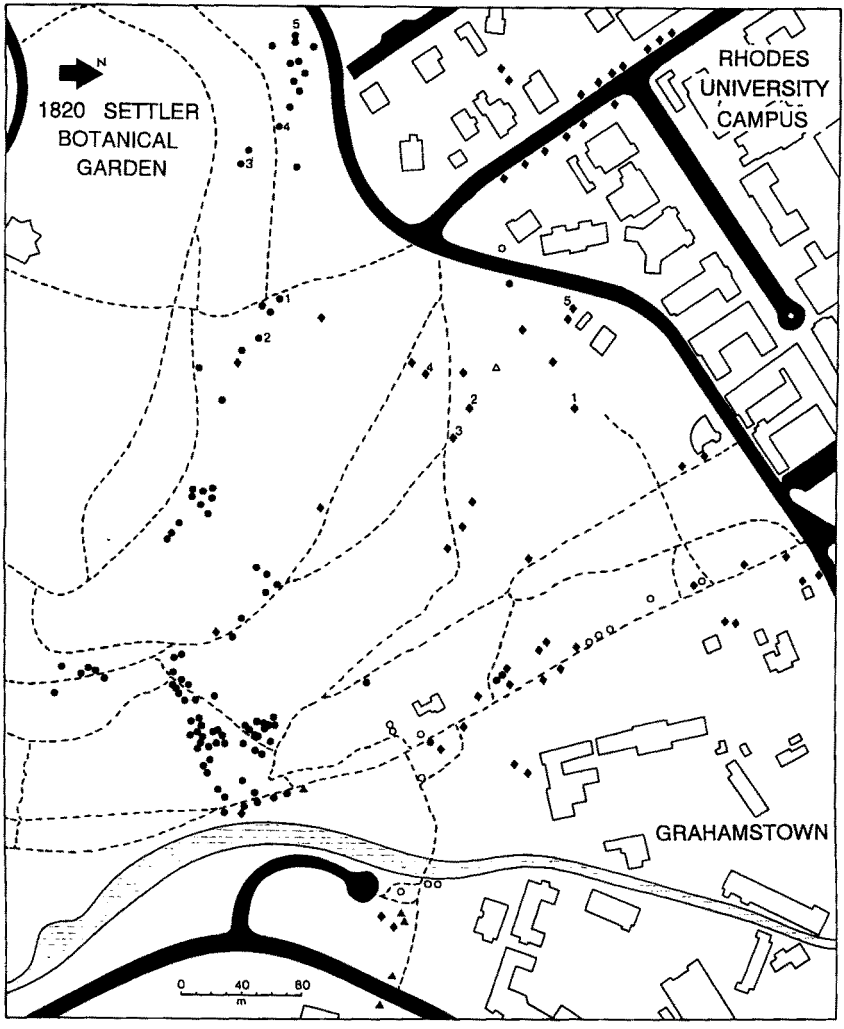

FIG. 1. Portion of the 1820 Settlers Botanical Gardens (Grahamstown, South Africa) showing the relative positions of *F. thonningii* (♦), *F. burtt-davyi* (●), and *F. sur* (△) trees. Additional exotic fig trees are represented by the open symbol (○). The numbers indicate those trees used to monitor the arrivals and departures of fig wasps.

TABLE 1. INDIGENOUS *Ficus* spp. PRESENT IN GRAHAMSTOWN BOTANICAL GARDEN, AND THE WASPS NORMALLY ASSOCIATED WITH THE TREES IN GRAHAMSTOWN

Ficus spp.	Pollinator	Nonpollinators
F. thonningii Bl.	*Elisabethiella stuckenbergi* Grandi	*Otitesella tsamvi* Wiebes
		Phagoblastus barbarus Grandi
		Sycoryctes sp.[a]
		Philotrypesis sp.[a]
F. burtt-davyi Hutch.	*Elisabethiella baijnathi* Wiebes	*Otitesella uluzi* Compton
		Otitesella sesquianellata van Noort
		Sycoryctes sp.[a]
		Philotrypesis sp.[a]
F. sur Forssk.	*Ceratosolen capensis* Grandi	*Sycophaga cyclostigma* Waterston
		Apocrypta guineensis Grandi
		Apocrytophagus spp.

[a]The *Philotrypesis* and *Sycoryctes* species recorded from *F. thonningii* and *F. burtt-davyi* cannot be distinguished at present and may not be host-tree specific.

were enclosed in cotton bags during their early prefemale phase. This prevented any pollination or oviposition by fig wasps. Single sticky traps were then placed in each tree to monitor arrivals of fig wasps and were replaced weekly.

The specificity of the volatile attractants emanating from the figs of *F. thonningii* and *F. burtt-davyi* figs was investigated in two field choice experiments. In the first experiment a 3 × 3 array of sticky traps was used as before, but with the cotton bags containing either 25 receptive-phase figs of *F. thonningii* (three bags) or 25 receptive-phase figs of *F. burtt-davyi* (three bags). The last three empty bags again acted as controls. Two replicate trials were conducted in December 1989 and January 1990.

In a long-term experiment monitoring the specificity of wasp attraction, the arrivals of wasps at *F. thonningii* and *F. burtt-davyi* trees in the Botanical Gardens were monitored over a two-year period. Single sticky traps were placed in five individuals of each tree species. In *F. burtt-davyi* the traps were positioned between 0.5 and 1.5 m above ground level, while in the taller *F. thonningi* they were placed at a height of approximately 2 m. The traps were replaced weekly and the numbers and identity of the trapped figs wasps were recorded. The relative positions of the trees that contained traps are indicated in Figure 1.

RESULTS

Significantly more *E. stuckenbergi* females, the pollinators associated with *F. thonningii* in the Grahamstown area, were recorded from sticky traps placed near receptive-phase *F. thonningii* figs than on traps near pollinated figs or the

control bags (Mann-Whitney U: $P < 0.01$) (Table 2). There was no difference between the number of *E. stuckenbergi* trapped on the control sticky traps and those traps placed near the pollinated figs ($P > 0.05$) (Table 2). In comparison with the pollinator species, low numbers of nonpollinating fig wasps were recorded from the traps. A similar preference for unpollinated figs of *F. thonningii* was shown by the nonpollinating species, *Phagoblastus barbarus* Grandi ($P < 0.05$), *Philotrypesis* sp. ($P < 0.05$), and *Otitesella* spp., although too few examples of the last genus were trapped for statistical significance to be recorded.

In the experiment where we examined how long *F. burtt-davyi* figs remained attractive to their pollinators, *E. baijnathi*, figs on the control tree were rapidly pollinated, and within two weeks wasps were no longer recorded from the sticky traps placed at this tree (Figure 2). In contrast, large numbers of wasps continued to arrive at the *F. burtt-davyi* tree with bagged figs for a period of five weeks (Figure 2). Unpollinated receptive figs therefore remained attractive to their pollinating wasps for an extended period if pollination was prevented. Far fewer wasps were collected on the control tree, presumably because they avoided the traps by entering the figs. In addition, it is likely that shortly after a wasp enters a fig the volatile attractant ceases to be produced, and the fig no longer attracts further wasps. Consequently, both the number of wasps and the time they spend near the control tree would be corresponding less than on the bagged tree.

The specificity of wasp attraction was confirmed when individuals were provided with a choice between the receptive figs of two closely related *Ficus* species. The ratios of wasps on different treatments were similar during the two trapping periods, and the data have therefore been combined for analysis. Significantly more *E. stuckenbergi* and *P. barbarus* (wasp species associated with *F. thonningii*) were trapped near receptive *F. thonningii* figs than on traps near *F. burtt-davyi* figs or the controls (Mann-Whitney U: $P < 0.001$) (Table 3). Likewise, significantly more *E. baijnathi* were trapped near figs of its host species, *F. burtt-davyi* ($P < 0.001$). No preferences were shown by the *Philotrypesis* sp., a species that may be associated with both *F. thonningii and F. burtt-davyi* ($P > 0.05$).

Of the five *F. burtt-davyi* trees monitored, one never produced a fig crop, three produced a single crop, and one had two crops of figs. Similarly, one *F. thonningii* did not produce any figs over the observation period while one tree had two crops, two trees three crops, and the final tree four crops. Crop duration during the observation period varied from as little as eight weeks in summer to over 20 weeks during winter. The development of the figs on any one tree was generally well synchronized, but the trees fruited at different times of the year.

Most of the wasps trapped on these trees belonged to species known to be specifically associated with that *Ficus* species (Table 4). *F. sur* is the third indigenous fig species growing in the botanical gardens, and only small numbers

TABLE 2. FIG WASPS TRAPPED NEAR COTTON BAGS CONTAINING POLLINATED OR UNPOLLINATED (Receptive) *F. thonningii* FIGS[a]

Wasp species trapped	Number of wasps trapped									Mann-Whitney *U* comparisons			
	Receptive figs			Postpollinated figs			Control			Control/ receptive figs		Control/ post-pollinated figs	
	Traps (*N*)	Mean/trap	Range	Traps (*N*)	Mean/trap	Range	Traps (*N*)	Mean/trap	Range	*U*	*P*[b]	*U*	*P*[b]
E. stuckenbergi	6	162.6	5–638	6	4.8	1–14	6	1.8	0–4	36.0	**	27.0	NS
P. barbarus	6	3.3	0–7	6	0.3	0–1	6	0		32.5	*	21.0	NS
Philotrypesis sp.	6	4.3	0–8	6	0.7	0–1	6	0		33.0	*	21.0	NS
Otitesella spp.	6	2.5	0–11	6	0		6	0					

[a]The control bags were empty. Combined results from two trials.
[b]NS = not significant. *$P < 0.5$; **$P < 0.01$.

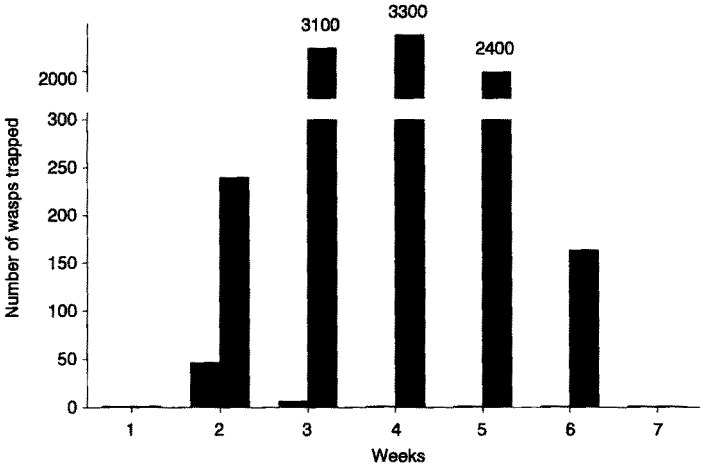

FIG. 2. The effect of bagging prereceptive (= pre-female phase) figs (hatched bar) of *F. burtt-davyi* on the numbers of pollinating wasps, *E. baijnathi*, trapped. The solid bars indicate the number of wasps trapped on a similar tree which remained unbagged.

of the wasps associated with this species were recorded from traps placed in the *F. thonningii* trees and none from traps placed in *F. burtt-davyi* trees (Table 4).

Few wasps were trapped in the census trees when they were not bearing fruit—the intercrop period (Table 5). At times when the trees were bearing figs, the first wasps trapped comprised those adult female wasps that had been attracted to the tree to oviposit. This was then followed by a period when few wasps were trapped. A few weeks later a large number of wasps, the progeny of the foundresses, were trapped as they emerged from the mature figs (Table 5, Figures 3 and 4). Only the wasps trapped during the first half of each crop period had therefore flown to the trees from elsewhere, and only the results from this period have been included in the following analyses.

The numbers of *E. stuckenbergi* and *P. barbarus* on *F. thonningii* were significantly higher during the first half of the crop periods than during the intercrop periods (Mann-Whitney U: $P < 0.001$) (Table 6), while there were no such differences in the trapping rates of *E. baijnathi*, the species that pollinates *F. burtt-davyi* ($P > 0.05$). The reverse situation was present on the *F. burtt-davyi* trees, where there were no increases in the numbers of *E. stuckenbergi* and *P. barbarus* trapped on the trees during receptive periods ($P > 0.05$), but numbers of *E. baijnathi* did increase ($P < 0.001$) (Table 6). Thus, during periods of fig receptivity the three species were only preferentially attracted to their own host trees (Table 6).

TABLE 3. FIG WASPS TRAPPED NEAR COTTON BAGS CONTAINING UNPOLLINATED (Receptive) FIGS OF *F. thonningii* OR *F. burtt-davyi*[a]

	Number of wasps trapped									Mann-Whitney *U* comparisons					
	Receptive figs *F. burtt-davyi*			Receptive figs *F. thonningii*			Control			Control/*F. thonningii*		Control/*F. burtt-davyi*		*F. thonningii*/*F. burtt-davyi*	
Wasp species	Traps (*N*)	Mean/trap	Range	Traps (*N*)	Mean/trap	Range	Traps (*N*)	Mean/trap	Range	*U*	P^b	*U*	P^b	*U*	P^b
E. stuckenbergi	6	3.2	0–6	6	53.8	31–66	6	2.5	0–4	36	***	21	NS	36	***
E. baijnathi	6	10.0	4–17	6	1.5	0–3	6	1.2	1–2	23.5	NS	36	***	36	***
P. barbarus	6	0.2	0–1	6	10.8	2–18	6	0.3	0–1	36	***	25	NS	36	***
Philotrypesis sp.	6	0.5	0–3	6	1.3	0–5	6	0		27	NS	21	NS	23.5	NS

[a]Control bags were empty.
[b]NS = not significant; ***$P < 0.001$.

TABLE 4. POLLINATING AND NONPOLLINATING WASPS CAUGHT IN STICKY TRAPS ON *F. thonningii* AND *F. burtt-davyi* TREES AND THEIR NORMAL HOST *Ficus*

Trap location	*Ficus* orgins of wasps			Host-tree indeterminate
	F. thonningii	*F. burtt-davyi*	*F. sur*	
F. thonningii	2120	13	22	696
F. burtt-davyi	1	1204	0	120

TABLE 5. FIG WASPS (All Species), TRAPPED AT *F. thonningii* AND *F. burtt-davyi* TREES DURING INTERCROP, RECEPTIVE (First Half of Crop Period), AND PRODUCER (Latter Half of Crop Period) STAGES

Tree	Number of crops	Total crop period (weeks)	Intercrop period (weeks)	Number of wasps trapped (mean/week)		
				Intercrop period	Receptive period	Producer period
F. thonningii						
1	1	21	98	2.37	18.66	13.29
2	2	42	77	1.35	14.29	25.74
3	1	38	81	2.10	15.32	8.35
4	1	63	56	0.91	8.32	22.10
5	0	0	119	0.75		
Total	5	164	431	1.25	12.79	15.39
F. burtt-davyi						
1	2	15	90	0.12	0.40	2.80
2	3	28	77	0.04	7.29	15.79
3	4	21	84	0.02	39.91	0.76
4	3	19	86	0.26	50.42	3.16
5	0	0	105	0.04		
Total	12	83	442	0.10	24.35	6.75

DISCUSSION

In the Grahamstown Botanical Gardens the overlap in the fruiting periods of *F. burtt-davyi* and *F. thonningii* and the close proximity of the trees meant that female adult wasps associated with the two species could potentially colonize the figs of either species. However, long-term monitoring of wasp arrivals at *F. thonningii* and *F. burtt-davyi* trees showed that the trees' pollinators were only attracted to their respective host trees. The two wasp species were thus able to distinguish their own host figs in the presence of receptive figs of the

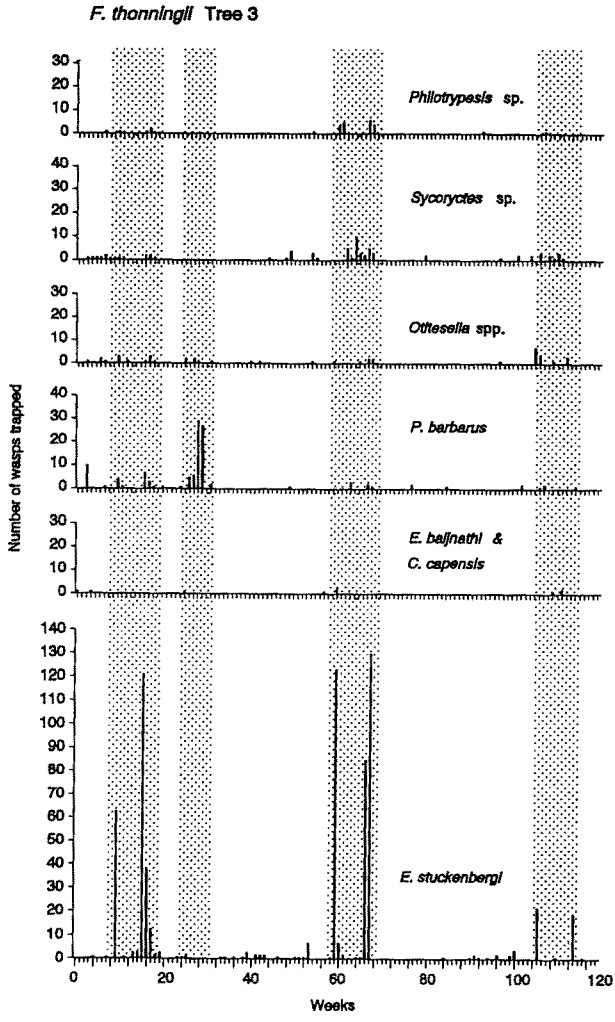

FIG. 3. Identity and numbers of fig wasps trapped at a *F. thonningii* tree. The shaded areas represent those periods when figs were present on the tree.

other species. These observations were confirmed by placing figs in cotton bags and demonstrated that the pollinators of *F. thonningii* were specifically attracted to their host figs only when the figs were ready to be pollinated, just as *E. baijnathi* was to its host, *F. burtt-davyi* (van Noort et al., 1989).

P. barbarus was the only nonpollinating wasp recorded on the traps in

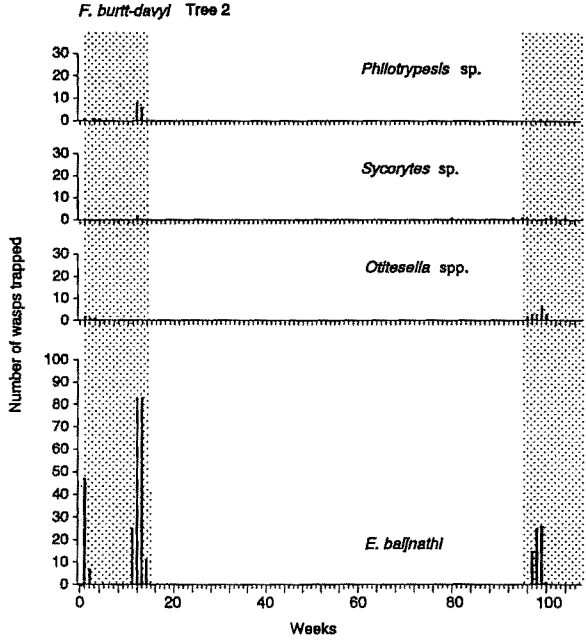

FIG. 4. Identity and numbers of fig wasps trapped in a *F. burtt-davyi* tree. The shaded areas indicate those periods figs were present on the tree.

large numbers. Like *E. stuckenbergi*, this species was also only attracted to receptive-phase figs of its host tree (*F. thonningii*). *P. barbarus* females enter the figs to oviposit at the same time as the pollinators and are also probably attracted by volatiles that are released during the receptive period (Ware et al., 1993).

Besides the volatile attractants, other attributes of the fig may also help to maintain the isolation of the species. The surface chemistry of the fig may also act as a recognition cue (Ware and Compton, 1992), while the ostiole is considered to act as a filter, preventing nonadapted wasps from entering "wrong" figs (Janzen, 1979). Modifications of the heads and bodies of the pollinators facilitate their entry into the figs (Ramirez, 1974). The development of similar mandibular appendages within the Sycoecinae, seed predatory fig wasps that are also often *Ficus* species-specific (van Noort, 1992), and the convergence of their head shape with that of the pollinators, implies that the ostiole has produced similar evolutionary pressures on the two lineages (van Noort, 1992). However, fig wasps have been recorded entering the "wrong" figs, which demonstrates the barrier is not an efficient one (Galil and Eisikowitch, 1969; Ramirez, 1970;

TABLE 6. COMPARISONS OF NUMBERS OF WASPS TRAPPED DURING INTERCROP PERIODS WITH NUMBERS TRAPPED DURING FIRST HALF OF EACH CROP PERIOD (Includes Receptive Female Phase of Fig Development)

	Number of wasps trapped (mean/week)											
	E. stuckenbergi				*P. barbarus*				*E. baijnathi*			
			Mann-Whitney				Mann-Whitney				Mann-Whitney	
Tree	Receptive period	Intercrop period	*Z*	P^a	Receptive period	Intercrop period	*Z*	P^a	Receptive period	Intercrop period	*Z*	P^a
F. thonningii												
1	29.29	0.5	−2.899	***	0.14	0.25	−2.787	**	0	0.01	0.227	NS
2	6.85	0.42	−3.436	***	3.15	0.49	−3.676	***	0	0.05	0.969	NS
3	11.00	0.54	−1.019	NS	0.60	1.05	−1.719	NS	0.05	0.03	−0.711	NS
4	9.71	0.36	−3.903	***	1.93	0.13	−3.295	***	0	0	1.000	NS
5		0.37				0.20				0.09		
Total	11.40	0.44	−5.905	***	2.03	0.41	−6.291	***	0.02	0.20	0.413	NS
F. burtt-davyi												
1	0	0	1.000	NS	0	0	1.000	NS	0.13	0.06	−1.103	NS
2	0.07	0	0.260	NS	0	0	1.000	NS	5.29	0	−5.586	***
3	0	0	1.000	NS	0	0	1.000	NS	38.00	0.02	−5.548	***
4	0	0	1.000	NS	0	0	1.000	NS	51.00	0.17	−4.486	***
5		0				0				0.03		
Total	0.02	0	0.311	NS	0	0	1.000	NS	22.67	0.06	−9.473	***

[a]NS = not significant; $**P < 0.01$; $***P < 0.001$.

Bronstein, 1987; Michaloud, 1988; Compton, 1990; Ware and Compton, 1992; Compton et al., 1994).

Not only do the volatiles making up the olfactory profiles of the individual *Ficus* species differ but, in all the species investigated, the additional volatiles that were present when the figs are ready to be pollinated also differed between species (Ware et al., 1993). These results indicate that these latter chemicals are likely to be the means by which fig wasps are able to locate their particular host *Ficus* and, like orchids pollinated by euglossine bees, the volatiles effectively act as prepollination isolation mechanisms.

The volatile attractants that form the basis of the species-specific pollinator wasp–*Ficus* interactions may be a key element in the radiation of both *Ficus* species and their pollinators. Two events, hybridization and mutation, are likely to be the main causes resulting in the alterations of the attractants. Although speciation through ploidy changes is common in plants (Stace, 1975), few *Ficus* tetraploids have been recorded. Chromosome counts of *Ficus* spp. indicate that they are mostly diploid ($2N = 26$) (Condit, 1933, 1964; Ohri and Khoshoo, 1987) and only four tetraploid "species" have been reported (Condit, 1964). In two cases, *F. burkei* Miq. and *F. hochstetteri* A. Rich. (both $2N = 52$), tetraploidy has apparently not influenced the chemical composition responsible for pollinator attraction as they still share the same pollinator species with *F. thonningii* ($2N = 26$). The other two tetraploid ($2N = 52$) species, *F. glumosa* Delile (= *F. sonderi* Miq) and *F. stuhlmannii* Warb. (Condit, 1964), are sympatric and are both pollinated by their own specific wasp species (Berg and Wiebes, 1992). In the last two cases the doubling of the chromosome number, whether through autotetraploidy or hybridization followed by a ploidy change, was probably associated with a change in the composition of the volatile attractants. However *Ficus* hybrids are rare, and they often appear incapable of developing into mature trees (Ramirez, 1970; Ware and Compton, 1992); nothing is known of their reproductive ability once they have reached maturity. It follows, therefore, that the radiation of *Ficus* species, and the changes in their chemical volatile attractants, rarely involve alletetraploidy.

One apparent case of host-plant switching by pollinating fig wasps has occurred. This involved the two agaonines, *C. galili* Wiebes and *C. arabicus* L., which share one fig species, *F. sycomorus* L. The true pollinator is *C. arabicus*, while *C. galili* acts as a "cuckoo" by utilizing the ovules for larval development without effecting pollination (Galil and Eisikowitch, 1967; Compton et al, 1991). They are not sister species and *C. galili* is therefore not derived from *C. arabicus* (Wiebes, 1989). The "cuckoo" is equipped to carry pollen (has corbiculae or pollen baskets), and its ancestors evidently acted as a pollinator of some unknown *Ficus* species before colonizing *F. sycomorus*. Perhaps the ancestors of *C. galili* were initially accidental colonizers of *F. sycomorus* that then came to recognize the attractive volatiles of their new host species.

The potential of pollinator wasps to host switch was demonstrated when *E. stuckenbergi*, the pollinating species normally associated with *F. thonningii*, was recorded mistakenly entering the figs of *F. lutea*. Here they not only pollinated the flowers but successfully reproduced (Compton, 1990; Ware and Compton, 1992). *Ceratosolen capensis* (Grandi), pollinator of the more distantly related *F. sur* Forssk., also successfully entered the figs of *F. lutea* but were unable to reproduce. These observations suggest that reproduction is more likely if those wasps making mistakes enter figs that are closely related to their original host species. Like the pollinators, some nonpollinating sycocine fig wasps enter the figs to oviposit and probably also respond in a similar way to the volatile cues of their host species. Some of these species have also been observed to make "mistakes" and even successfully to reproduce in their adopted host (Ware and Compton, 1992).

Bronstein (1992), in discussing the proximate factors that determine whether or not a fig tree will be pollinated, suggested that temporary localized wasp extinctions could be a major factor limiting fig production. This is because in small populations of trees there may be no receptive figs for the short-lived pollinating wasps to colonize. However, if they remain unpollinated, the figs of *F. burtt-davyi* are able to maintain their attractiveness to pollinators for extended periods. This could potentially overcome local shortages of pollinators by allowing trees to "wait" for pollinators for five weeks or more. Another consequence of the extended receptive period is that a smaller number of fig trees can maintain the wasp populations in each local area (Bronstein et al., 1990). Where the fig crop of a *Ficus* species is asynchronous, then even smaller fig tree populations will be required to maintain the pollinator populations.

A casting flight behavior among the pollinators, which is typical of insects approaching an attractive odor source, was observed when the wasps were near receptive figs of their specific *Ficus* hosts (Ware and Compton, 1994). Gas chromatograph analysis of volatiles produced by the figs of these species has shown that additional compounds are released during their receptive phase of development, and these are likely to be the basis for the observed flight behavior of the wasps and the specificity of attraction (Ware et al., 1993). The experiments detailed here confirmed the specificity of wasp responses to the volatile attractants released by two closely related *Ficus* species in the presence of one another and have shown that figs normally remain attractive for a short period, unless pollination is prevented. However, only isolation and bioassay of the attractant volatiles and/or electroantennogram studies will allow this link to be confirmed.

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CHEMICAL SIGNALS INVOLVED IN SPACING BEHAVIOR OF BREEDING FEMALE BANK VOLES (*Clethrionomys glareolus* Schreber 1780, Microtidae, Rodentia)

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Abstract—In order to study the mechanism involved in the seasonal territoriality of breeding bank voles, the social behavior and scent marking of paired females were observed throughout a reproductive cycle. Initially unfamiliar females were kept in large laboratory pens provided with individual burrows. After a brief period of hostility, females behaved in a friendly manner towards each other, sharing the same nest even in the presence of a male and until the middle of pregnancy. They scarcely marked with urine. Continuous olfactory assessment appeared to play an important role in maintaining the friendly interactions. In late pregnant and lactating females, on the contrary, the odor of a familiar female triggered aggressiveness and scent marking with urine and probably with flank glands. These reactions may be interpreted as spacing behavior. Moreover, the interaction between females may inhibit reproduction in one of them. These results are discussed in relation with the available ecological data.

Key Words—Spacing behavior, aggressivity, scent marking, urine, olfactory communication, maternal behavior, reproduction, voles, *Clethrionomys glareolus*, Rodentia.

INTRODUCTION

In the winter, during the nonbreeding season, female bank voles are distributed in clusters (Bujalska, 1973; Ylönen and Viitala, 1985) and nest communally (Gebczynski, 1969). Early in the spring, females disperse evenly; later, when

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breeding, they hold exclusive territories (Bujalska, 1973, 1985; Wiger, 1982; Bondrup-Nielsen and Karlsson, 1985).

The mechanism involved in their spacing behavior is unknown. In the red-backed vole (*Clethrionomys rufocanus*), it can be inferred from the scarcity of wounds that breeding females are not very aggressive towards their familiar neighbors. This suggests that they would signal their breeding territories by chemical and auditory cues rather than aggressively defending them (Viitala and Hoffmeyer, 1985). On the other hand, female bank voles are known to react vigorously to the nearness of an unknown conspecific by marking with urine (Rozenfeld and Rasmont, 1987). It may therefore be hypothesized that urine marks are used by residents to assert territorial rights respected by their close neighbors.

In this paper, we report laboratory experiments designed to test these hypotheses. Initially unfamiliar, nonbreeding females were paired for 37 days in large pens provided with individual burrows. Their social behavior and scent marking were observed during a reproductive cycle: from sexual quiescence, through receptivity, mating and pregnancy, until the onset of lactation.

In bank voles, as in other microtine species, females are reflex or induced ovulators (Clarke et al., 1970; Westlin and Nyholm, 1982). Therefore, in the first part of our experiments, we presumed that females, kept in complete isolation from males and their odors, would be in sexual quiescence. After their behavior had been assessed, the paired females were given the opportunity to mate with a dominant male.

We expected that: (1) during the familiarization period, nonbreeding females would behave in a nonaggressive way, perhaps share the same nest as in winter and scent mark at a low level; and (2) with the onset of estrus induced by physical contact with the male, and later with the onset of pregnancy, a reproductive female would become intolerant, nest on her own, and use chemical signals to assert the occupancy of her exclusive burrow.

METHODS AND MATERIALS

All the voles were adults of the first generation born in the laboratory to parents caught in central Belgium. They were sexually experienced.

Eighteen parous females 9–13 months old were maintained in a nonbreeding condition by keeping them for two months in a room completely isolated from males and their odors. No vaginal smears were taken in order to prevent the likelihood that mechanical vaginal stimulation might influence fertility (Westlin and Gustafsson, 1984).

The fact that females are more attracted by dominant males than by subordinate males (Hoffmeyer, 1982) made us choose dominant males as sires.

Nine pairs of initially unfamiliar males were kept for four weeks in large observation pens. As done previously (Rozenfeld et al., 1987), the relative status of each male was determined from the observation of their agonistic behavior and from the numbers of urine marks left on a flooring filter paper and made visible under UV light (Desjardins et al., 1973).

Experimental Enclosures. Each individual enclosure consisted of an aluminum observation pen (60 × 60 × 50 cm) with a front glass window. The pen was connected through a glass tube to a plaster burrow. Each burrow was made of two Y-shaped elementary burrows (described in Rasmont and Rozenfeld, 1987). In the present configuration, this double burrow had a single entrance opening into a nest chamber. From there, two other galleries led to a second chamber, the rear outlet of which was closed with wire mesh.

The floor of each enclosure was lined with filter paper to collect urine marks. Each pen was provided with soft paper as nesting material, a drinking bottle, a trough with commercial hamster food and fresh vegetables (carrots or lettuce). Small open glass vials (5 cm high × 5 cm wide) were provided in two corners. The animals used them as latrines: in this way, the urine puddles that otherwise would have blurred the fine traces were considerably reduced (Rozenfeld et al., 1987). According to the different phases of the experiment, these observation pens were arranged in three configurations: completely separated (Figure 1, phase 1), fused into a single large enclosure (Figure 1, phases 2 and 4) or a third pen connected to the large enclosure (Figure 1, phase 3).

Experimental Design. The experiments included nine pairs of initially unfamiliar females and nine dominant males and was organized in four phases (Figure 1).

During phase 1 (= isolation), each nonbreeding female, marked by fur-clipping, was kept alone in an isolated pen.

For phase 2 (= free interaction and familiarization), two pens were merged. The behavior of both females was video recorded on days 0, 3, 5 and 9 (D_0, D_3, D_5, D_9 in Figure 2).

At the onset of phase 3 (= cohabitation with a male), i.e., on day 10, the pen of a dominant male was connected to the enclosure of the females. The three individuals were allowed to interact for six days. Their behavior was recorded on D_{10}, D_{13}, and D_{15}.

During phase 4 (pregnancy and lactation), the pen with the male was removed and the females remained together until parturition. Their behavior was observed daily but we only analyzed the pictures recorded 10 days before parturition (P_{-10}), four days before parturition, when the first aggressive behavior appeared (P_{-4}), and on the day of birth (P_0).

Social Behavioral Patterns. All observations started at the same hour of the day and all recordings lasted 3 hr.

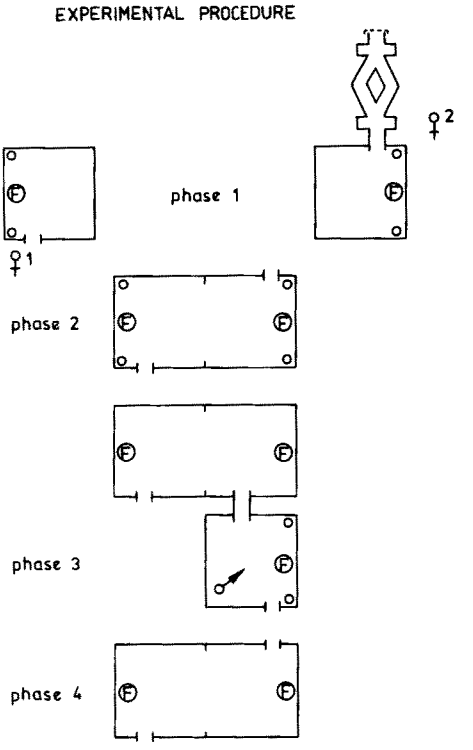

FIG. 1. Configuration of chambers in the four phases of study. F: food and water.

Four categories of behavior were analyzed; agonistic, friendly, scent marking, and sexual events.

Agonistic events include positive aggressive acts such as: attack, fight, chase, and threat and negative acts such as retreat, avoidance, and retaliation (the typical defensive reaction of a resident threatened in his burrow). These acts have been defined by Clarke (1956), Johst (1967), and Mihok (1976). We analyzed only positive aggressive acts.

We regard as friendly behavior (= amicable behavior for Perrin, 1981), all the interactions that include a close contact between females without inducing an agonistic response. Most of these interactions were observed in a burrow. Three friendly behaviors were recorded: nesting together, mutual sniffing, and mutual grooming. When nesting together, in the same chamber, females lie over one another or huddle side by side. Mutual sniffing is the very close sniffing of various body regions of another female: muzzle, ears, flanks, anal region, per-

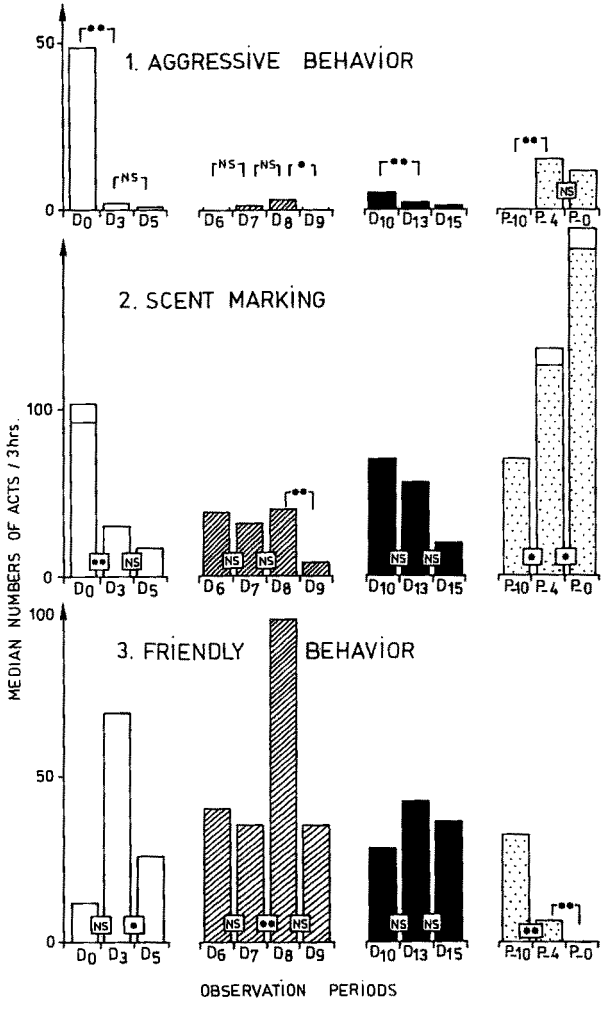

FIG. 2. Behavior of nine paired females throughout a reproductive cycle: sexual quiescence (open columns), cohabitation with a dominant male (hatched columns), pregnancy and lactation (solid columns), flank scratching (stippled columns). D_0, D_3, . . . : days of observation. P^{-10}, . . . : 10 days before parturition; P_0: day of parturition. NS: not significant, *: significant, **: highly significant.

ineal region, or nipple region. This behavior was more frequent when one female came back to the common nest after having been away. Mutual grooming is defined as one female scratching and licking the fur of another, while the groomed individual would often lie flat on the floor with half-closed eyes. When scent marking, two activities were observed: deposition of small amounts of urine and flank scratching. As dominant males do (Rozenfeld and Rasmont, 1991), a female repeatedly scratches her flanks with the hind feet. Mating behavior includes courtship, during which a male pursues a female with his head resting on her rump and, as Christiansen and Døving (1976) already described, olfactory investigation, mounting, and lordosis.

Counting Urine Marks. To differentiate between the marks of each female, we used the same method as for males (Rozenfeld et al., 1987). Before each observation period females were isolated for two hours in their own burrow with a standard piece of apple stained with 5 ml of a 1/1000 solution of either methylene blue (medicinal grade, Hoechst) or fluorescein sodium (Merck). The flooring paper was renewed at the onset of an observation collected at the end, and the stained marks were tallied.

Statistical Tests. As a central measure of each class of events, we used the median, because we cannot make any assumption about the distribution of our data. The statistical significance between the classes was tested with the Wilcoxon matched-pairs signed-rank test. We used the AMSTAT package (Coleman and Coleman, 1986) on an AMSTRAD PCW 8512 computer. The differences are rated as nonsignificant, for $\alpha > 0.05$, as significant (*) for $\alpha \leq 0.05$ and as highly significant (**) for $\alpha \leq 0.01$. The single-sided values of t were considered where we tested the hypothesis of a directional change, the double-sided values were used to test the significance of an unpredictable change (Siegel and Castellan, 1988).

RESULTS

Social Behavior and Scent Marking of Paired Females in Sexual Quiescence (Phase 2). In Figure 2, open columns show the median numbers of aggressive acts (2.1), scent marking (2.2), and friendly acts (2.3) performed by the nine pairs of females during the first nine days of interactions.

On day 0, the first encounters between unfamiliar nonbreeding females were always agonistic. The most frequent aggressive acts were threatening approach that induced flight or defensive spar. Neither attack nor fight was observed.

Immediately after an agonistic interaction, the threatening female marked with small urine drops and scratched her flanks. Even without any interaction, both females marked the common environment with urine. After 2 hr, some pairs became friendly.

On day 3, aggressivity and urine marking had decreased in a highly significant way and flank scratching had completely disappeared. In contrast, the number of friendly acts (mutual olfactory investigation and grooming) was high, although the increase from D_0 to D_3 is not statistically significant.

Besides the specific behavior patterns that we analyzed, females spent much time together in the same burrow. They shared the same nest, which was always built in the front chamber (i.e., the chamber closest to the entrance). The rear chamber of the common burrow as well as the unoccupied burrow were used as solitary resting places. In the field, bank voles are known to use different kinds of shelters in their home range as resting places (Albov et al., 1979; Mironov, 1990). Urine marks of both females were frequently deposited in front of the common burrow and along the wall near the food sources.

On day 5 and later on day 9, there was no further difference in behavior.

Behavior of Paired Females during 6 Days of Cohabitation with a Dominant Male (Phase 3). In this phase, we tested the hypothesis that the social interaction of familiar females would be modified by the presence of a male. In Figure 2, hatched columns represent the behavioral interactions between paired females in the presence of a dominant male. On the first day of cohabitation with a dominant male (D_{10}), aggressivity between the females and urine marking increased in a highly significant way, while the number of friendly acts did not vary significantly.

On days 13 and 15, the only change in female–female interactions was that aggressivity significantly decreased.

As our recorder does not allow time-lapse recording, only four matings could be observed.

Outside the mating period, both females in each pair were usually intolerant towards the male. The median numbers of aggressive acts were: 11 (range: 0–29) on day 10, 7 (range: 2–13) on day 13, and 7 (range: 0–11) on day 15. Throughout his presence, the male nested on his own, while the females kept nesting together.

Behavior of Breeding Females after Withdrawing the Male (Phase 4). In the last phase, the male was removed and the females remained together in their home pen until they were expected to give birth. Females were observed daily for 21 more days. We analyzed their behavior at the middle and at the end of pregnancy, i.e., 10 days (P_{-10}) and 4 days (P_{-4}) before parturition and, finally, on the day of delivery (P_0). The results are illustrated in Figure 2 (solid columns). In two pairs, both females gave birth, in the seven others, only one female did so.

By the middle of pregnancy, females in each pair remained friendly, scent marked with urine at a low level, and shared the same nest.

Four days before parturition, friendly interactions decreased while aggressivity and scent marking markedly increased. In seven pairs, aggression was

displayed by gravid females towards nonpregnant ones. In the two other pairs, one of the females, more advanced in pregnancy by two days, behaved aggressively towards the other.

The frequency of aggressive acts was lower than when the females, then unfamiliar, first met, and their pattern differed markedly. The dominant female responded with attacks, fights, and chases to the attempts of the submissive one to entering the previously common burrow. Eventually, the ousted female nested on her own, in the other burrow.

After most aggressive episodes, the resident female would scratch her flanks and mark with urine. She profusely marked the surroundings of her own burrow and of the food source located next to her nest. In the seven enclosures where only one female was pregnant, 66% of all the urine marks were made by the pregnant individual.

This proportion rose to 73% (**) on the day of parturition. The resident females reacted aggressively to the frequent attempts of the subordinate to intrude into their burrow. They also moved their nest to the more remote nesting chamber of the same burrow and closed the entrance with pieces of the flooring paper.

DISCUSSION

Ecological evidence suggests that in the open, mature female bank voles are solitary during the breeding season. Furthermore, the intolerance of pregnant or lactating females towards conspecifics has been experimentally ascertained in several families of rodents. Most of the experiments, however, involved brief encounters between an intruder and females in different reproductive states (see review by Ostermeyer, 1983). In the present experiments, the behavioral evolution of paired females, kept together in a complex environment throughout a reproductive cycle, sheds some light on the mechanisms involved in their territoriality.

Until late pregnancy, familiar females established friendly relations and nested together. This relationship appeared to be continuously reinforced through mutual body sniffing and touching. Olfactory investigation was clearly focused on scent gland areas already described in bank voles or supposed to exist in comparison with other rodent species (Brown, 1987).

As a first conclusion, we suggest that the odors of a familiar female act upon another female in a way that depends on her own status: as an aggression-inhibiting signal before pregnancy and as a releaser of aggressive behavior and scent marking later in pregnancy and early in lactation.

A lactating female responds swiftly and specifically to the attempted intrusions of another female by aggressiveness, scent marking, moving her young to a safer place, and fencing off the entrance. The latter point matches an obser-

vation of Mironov (1990), who reported that in the field, during the breeding season, bank vole females cover the entrance of their burrows with dry leaves whenever they leave or return home. The function of these various aspects of the young mother's behavior is clearly to protect the offspring.

When comparing our results with observations in the open, it should be kept in mind that, in the confined environment of the laboratory and without any competition for food, the breeding territory of each female is reduced to her nesting place. In this situation, the maternal responses may also be regarded as territorial behavior.

Scent marking with urine and probably also with flank gland secretions dispersed by scratching was often associated with agonistic interactions. This correlation was also observed during male–male interactions (Rozenfeld et al., 1987; Rozenfeld and Kyriamarios, unpublished) and is consistent with the "scent matching" hypothesis of Gosling (1982). Learning to associate the scent marks with the odor of the dominant vole would progressively enable the subordinate to flee in anticipation of an attack.

Finally, the scent marks of the mother may also play an important role in mother–offspring interactions. In the bushy-tailed woodrat, *Neotoma cinerea* (Escherich, 1981), and in the Mongolian gerbil, *Meriones unguiculatus* (Wallace et al., 1973; Yahr, 1976), mothers increase their ventral scent-marking during pregnancy and lactation. The secretions of this gland are attractive to pups and orient them towards their mother and the nest material (Gerling and Yahr, 1982).

The fact that in seven of nine pairs, the reproduction of one female was repressed is comparable to the result obtained in prairie voles (*Microtus ochrogaster*) when two females were paired with a single male (Carter and Getz, 1985). This phenomenon is probably not a laboratory artifact since it was observed in the field, in an island population of bank voles (Bujalska, 1970, 1985) and in an enclosed population of red-backed voles (Kawata, 1987).

At least two hypotheses may explain this inhibition: (1) tactile stimuli or pheromones picked up during female–female contacts may interfere with the physiology of breeding and/or (2) a dominant male could select his mate. In the prairie vole, the reproductive maturation of a virgin female, recently stimulated by a male, may be inhibited by a chemical signal (Batzli et al., 1977) conveyed by the urine of another female (Getz et al., 1983).

These findings are consistent with the hypothesis that pregnancy failure is one of the density-dependent factors that may temporarily decrease reproductive success of a female, until she has acquired an exclusive range in which to breed successfully (Kawata, 1987).

As a general conclusion, we think that aggressiveness and scent marking are two main aspects of the spacing behavior of breeding females. Under laboratory conditions, they are triggered in late pregnancy.

Acknowledgments—We are very grateful to Professor Raymond Rasmont for his valuable help in the preparation of this manuscript.

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Journal of Chemical Ecology, Vol. 20, No. 3, 1994

Book Review

Phytochemical Dictionary: A Handbook of Bioactive Compounds from Plants. Jeffrey B. Harborne and Herbert Baxter (eds.). London and Washington, D.C.: Taylor & Francis, 800 pp, $350.00.

Judging *Phytochemical Dictionary: A Handbook of Bioactive Compounds from Plants* by its cover and overall quality of production, one must conclude that this book is nothing short of a masterpiece. If, however, one attempts to extract useful information from it, one quickly realizes that this book, while still quite good, has some serious flaws. Do the book's good points overshadow its flaws, and is it destined to become a great handbook in the tradition of an established handbook such as the *Merck Index*? Of course, only time will tell, but I believe that the information offered is too limited to merit much more than occasional use.

Presented in this book is information on 2793 compounds from plants (from abietic acid to zygadenine), and almost all possess some biological significance. The manner in which the editors have arranged the 2793 entries is the first of the book's flaws, albeit a minor one. Unlike most dictionaries, which simply list the entries alphabetically, the editors chose to group the entries first by a major compound class [i.e., carbohydrates and lipids, nitrogen-containing compounds (excluding alkaloids), alkaloids, phenolics, or terpenoids]; then each major class is further divided into subclasses (for example, the terpenoids are classified as monoterpenoids, iridoids, sesquiterpenoids, sesquiterpene lactones, diterpenoids, triterpenoid saponins, steroid saponins, cardenolides and bufadienolides, phytosterols, cucurbitacins, nortriterpenoids, miscellaneous triterpenoids, or carotenoids). The entries are listed alphabetically within each subclassification.

This classification system results in 60 separate alphabetical listings (chapters) of compounds, a system that denies a reader the ability to simply look up a compound. As an example, let us try to obtain information on quinine, a rather well-known compound found in the bark of Cinchona trees. First, the user must know that quinine is an alkaloid, a pretty well-known fact although not universally known. Next, the user must know that quinine is a quinoline alkaloid. Let us assume that the user is quite knowledgeable about alkaloids so that the above required knowledge is not a problem. Now, our user must know that quinoline alkaloids start on page 267. The only way to know this is to look in the table of contents. Of course, the user could find quinine in the index (entry number

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1038, no page listed) and leaf through until entry number 1038 is found on page 279. In fact, unless one is interested in perusing the handbook, the index is the only practical way to find something.

This inconvenience is hardly bothersome to a user looking for one or two compounds, but it becomes increasingly annoying as the number of compounds being sought increases. The editors should have used a dictionary format (after all, they call it a dictionary) in which all of the entries are listed alphabetically; the class (and subclass) could easily have been included as part of the listing. In doing away with the chapters, the editors could have collected their brief introductions to each compound type and presented them together as an introduction to the dictionary portion of the book.

The individual entry for each compound is obviously the purpose of the book. Each entry lists the compound's name and a list of synonyms. These synonyms are cross-linked in the index. Following its name is a stereochemically accurate structural formula for nearly all compounds listed. These structural formulas are clear and well presented; they are arguably the most useful feature of the book. Below the structural formulas are molecular formulas and molecular weights (calculated to two decimal places) for nonpolymer listings. Each entry usually ends with two paragraphs of information about the compound. The first paragraph covers the occurrence of the compound in the plant kingdom; the second paragraph describes its biological significance with a special emphasis on medical or veterinary uses. These paragraphs tend to be short and suggestive rather than long and exhaustive. Conspicuously lacking in these entries are any other chemical data, and I am personally disappointed to find no spectral data.

So far, I have described a generally useful handbook of plant compounds, which has very readable type and beautifully presented structures of the compounds. The flaw that I have mentioned is minor and probably will not bother many people as it bothers me. There are, unfortunately, two other flaws which I think should have a much greater impact on whether this book will find its way to the book shelves of the pharmacists, pharmacognosists, food scientists, nutritionists, chemical ecologists, natural product chemists, and phytochemists for which the editors target this book.

Although the entries are useful, they lack one of the most important types of information that professionals can use: references to primary literature sources. The editors, writing in the introduction, gloss over this glaring omission with a single sentence, "References to the primary literature have not been included in each entry, because they are generally accessible in other reference works." In other words, if one finds the compound of interest and requires more detailed information, one must start over with some other secondary source in order to get references to primary literature. As useful as the information contained in the book is, the book is a dead end.

The other serious shortcoming of this book concerns the index. Each com-

pound entry is indexed only by its name and its synonyms (indispensable to this book) but not by occurrence (among plant genera or species) or by biological properties. This shortcoming became painfully clear to me the very first time I opened the book. A colleague had asked me if I knew of other compounds from the neem tree, *Azadirachta indica*, besides azadirachtin, which possess similar activity. A phytochemical dictionary seemed an ideal starting place for a search. To my dismay, there were no species or genera listed in the index and I could find no simple way to find the information unless I had a compound name. Thus, my first encounter with this book proved fruitless simply because the book is only indexed with the names of the compounds.

Overall, this book is exceptionally well produced (a pleasure to look at), it contains a wealth of information, and, with revision, may become an indispensable tool to anybody interested in quick information about phytochemicals. Currently, however, its lack of references to the primary literature and its parsimonious index greatly diminish its usefulness. I look forward to the second edition, which should include references to the primary literature and a greatly expanded index.

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Erratum

EVIDENCE FOR SEX PHEROMONES PRODUCED BY MALES AND FEMALES IN *Blatta Orientalis* (DICTYOPTERA, BLATTIDAE)

DEHBIA ABED, REMY BROSSUT, and JEAN-PIERRE FARINE

The above paper appeared in Volume 19, Number 12, pp. 2831–2853. Due to an inadvertent error, the incorrect figure printed as Figure 3 on p. 2838. The correct figure and caption are printed on the following page.

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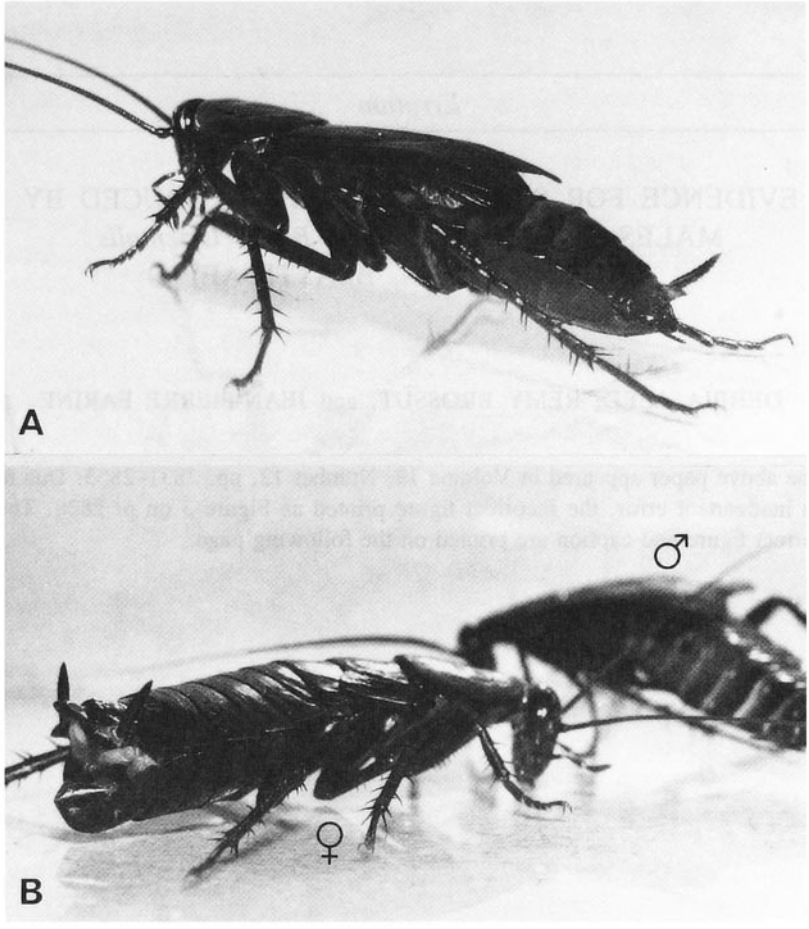

FIG. 3. *Blatta orientalis:* Courtship behavior. Calling posture of male: the extended abdomen fully exposes the tergites (A). After contact with a male, the female adopts a calling posture. The genital atrium is opened widely (B). The two turgescent whitish structures are the atrial glands.

Journal of Chemical Ecology, Vol. 20, No. 3, 1994

Erratum

NONFLORAL SOURCES OF CHEMICALS THAT ATTRACT MALE EUGLOSSINE BEES (APIDAE: EUGLOSSINI)

W. MARK WHITTEN, ALLEN M. YOUNG, and DAVID L. STERN

The above paper appeared in Volume 19, Number 12, pp. 3017–3027. Due to an inadvertent error, the incorrect figure printed as Figure 1 on p. 3020. The correct figure and caption are printed on the following page.

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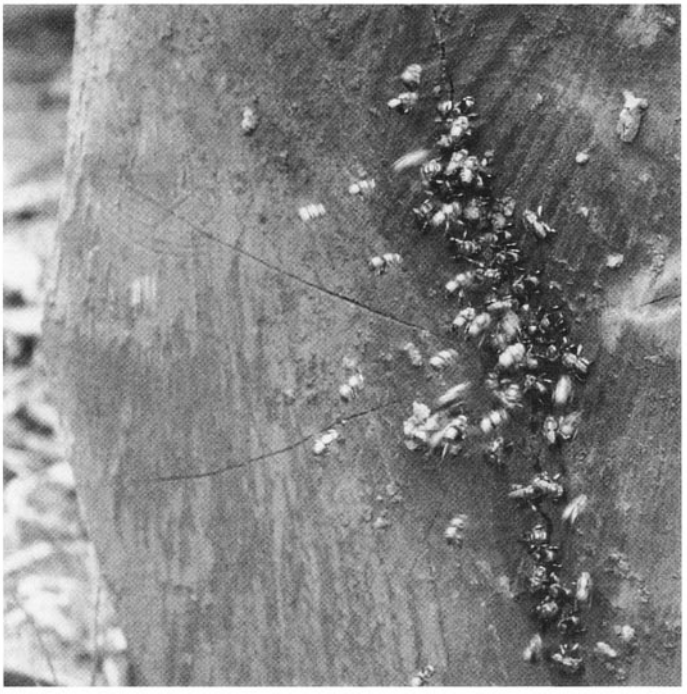

FIG. 1. Male *Euglossa purpurea* collecting volatiles from crack in sawn end of skatole-producing *Ocotea* log in Sarapiqui District, Costa Rica.

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NEW SESQUITERPENES FROM *Maytenus species* (Celastraceae). TAXONOMIC AND CHEMOTAXONOMIC CONSIDERATIONS CONCERNING CHILEAN *Maytenus*

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Abstract—Three new dihydro-β-agarofuran sesquiterpenes from two species of *Maytenus* were isolated and their structures were elucidated by means of ^{1}H and ^{13}C NMR studies. The differences and similarities noted in the chemical content of the dihydro-β-agarofuran sesquiterpenes from the four *Maytenus* species from Chile are in line with the taxonomic characterization of these species; their geographical distribution is also given.

Key Words—Chilean *Maytenus*, Celastraceae, dihydro-β-agarofuran sesquiterpenes, chemotaxonomic relationships.

INTRODUCTION

Nine species of the genus *Maytenus* (Celastraceae) are found in the Antarctic Andean woodland covering parts of Argentina and Chile: *M. spinosa* Gris., *M. scutioides* Gris., *M. viscifolia* Gris., *M. vitis-idaea* Gris., *M. ilicifolia* Mart. et Reissek, *M. Boaria* Molina, *M. disticha* Hook, *M. chubutensis* Speg., and *M. magellanica* Lam. These plants were researched intensively from the botanical

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point of view (Lourteig and O'Donnell, 1955), and the taxonomic differences between some of the species were so slight that it proved difficult to determine their proper botanical classification. Polyester dihydro-β-agarofuran sesquiterpenes are found only in the Celastraceae and are considered to be chemotaxonomical markers (Brüning and Wagner, 1978), while nonesterified sesquiterpenes with the same skeleton occur as natural products only in the fungus *Aquilaria agallocha* (Varma et al., 1965) and some species of *Myrtales* and *Santalaceae* (Brüning and Wagner, 1978).

This paper reports the isolation and structural elucidation of two new dihydro-β-agarofuran-type sesquiterpenes isolated from *M. magellanica* (Lourteig and O'Donnel, 1955) known locally as "black mayten" and one new sesquiterpene isolated from *M. boaria*, "mayten," a species that has several popular applications (Farga and Lastra, 1988). This paper also gives details of the four species of *Maytenus* found in Chile, *M. chubutensis*, *M. disticha*, *M. magellanica*, and *M. boaria* (Lourteig and O'Donnell, 1955), their geographical distribution and sesquiterpene constituents, which are in agreement with their diverse taxonomic characteristics.

METHODS AND MATERIALS

Experimental Procedures. IR spectra were taken on a PE 681 spectrophotometer and ^{1}H and ^{13}C NMR on a Bruker WP-200 SY in $CDCl_3$ at 200 and 50 MHz, respectively, with TMS as internal reference. The HMBC was run on a Bruker at 400 MHz. Optical rotations were measured on a PE 550-SE and CD spectra on a Jasco J-600 spectropolarimeter. MS were recorded on a VG Micromass ZAF-2F and a Hewlett-Packard 5995. UV spectra were collected on a Perkin-Elmer model 550-SE.

The plants, *M. magellanica* and *M. boaria*, were gathered in January 1987 in the Novena region, Province of Temuca, on the slopes of the Osorno volcano in Chile, and in the National Park Vicente Perales Rosales, respectively. Voucher specimens (Nos. 93-5341C and 87-43042, respectively) are on file with the Facultad de Ciencias, Universidad de Chile, Santiago.

The aerial part of *M. magellanica* and *M. boaria* (4 kg and 2 kg, respectively) was extracted with EtOH (10 dm^3) at room temperature for a week. The extracts (250 g and 153 g) were repeatedly chromatographed on Sephadex LH-20 using a mixture of *n*-hexane–$CHCl_3$–MeOH (2 : 1 : 1) and silica gel with different mixtures of *n*-hexane, ethyl acetate, and benzene as eluants to afford the following products: **25** (4 mg) and **26** (4 mg) from *M. magellanica* and **34** (6 mg) from *M. boaria*.

1α,9β-Dibenzoyloxy-2β,6β,8β-triacetoxy-4β-hydroxy-dihydro-β-agaro-

furan (25). Oil; molecular formula, $C_{35}H_{40}O_{12}$ [found, (M^+-$COCH_2$), 610.2399; requires, 610.2384]; $[\alpha]_D^{20}$ = +31.7° ($CHCl_3$, c 0.06); IR ν_{max} ($CHCl_3$) cm^{-1}: 3551, 3447, 3018, 2930, 1737, 1602, 1452, 1369, 1281, 1236, 1210, 1096; UVλ_{max} (EtOH) nm: 228, 274, 282; ^{1}H NMR (200 MHz) δ: 1.49 (3H, s), 159 (3H, s), 1.66 (3H, s), 1.68 (3H, s), 1.79 (3H, s), 1.82 (3H, s), 2.16 (3H, s), 2.44 (1H, d, J = 3.2 Hz, H-7), 3.03 (1H, s, HO−), 5.13 (1H, m, H-2), 5.35 (1H, d, J = 6.2 Hz, H-9), 5.59 (1H, s, H-6), 5.62 (1H, dd, J = 3.2, 6.2 Hz, H-8), 5.91 (1H, d, J = 10.5 Hz, H-1), 7.28–8.02 (10H, m); ^{13}C NMR (100 MHz) δ: 20.51 (q, C-15), 24.83 (q, C-14), 26.52 (q, C-12), 30.25 (q, C-13), 44.23 (t, C-3), 50.63 (s, C-10), 53.84 (d, C-7), 68.57 (d, C-8), 68.65 (d, C-2), 70.84 (s, C-4), 71.84 (d, C-9), 72.36 (d, C-1), 77.32 (d, C-6), 85.28 (s, C-11), 91.05 (s, C-5); EI-MS *m/z* (rel. int. %): 610 [M^+-$COCH_2$] (1), 592 (1), 550 (1), 532 (1), 515 (4), 490 (1), 455 (2), 428 (1), 410 (1), 335 (1), 105 (100).

2β,8β-Diacetoxy-1α,6β,9β-tribenzoyloxy-4β-hydroxy-dihydro-β-agarofuran (26). Oil; molecular formula, $C_{40}H_{42}O_{12}$ [found, [M^+], 714.2711; requires, 714.2746]; $[\alpha]_D^{20}$ = +45.4° ($CHCl_3$, c 0.37); IR ν_{max} ($CHCl_3$) cm^{-1}: 3520, 2920, 2840, 1710, 1265, 1105, 1070, 1025, 710; UVλ_{max} (EtOH) nm: 229, 272, 280; ^{1}H NMR (200 MHz) δ: 1.52 (3H, s), 1.59 (6H, s), 1.70 (3H, s), 1.78 (3H, s), 1.82 (3H, s), 2.15 (2H, m, H-3), 2.65 (1H, d, J = 2.0 Hz, H-7), 3.25 (1H, s, HO−), 5.15 (1H, m, H-2), 5.40 (1H, d, J = 6.4 Hz, H-9), 5.72 (1H, s, H-6), 5.75 (1H, dd, J = 2.0, 6.4 Hz, H-8), 5.95 (1H, d, J = 10.0 Hz, H-1), 7.40–8.25 (15H, m); ^{13}C NMR (100 MHz) δ: 20.85 (q, C-15), 24.79 (q, C-14), 26.56 (q, C-12), 30.36 (q, C-13), 44.51 (t, C-3), 50.94 (s, C-10), 53.61 (d, C-7), 68.57 (d, C-2), 68.57 (d, C-8), 71.14 (s, C-4), 71.90 (d, C-9), 72.42 (d, C-1), 78.16 (d, C-6), 85.36 (s, C-11), 90.96 (s, C-5); EI-MS *m/z* (rel. int. %): 714 [M^+] (1), 699 (1), 654 (1), 592 (1), 577 (7), 517 (2), 428 (1), 231 (3), 189 (3), 105 (100).

(1R, 2S, 3S, 4S, 5S, 6R, 7R, 9S, 10R)-2-acetoxy-1,9-dibenzoyloxy-3,4-6-trihydroxy-dihydro-β-agarofuran (34). Amorphous solid; $[\alpha]_D^{20}$ = +76.5 ($CHCl_3$, c 0.23); IR ν_{max} ($CHCl_3$) cm^{-1}: 3447, 1734, 1652, 1450, 1367, 1282, 1176, 1113, 1070, 1026, 979, 710; ^{1}H NMR (200 MHz) δ: 1.54 (3H, s), 1.60 (6H, s), 1.77 (3H, s), 1.86 (3H, s), 2.17 (3H, m), 3.18 (1H, d, J = 3.0 Hz), 4.22 (1H, s, HO−), 4.56 (1H, s, H-6), 5.04 (1H, d, J = 6.4 Hz, H-9), 5.35 (1H, dd, J = 3.0, 11.3 Hz, H-2), 6.19 (1H, dd, J = 11.3 Hz, H-1), 7.22–7.92 (10H, m); EI-MS *m/z* (rel. int. %): 568 [M^+] (1), 553 (16), 550 (1), 534 (16), 508 (6), 493 (5), 479 (4), 430 (6), 386 (15), 264 (8), 248 (11), 220 (8), 202 (7), 166 (8), 130 (10), 105 (100), 77 (35), 43 (33).

Acetylation of 34. Acetic anhydride (four drops) was added to compound **34** (2 mg) dissolved in pyridine (two drops) and the mixture was left at room temperature for 16 hr; EtOH (3 × 2.0 ml) was added and carried almost to

dryness in a rotavapor and this process was repeated with C_6H_6 (3 × 2.0 ml) to give **16** (2 mg).

RESULTS AND DISCUSSION

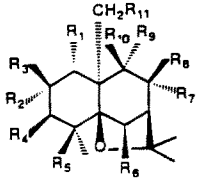
Earlier papers have described our findings from the study of the major constituents of the four Chilean *Maytenus* (González et al., 1989, 1990a,b, 1992a,b). Here we report the isolation of minor constituents from *M. magellanica* (**25** and **26**) and *M. boaria* (**34**), completing our phytochemical analysis of these species and allowing certain conclusions to be drawn about their sesquiterpene composition.

After repeated chromatography of the ethanol extract of the aerial part of *M. magellanica* on Sephadex LH-20 and silica gel, compounds **25** and **26** were obtained; *M. boaria* yielded **34.**

Compound **25** had the molecular formula $C_{35}H_{40}O_{12}$ (HR-EI-MS) with IR absorption bands for a hydroxy group (3551 cm^{-1}) and ester groups (1737 cm^{-1}). The electron impact mass spectrum showed fragments at *m/z* 105 and (M^+-60), suggesting that benzoate and acetate groups were present in the molecule. This was confirmed by the ^1H and ^{13}C NMR spectroscopic data, which included signals for 10 aromatic protons between 7.28 and 8.02 δ, three acetate methyls as singlets at 1.79, 1.82, and 2.16 δ, two carboxylic benzoate carbons at 165.2 and 166.0 δ, and three acetate carboxylic carbons at 169.0, 170.0, and 170.5 δ; all these data indicated that **25** was a polyester sesquiterpene with a dihydro-β-agarofuran skeleton.

The substituent positions were determined as 1α, 2β, 4β, 6β, 8β, and 9β from a careful study of the coupling constants and COSY experiments and were confirmed by a Roesy experiment. The regiosubstitution characteristics were ascertained by a long-range correlation ^1H-^{13}C NMR spectrum with inverse detection (HMBC), thus the 1-H (5.91 δ) and 9-H (5.35 δ) were clearly three-bond-coupled with the carboxylic carbons of two benzoates; the 2-H (5.13 δ), 6-H (5.59 δ), and 8-H (5.62 δ) were three-bond-coupled with the carboxylic carbons of three acetates, which positioned the benzoate groups at C-1 and C-9, the acetate groups at C-2, C-6, and C-8, and the hydroxy group at C-3. The structure of **25** is 1α,9β-dibenzoyloxy-2β,6β,8β-triacetoxy-4β-hydroxy-dihydro-β-agarofuran (Figure 1).

A detailed study of the spectroscopic data of compound **26** (Figure 1), which had the molecular formula $C_{40}H_{42}O_{12}$ (HR-EI-MS), showed it to be related to **25** with the most notable differences in ^1H NMR being the existence of 15 aromatic protons between 7.40 and 8.25 δ instead of 10 as in **25,** the disappearance of one of the acetate methyls at 2.16 δ and the corresponding shift of the H-6 from 5.59 δ to 5.72 δ, establishing its regiosubstitution, which was



M. chubutensis

	OH	OAc	OBz	
1		1α,2α,6β,8α,15	9β	
2	15	1α,2α,6β,8α	9β	
3	8α	1α,2α,6β,15	9β	
4	8α,15	1α,2α,6β	9β	
5		1α,2α,6β,15	9α	8(C=O)
6	15	1α,2α,6β	9α	8(C=O)
7	15	1α,2α,6β	9β	
8		1α,2α,6β,15	9β	
9	8α,15	1α,2α,6β	9α	
10	15	1α,2α,6β,8α	9α	

M. magellanica

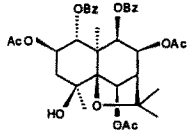
	OH	OAc	OBz
15	3β,4β	2β,6β	1α,9β
16	4β	2β,3β,6β	1α,9β
17	2β,4β	3β,6β	1α,9β
18	3β,4β	2β	1α,9β
19	4β	2β,3β	1α,9β
20	3β	2β,6β	1α,9β
21	4β	2β,3β	1α,6β,9β
22	4β	2β,3β,8β	1α,9β
23	3β,4β	2β,8β	1α,9β
24	4β	2β,3β	1α,8β,9β
25	4β	2β,6β,8β	1α,9β
26	4β	2β,8β	1α,6β,9β
27	4β	2α,6β,8β	1α,9β
28	4β	2β	1α,6β,9β
29	4β	2β,6β	1α,9β
30	4β	2β	1α,9β
31	4β		1α,9β
32	4β	6β	1α,9β

M. disticha

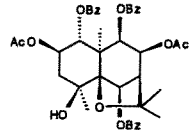
	OH	OAc	OBz
1, 2			
11	4β	6β,8β	1α,9β
12	15	1α,6β	9β
13	8α	1α,6β,15	9β
14		1α,6β,8α,15	9β

M.boaria

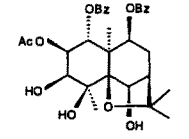
	OH	OAc	OBz	
	6,8,13, 15-18, 20,25			
33	15	1α,6β,8α	9α	
34	3β,4β,6β	2β	1α,9β	
35	3β,4β	2β,6β	1α	9β(OCinn)
36	3β,4α			



25



26



34

FIG. 1. Sesquiterpenes from *Maytenus chubutensis*, *M. disticha*, *M. magellanica*, and *M. boaria*.

confirmed by HMBC and Roesy experiments. Compound **26** is 2β,8β-diacetoxy-1α,6β,9β-tribenzoyloxy-4β-hydroxy-dihydro-β-agarofuran. Compounds **25** and **26** had the basic 2β,4β-dihydroxy-8-epi-celapanol polyhydroxy skeleton (Wagner et al., 1975).

Compound **34** (Figure 1) had a molecular ion of *m/z* 568 with fragments at *m/z* 105 (M^+-60) and (M^+-18) (EI-MS), suggesting that benzoate, acetate, and hydroxy groups were to be found in the molecule, and this was confirmed by the ^1H NMR spectroscopic data, which included signals for 10 aromatic protons between 7.22 and 7.92 δ and one acetate methyl at 1.86 δ. When **34** was acetylated under standard conditions, it afforded a product with physical and spectroscopic data superimposable on those of another compound earlier isolated from the same species, **16** (González et al., 1992a) and provided with a firm absolute configuration; that of **34** was therefore (1*R*,2*S*,3*S*,4*S*,5*S*,6*R*, 7*R*,9*S*,10*R*)-2-acetoxy-1,9-dibenzoyloxy-3,4,6-trihydroxy-dihydro-β-agarofuran and its basic polyhydroxy skeleton, magellanol (González et al., 1992a).

The most notable features of the geographical distribution of *M. chubutensis*, *M. disticha*, *M. magellanica*, and *M. boaria* were recorded by Lourteig and O'Donnell. Three of the four species are found in Tierra del Fuego, at the extreme south of the American continent, while *M. chubutensis* grows much further north near Santiago de Chile and, at its most southerly, in the Aysen region near Cohiaiqué. *M. boaria* is the most extended of the species, throughout the length of the Chile from Arica on the northern border with Peru to Tierra del Fuego; the four species are most abundant in a wild state in the national parks in both Argentina and Chile.

From the taxonomical point of view, the four species have the characteristics of the genus *Maytenus*, subgenus *Eu-maytenus* Loes, observed both in the descriptions given by Lourteig and O'Donnell and in the herbarium specimens. Table 1 lists the most characteristic differences and similarities of the four species; careful analysis of these data indicates that *M. chubutensis* and *M. disticha* have similar biotypes (shrub or subshrub), branches (pilose), and leaves (distichous), while *M. magellanica* and *M. boaria* are alike in aspect (treelike), with glabrous branches and nondistichous leaves; their leaves and flowers are visibly larger than those of *M. chubutensis* and *M. disticha*. On the other hand, characteristics such as pedunculate inflorescence, pentameric flowers, sepal morphology, and aril brevity clearly differentiate *M. magellanica* from the other three taxa.

Analysis of the sesquiterpenes of the four species of *Maytenus* under study has given 36 new polyester sesquiterpenes (Figure 1). *M. chubutensis* biosynthesizes metabolites **1–6** (González et al., 1990a) and **7–10** (González et al., 1989), *M. disticha* **1, 2,** and **11–14** (González et al., 1990b), and *M. magellanica* **15–32** (González et al., 1992b). None of these latter substances occur in *M. disticha* or *M. chubutensis*. *M. boaria* (González et al., 1992b) yielded metabolites **6, 8, 13, 15–18, 20, 28,** and **33–35.** Thus, *M. chubutensis*, *M. disticha*, and *M. boaria* have some sesquiterpenes in common, while *M. magellanica* is chemically correlated only with *M. boaria* and so the species *M. magellanica*, which is taxonomically most different from the others, is also

TABLE 1. DIFFERENCES AND SIMILARITIES OF FOUR CHILEAN *Maytenus*

	Maytenus chubutensis	*Maytenus disticha*	*Maytenus magellanica*	*Maytenus boaria*
Biotype	Shrub or subshrub	Shrub or subshrub	Bush or tree	Tree
Branches	Pilose, short rigid hairs	Pilose, erect hairs	Glabrous	Pendula, glabrous
Leaves	Distichous	Distichous	Not distichous	Not distichous
	Petiole 0.5–3 mm, pubescent	Petiole 0.3–1 mm, glabrous	Petiole 2–7 mm, glabrous	Petiole 2–5 mm
	Limbus 0.4–1.8 × 0.2–1.3 cm elliptical or obovate to soborbiculate, upper side with fine hairs (short rigid hairs), underside slightly pubescent occasionally dentate edges	Limbus 0.3–1.5 × 0.2–0.6 cm elliptical or oblong obovate glabrous or, occasionally, slightly pilose on upper side smooth edges	Limbus 1.5–8 × 1–3.5 cm lanceolate or elliptical (rarely obovate) upper and undersides glabrous saw-toothed edges	Limbus 1.8 × 0.4–2 cm lanceolate or elliptical (rarely obovate) dentate edges
Inflorescence	No peduncle	No peduncle	Peduncles 0.5–3 mm	No peduncle
Flowers	Tetramer unisexual (also hermaphrodite)	Tetramer, unisexual (also hermaphrodite)	Pentamer (seldom quatrimer) unisexual or hermaphrodite	Tetramer
	Masculine flowers oval sepals oval petals 1.5–2 mm	Masculine flowers oval sepals oval petals 1.2–1.5 mm	Masculine flowers suborbiculate sepals elliptical or subobovate petals 2.5–3 mm	Masculine flowers oval sepals elliptical or subobovate petals 2.5–3 mm
	stamens 1–1.3 mm anthers 0.4–0.5 mm tetra or pentagonal disk	stamens 0.8–1 mm anthers 0.2–0.3 mm tetragonal disk	stamens 1.8–2.2 mm anthers 0.6–0.8 mm pentagonal disk	stamens 1.8–2.3 mm anthers 0.8–1 mm pentagonal disk
	Feminine flowers notable style stamens 0.5 mm anthers 0.2–0.3 mm	Feminine flowers short style stamens 0.5 mm–0.7 mm anthers 0.2 mm	Feminine flowers 0.5 mm style stamens 0.8–1.5 mm anthers 0.3–0.5 mm	Feminine flowers short style stamens 0.5–0.7 mm anthers 0.3–0.4 mm
Seeds	Completely covered by aril	Completely covered by aril	With short aril no more than lower third (aril only at base)	Completely covered by aril

different in chemical composition. Lourteig and O'Donnel (1955) proposed that all four species be included in the section *Microphylla* Loes. with *M. magellanica* in transition towards the *Pachyphylla* Loes. section. Our chemical findings reinforce the purely taxonomical considerations and attest to the singularity of *M. magellanica*.

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INFLUENCE OF WHITE PINE WATERING REGIMES ON FEEDING PREFERENCES OF SPRING AND FALL ADULTS OF THE WHITE PINE WEEVIL *Pissodes strobi* (PECK)

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Abstract—Spring and fall adults of the white pine weevil, *Pissodes strobi* (Peck), were exposed in no-choice and two-choice tests to bark from water-stressed and non-water-stressed white pine (*Pinus strobus* L.), which had also been exposed or not exposed to weevil attack. This experiment demonstrated that the weevils could discriminate between bark from water-stressed white pine and preferred bark from the nonstressed plants. The weevils also preferred bark from nonstressed plants that were previously exposed to weevil damage. Spring and fall adults displayed the same feeding preferences. No sex differences were found in feeding preferences. Less nitrogen, phosphorus, and potassium were found in bark from the nonstressed plants, and the potassium level was higher in damaged plants. We expect that the biological performance of the weevil should be favored by vigorously growing plants rather than by stressed plants.

Key Words—*Pissodes strobi*, white pine weevil, Coleoptera, Curculionidae, *Pinus strobus*, white pine, plant vigor, water stress.

INTRODUCTION

In a natural environment, plants are subjected to a number of biotic and abiotic stresses that can influence their growth and development (Heinrichs, 1988; War-

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ing, 1991). Plant stress resulting from drought or poor growing conditions can have positive effects on insect development (Goyer and Benjamin, 1972; Hodges and Lorio, 1975; Munster-Swendsen, 1984; Mattson and Haack, 1987a,b). It has been proposed that plant stress resulting from water deficit or nutrient imbalances can lead to improvement of the nutritional quality of the plant and/or a decrease in individual plant resistance that results in enhanced insect performance (White, 1974, 1984; Rhoades, 1979, 1983; Cates et al., 1983; Larsson et al., 1983; Waring and Pitman, 1985; Mulock and Christiansen, 1986; Mattson and Haack, 1987a,b). However, insect responses to plants growing under stressful environmental conditions are poorly documented (Louda and Collinge, 1992). Moreover, as indicated by Mattson and Haack (1987a), the stress theory is largely based on circumstantial evidence. The review made by Waring and Cobb (1992) illustrates the diversity of insect responses to water stress. However, for some insect species, it has been demonstrated that a nonstressed or vigorous plant constitutes a more adequate host (Miles et al., 1982; Myers, 1985; Wagner, 1986; Waring and Price, 1988; Price, 1989; Wagner and Frantz, 1990).

The white pine weevil [*Pissodes strobi* (Peck)] is a native insect that attacks the leader of several pine and spruce species, although white pine (*Pinus strobus* L.) and Norway spruce [*Picea abies* (L.) Karst] are preferred in eastern North America (Belyea and Sullivan, 1956). Most of the studies and field observations on the white pine weevil show a positive relationship between vigorous pine growth and weevil attack. The insects of this species attack young plantations more frequently than old ones and prefer plants with long terminal shoots and large leader diameters (MacAloney, 1930; Prebble et al., 1951; Kriebel, 1954; Holst, 1955; Connola, 1965; Wilkinson, 1983). Lavallée et al. (1993b) demonstrated that weevil development, expressed as the weight of emerging summer adults, can be affected by bark quality, as adult weight is heavier on well-hydrated white pine leaders than on severely water-stressed ones. Some investigators have reported that damage can be more pronounced when white pines are growing in nutrient-deficient or poorly drained soils (Maughan, 1930; Connola and Wixson, 1963; Dirks, 1964; Xydias and Leaf, 1964; Connola and Birmingham, 1978). On Sitka spruce [*Picea sitchensis* (Bong.) Carr.], a common host of the white pine weevil in western North America, Warkentin et al. (1992) have shown that more serious damage occurs in plantations located in geographic areas where trees are subjected to more important midday air moisture stress. To understand these apparent contradictions in the white pine weevil–host relationship, it is important to examine the sensitivity of the adult weevil to the growing conditions of its host, as assessed by its feeding preferences.

The objectives of this study were to determine: (1) if the white pine weevils could discriminate between bark from white pine plants grown under different water regimes, (2) if weevils could discriminate between bark from attacked

and intact hosts, and (3) if the age or sex of the adults had any influence on feeding preferences.

METHODS AND MATERIALS

Insect Collection and Rearing. Adult insects were collected on white pine leaders during the first week of May 1991 in two white pine plantations (Saint-Luc-de-Champlain (46°30'N, 72°30'W) and Sainte-Béatrix (46°13'N, 73°38'W)). The insects were sexed according to techniques described by Lavallée et al. (1993a) and kept at 5°C on an artificial diet (see below). These adults are referred to here as spring adults. In July 1991, white pine leaders infested during the spring were harvested at the Saint-Luc plantation and stored in cages at room temperature (20°C). The insects that emerged from these leaders were then placed on an artificial diet and kept at 5°C. These insects are subsequently referred to as fall adults.

To facilitate insect rearing and to avoid the possible effects of chemical variations of natural diet on feeding preferences, weevils were kept on an artificial diet (Trudel et al., in press) to which was added 2% (w/v) oven dry white pine bark collected in the spring. The diet was dispensed into Petri dishes (100 × 15 mm) and the diet surface was covered with a piece of Whatman No. 1 filter paper while still hot. The diet was replaced bimonthly to avoid contamination.

Plant Treatments and Monitoring of Soil Water Moisture. Five- and 6-year-old white pine was used in 1989 and 1990, respectively. Trees were planted in a sandy loam and subjected to two watering regimes, with and without insect attack. In 1989 and 1990, the experiments were conducted in a greenhouse in which the plants were placed on May 29 for both years and watered daily until June 10, 1989, and June 15, 1990. Each year, 64 white pine were arranged in four randomized blocks, with or without insects, two watering regimes, and four sampling dates. Four plants in each block per watering regime were selected randomly; two male and two female weevils were introduced into a screened plastic bag placed over the leaders of the previous year to allow feeding, mating and oviposition. In 1989, insects were allowed to feed, copulate, and lay eggs from June 1 through June 14, and in 1990 insects were left on plants from May 31 through June 18. Gypsum blocks (Hoskin Scientific, Montréal) were used to measure soil water potentials. In the dry treatment, soil water potentials were maintained close to −0.15 MPa, whereas for the wet treatment, soils were kept above −0.03 MPa. In the dry treatment, plants received 100 ml of water when necessary to raise the soil water potential. Plants in the wet treatment received at least 300 ml of water on a daily basis. Being normally distributed, soil water potential values between treatments were compared using an F test in the general linear model procedure (GLM) (SAS Institute, 1989).

Bark Collection and Measurements on Greenhouse Seedlings. Every two weeks over an eight-week period, 16 plants were removed from the greenhouse for bark collection and seedling water potential measurements, starting on June 15 and June 20, respectively, for each year. To measure differences in the water potential related to the different watering regimes, the midday xylem water potential was taken (Ruiz-Sanchez et al., 1988). The midday stress may reflect more accurately the living conditions of insects on the plants, given environmental conditions prevailing at that time of the year. Moreover, Sucoff (1972) demonstrated with red pine that midday stress shows less variation during hours of high evaporation demand. Then, at each sampling date, plants were brought to the laboratory around noon; three twigs per seedling were cut with a razor blade to measure the xylem water potential using a Scholander pressure chamber (model 1000, PMS Instruments, Corvallis, Oregon). Bark water content (BWC) was recorded on a dry weight basis {BWC = 100* × [(fresh weight − dry weight)/dry weight]} after a drying period of 48 hr at 70°C. The bark was then ground in a Wiley Mill (20 mesh) and kept at −20°C for feeding preference tests and chemical analyses.

The chemical analyses (nitrogen, phosphorus, and potassium) were performed on bark of each seedling from the 1989 greenhouse test according to the techniques of Kalra and Maynard (1991). The bark mixtures from the 1990 greenhouse test that were used in the feeding preference tests were also analyzed for their chemical content. Differences in chemical content among treatments were compared by analysis of variance using the GLM procedure (SAS Institute, 1989).

Feeding Preference Tests. The feeding preference tests were run with bark from the 2-year-old leader section harvested during the 1990 greenhouse test. Feeding tests were completed using a design modified from that of Alfaro et al. (1979). A 3.7-mm-thick lining of white styrofoam with four equidistant wells of 1 cm in diameter was placed in a 50-mm × 9-mm Petri dish. A few drops of 2% (w/v) neutral agar (USP, 100 mesh, Bioserv) were dispensed into each well to seal the bottom. Two types of feeding test were performed. In the two-choice tests, bark from each treatment was added to the agar at 1% per volume (Alfaro et al., 1979) and dripped into diagonally opposed wells. In no-choice feeding tests, bark was added at 1% (w/v) and dispensed into the four wells. A filter paper (1.1 cm diameter, Reeves Angel No. 202, Whatman) was placed on each well while the agar was still hot. One-millimeter holes were drilled in the Petri cover to prevent condensation.

Before beginning a feeding preference test, insects were starved for 24 hr at 25°C under a 16-hr photoperiod. For each test, two females or two males were placed per Petri dish during 24 hr under the same light and temperature conditions. The number of feeding punctures made by the insects, which has been shown to be a good indicator of food preference (Alfaro et al., 1979;

Piskornik et al., 1989), was used to compare insect response to bark from the different treatments.

Preference for Bark from Water-Stressed and Non-Water-Stressed Seedlings. In a series of two-choice tests, spring or fall adults of both sexes were exposed to barks from water-stressed and non-water-stressed plants. We used bark from plants with or without insects from the first (June 20) or last (July 30) harvest. Each test was done with 15 Petri dishes and repeated twice, for a total of 240 observations per test. Numbers of feeding punctures were transformed to their square root to achieve variance homogeneity and normality of the residuals. All analyses were performed using the GLM procedure (SAS Institute, 1989).

In a second type of two-choice test, we simultaneously tested preference for bark from water-stressed and non-water-stressed plants with spring or fall adults of each sex and with bark from the four sampling dates (Julian dates 171, 185, 199, 211). A schedule of nine replications gave a total number of 576 observations. Statistical analyses were performed with the GLM procedure (SAS Institute, 1989).

In no-choice tests, we evaluated the absolute feeding on bark from stressed or unstressed plants with spring or fall adults of each sex and with bark from the four sampling dates. A schedule of four replications gave a total of 512 observations. Statistical analyses were performed using the GLM procedure (SAS Institute, 1989).

RESULTS

Soil and Xylem Water Potential. Soil water potential and xylem water potential were affected by the different watering regimes. Measurements of soil water tension in 1989 and 1990 are presented in Table 1. During the 1989 and

TABLE 1. SOIL WATER POTENTIAL (−MPa) DURING EGG LAYING AND LARVAL DEVELOPMENT PERIODS OF WHITE PINE WEEVIL IN 1989 AND 1990

Year	Period	Treatment		$P > F$
		Dry	Wet	
1989	Oviposition	0.041 (0.003)[a]	0.037 (0.003)	0.2732
1989	Postoviposition	0.172 (0.006)	0.036 (0.004)	0.0001
1990	Oviposition	0.035 (0.0001)	0.035 (0.0001)	0.5884
1990	Postoviposition	0.094 (0.002)	0.035 (0.002)	0.0001

[a] Means (SEM).

1990 egg-laying periods, no significant differences were observed between soil water tensions for the different treatments. For each year, during the larval development periods, all soil tensions were significantly different. The non-water-stressed plants had a higher water potential (> -1 MPa) on all sampling dates (Table 2). In the dry treatment, the stress imposed on plants increased gradually during this study period, suggesting a rising level of stress.

Water, Nitrogen, Phosphorus, and Potassium Content. Bark water content decreased from one sampling date to another in the dry treatment but remained unchanged in the wet treatment in 1989 and decreased only slightly in 1990 (Table 3). For both years, at the first harvest, the bark water content was not significantly different between treatments but became different thereafter. The bark from water-stressed plants had the highest content of nitrogen, phosphorus, and potassium (Table 4). The mean values for the three elements were slightly higher in the bark from infested plants. However, the nitrogen and phosphorus content of bark from damaged plants was not statistically different from that of intact plants ($P_N = 0.93$; $P_P = 0.78$), but the potassium content was higher in plants with insect damage ($P_K = 0.05$). The same pattern was observed in bark used in feeding tests in 1990. The bark from the water-stressed plants had the highest content in the three elements, but potassium was not significant ($P_N = 0.01$; $P_P = 0.03$; $P_K = 0.07$). If we consider the chemical content according to insect presence or absence, plants with insects had the highest content of each element but only the difference in potassium was nonsignificant ($P_N = 0.02$; $P_P = 0.04$; $P_K = 0.50$).

TABLE 2. MIDDAY XYLEM WATER POTENTIAL (−MPa) FROM NEEDLES OF WHITE PINES SUBJECTED TO WET AND DRY SOIL MOISTURE TREATMENTS FOR DIFFERENT SAMPLING PERIODS IN 1989 AND 1990

Year	Date[a]	Treatment		$P > F$
		Dry	Wet	
1989	166	1.31 (0.05)[b]	0.99 (0.04)	0.0001
	178	2.11 (0.08)	0.85 (0.06)	0.0001
	192	2.92 (0.18)	0.72 (0.04)	0.0001
	206	2.95 (0.14)	0.78 (0.02)	0.0001
1990	171	1.26 (0.07)	0.94 (0.03)	0.0001
	185	2.22 (0.03)	0.87 (0.02)	0.0001
	199	2.59 (0.11)	0.67 (0.02)	0.0001
	211	2.60 (0.19)	0.90 (0.01)	0.0001

[a]Julian date: 1989: 166, 178, 192, 206 = June 15, 27; July 11, 25. 1990: 171, 185, 199, 211 = June 20; July 4, 18, 30.
[b]Means (SEM).

TABLE 3. BARK WATER CONTENT (% DRY WEIGHT) OF WHITE PINE SUBJECTED TO WET AND DRY SOIL MOISTURE TREATMENTS FOR DIFFERENT SAMPLING PERIODS IN 1989 AND 1990

Year	Date[a]	Treatment		$P > F$
		Dry	Wet	
1989	166	147.2 (6.0)[b]	154.4 (6.0)	0.4171
	178	122.1 (4.8)	163.8 (4.8)	0.0002
	192	109.1 (12.4)	182.2 (13.7)	0.0041
	206	89.4 (9.1)	164.1 (9.1)	0.0015
1990	171	197.7 (9.2)	225.8 (9.2)	0.0581
	185	139.0 (4.2)	203.5 (4.2)	0.0001
	199	108.9 (4.1)	193.7 (3.7)	0.0001
	211	109.7 (6.2)	169.3 (4.4)	0.0160

[a]Julian date: 1989: 166, 178, 192, 206 = June 15, 27; July 11, 25. 1990: 171, 185, 199, 211 = June 20; July 4, 18, 30.
[b]Means (SEM).

TABLE 4. NITROGEN, PHOSPHORUS, AND POTASSIUM CONTENT OF BARK HARVESTED IN 1989 FROM WHITE PINE GROWN UNDER DRY AND WET TREATMENTS

Chemical element	Watering regime		$P > F$
	Dry	Wet	
Nitrogen (%)	0.92 (0.03)[a]	0.78 (0.03)	0.019
Phosphorus (mg/g)	1.25 (0.03)	1.06 (0.03)	0.001
Potassium (mg/g)	6.88 (0.25)	5.61 (0.25)	0.006

[a]Means (SEM).

Feeding Preference Tests. If we consider only bark from plants that were not subjected to previous insect attack and larval feeding, the feeding response of adult weevils was affected by the watering regime and also by the period when bark was harvested (Figure 1). With the first harvested bark (date 171) from plants without insect damage, spring adults did not differentiate between bark from wet and dry treatments ($P = 0.87$) (Figure 1a). There was no sex difference in the number of feeding punctures ($P = 0.75$), and there was no interaction between sex and treatment ($P = 0.73$). However, fall weevils preferred bark from non-water-stressed plants over the bark from water-stressed plants, but the difference is nonsignificant ($P = 0.07$) (Figure 1a). Again, there

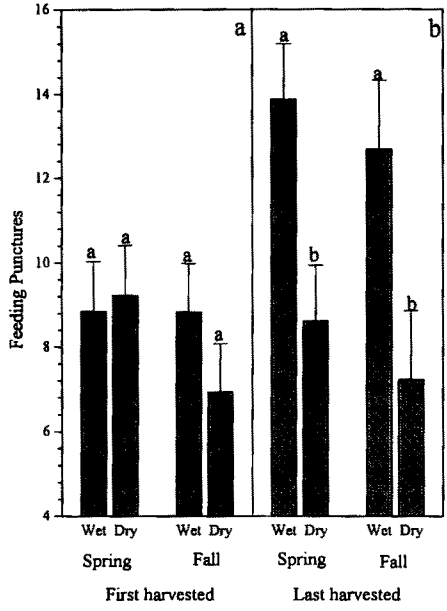

Fig. 1. Mean (+95% CI) number of feeding punctures on bark of non-water-stressed (wet) and water-stressed (dry) plants made by spring and fall white pine weevil adults on first harvested (Julian date 171) (a) and last harvested (Julian date 211) (b) bark from greenhouse-grown white pine that were not previously infested by insects; for each pair, values followed by different letters are significantly different (F test, $P < 0.05$).

was no difference between sexes in their preferences ($P = 0.24$), and there was no significant sex × treatment interaction ($P = 0.33$).

With the last harvested bark (Julian date 211) from plants without insect damage, spring and fall weevils of both sexes showed a significant preference for bark from wet treatments (Figure 1b). The spring and fall weevils made, respectively, 38% and 43% more feeding punctures on bark from the wet treatment than on bark from the dry treatment ($P_{spring} = 0.01$; $P_{fall} = 0.01$) (Figure 1b). For both ages, there was no difference between sexes in the number of feeding punctures ($P_{spring} = 0.33$; $P_{fall} = 0.33$) and the sex × treatment interaction was also not significant ($P_{spring} = 0.55$; $P_{fall} = 0.76$).

If we consider only the plants that supported insect attack and larval development, the feeding response of adults was affected by the watering regime and also by the period when bark was harvested. Spring and fall weevils made 26% and 60% more feeding punctures, respectively, on wet treatment bark ($P_{spring} < 0.01$; $P_{fall} < 0.01$) (Figure 2a). Female spring adults ate more than males

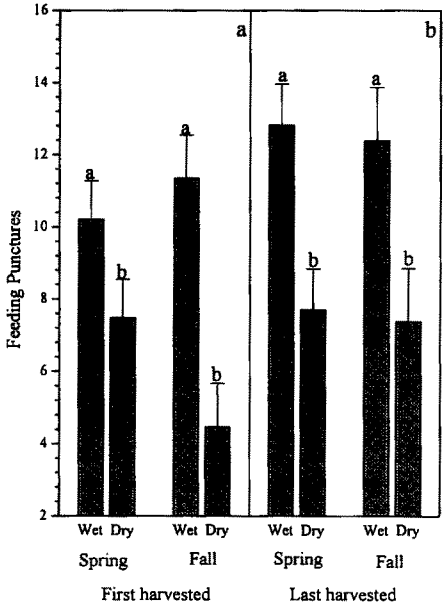

Fig. 2. Mean (+95% CI) number of feeding punctures on bark from non-water-stressed (wet) and water-stressed (dry) plants made by spring and fall white pine weevil adults on first harvested (Julian date 171) (a) and last harvested (Julian date 211) (b) bark from greenhouse-grown white pine that were previously infested by insects; for each pair, values followed by the different letters are significantly different (F test, $P < 0.05$).

($P < 0.01$) but there was no sex × treatment interaction ($P = 0.22$). With fall adults, males and females performed similarly ($P = 0.11$), and there was no significant sex × treatment interaction ($P = 0.69$). Furthermore, if we consider the first harvested barks, using plants infested by weevils, the preference for bark from the wet treatment was more pronounced (Figure 2a) than for bark from plants without insect damage (Figure 1a). With bark from the fourth harvest, spring and fall adults preferred bark from the wet treatment over the dry treatment ($P_{spring} < 0.01$; $P_{fall} < 0.01$) (Figure 2b). There was no difference in the number of feeding punctures between sexes of both ages ($P_{spring} = 0.96$, $P_{fall} = 0.89$). There was no sex × treatment interaction ($P_{spring} = 0.16$, $P_{fall} = 0.47$).

In a two-choice test, in which the four harvest periods, sex, and age were considered, wet bark was preferred over dry bark ($P < 0.01$) (Figure 3). Fall weevils made more feeding punctures than spring weevils ($P = 0.01$) (Figure 3). Females ate more than males ($P < 0.01$), and there was no age × treatment ($P = 0.30$). The effect of harvest date was not significant ($P = 0.72$).

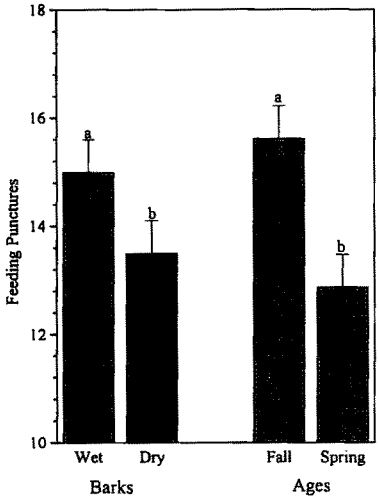

Fig. 3. Mean (+95% CI) number of feeding punctures made by spring and fall adult white pine weevils on bark from non-water-stressed (wet) and water-stressed (dry) white pine; for each pair, values followed by the different letters are significantly different (F test, $P < 0.05$).

In a no-choice test, when we considered simultaneously age, sex, and the four harvest dates, there was no difference in the preference for wet and dry bark ($P = 0.71$). The mean number of feeding holes were 17.6 (SEM = 0.87) and 17.2 (SEM = 0.87) for bark from wet and dry treatments, respectively. This indicates that, when forced to feed, the weevils will consume equal amounts of both types of bark. The effect of the harvest periods was not significant ($P = 0.62$). There was no significant interaction between bark and insect age ($P = 0.39$), and fall weevils did not eat more than spring weevils ($P = 0.07$). However, females consumed more than males ($P < 0.01$), but there was no sex × treatment interaction ($P = 0.53$).

DISCUSSION

The different watering regimes used in these tests affected the physiology of white pines as indicated by the xylem water potential. Although the effect of the water stress on precise physiological processes was not evaluated, a major impact on the plant may be suspected. Water stress can decrease growth directly by reducing turgor, photosynthesis, and translocation (Kaufmann, 1968). The plants under the wet watering regime had a higher water potential (> -1.0 MPa

on all sampling dates) and therefore can be considered vigorous (Cleary and Zaerr, 1980). For ponderosa pine seedings, photosynthesis was slowed or stopped at water potentials between −1.0 and −2.0 MPa, at values between −2.0 and −5.0 MPa seedling vigor declined, and at levels below −5.0 MPa seedlings died (Cleary and Zaerr, 1980). Kaufmann and Thor (1982), working on lodgepole pine, considered a midday xylem water potential of −2.3 MPa as a severe water stress. Running (1976) reported xylem water potentials ranging from −0.6 (predawn) to −1.5 MPa (midday) for Douglas fir and −0.7 (predawn) to −2.0 MPa (midday) for ponderosa pine.

These experiments demonstrate that the white pine weevil can discriminate between bark from water-stressed and non-water-stressed white pine plants, with a preference for the latter at a certain time of the year. This is in agreement with a number of field observations, which suggest that the most vigorous trees are preferred by the weevil (Graham, 1926; Plummer and Pillsbury, 1929; Kriebel, 1954; Holst, 1955; VanderSar and Borden, 1977). The possibility that some herbivores could attack the most vigorous hosts was recently suggested by Price et al. (1987a,b) and Price (1989) for several gall-forming sawfly species and their willow host plants. The bud-galling sawfly, *Euura mucronata* (Hartig) Man. (Churchill), attacks the longer shoots of its willow host, where establishment and survival are better (Price et al., 1987b). Other studies on herbivores have also shown that more vigorous growing plants can have positive effects on insect performances or susceptibility to insect attack (Miles et al., 1982; Craig et al., 1986, 1989, 1991; Wagner, 1986; Watt, 1986; Bultman and Faeth, 1987; McCullough and Wagner, 1987; Price et al., 1987a,b; Kimberling et al., 1990). If we consider the limited resources available for larvae under the bark of the leader, a careful selection by the ovipositing female appears biologically sound. Craig et al. (1989) mentioned that when the offspring of an insect complete all their subsequent development at the oviposition site, the females are under strong selective pressure to optimize oviposition site selection. Females of the white pine weevils can also select for vigorously growing trees with larger leaders to assure abundant food resources to their brood, as is observed with gall-forming insects (Price and Clancy, 1986; Craig et al., 1989; Price, 1989).

The lack of significant feeding response with early season bark from non-infested plants may reflect the physiological condition of plants that are still similar considering that the watering treatments are just starting. However, with the white pine weevil in Sitka spruce, Alfaro et al. (1979) demonstrated a concentration-dependent feeding response. Thus, using higher concentrations of bark to compare the preference of the weevil in a two-choice test may enhance the differences between the two types of bark.

Results of the no-choice tests indicate that when forced to feed on only one type of bark, weevils ate equal amounts of bark from water-stressed and non-

water-stressed plants. This can explain why in inadequate stands, where trees are less vigorous, the weevils are still able to feed on these hosts. However, field observations related to more pronounced weevil damage on inadequate sites for white pine growth (Maughan, 1930; Connola and Wixson, 1963; Dirks, 1964; Connola, 1965; Connola and Birmingham, 1978) are difficult to explain without biological data on the insect. More damage does not necessarily indicate more significant feeding and oviposition on trees growing under stressful conditions. Because the female can lay her eggs on one or more leaders (Plummer and Pillsbury, 1929; MacAloney, 1930), it is possible that on sites where tree growth is reduced, the smaller size of the previous year's leader could cause females to oviposit on more trees and, in so doing, contribute to an increase in the number of trees attacked yearly. It also is possible that because the tree is stressed, the same amount of feeding may appear to be more damage.

The preference for bark from the nonstressed plants was accentuated on attacked plants (Figure 2a) relative to nonattacked plants (Figure 1a) with the first harvested bark. We can hypothesize that the attack by weevils will modify bark chemistry and could lead to an increase in feeding activity by other attacking weevils. Lewis (1979) mentions that *Melanoplus differentialis* (Thomas) nymphs frequently feed on portions of leaves affected by previous insect feeding. For another weevil species, Ericsson et al. (1988) also observed that *Hylobius abietis* (L.) prefers to attack wounded Scots pine seedlings. For the white pine weevil, the biological advantage of this situation could be to allow sufficient oviposition by a number of females to assure the formation of a feeding ring under the bark which would efficiently kill the leader (Wallace and Sullivan, 1985).

The almost identical feeding preference of both ages of weevils is surprising. Considering that it is spring adults that lay eggs, we expected better discrimination with this age group compared with fall weevils. If we consider that weevil host selection and oviposition is done early in the spring (Dixon and Houseweart, 1983), it can be advantageous for this insect to also select its host tree during the summer and fall periods when weather is still favorable, and thus allow adult dispersion. The new adult generation is active during a period of two months before individuals enter into quiescence (Sullivan, 1959). MacAloney (1930) observed that feeding may take place even after heavy snow has fallen, if the weather warms up again. Consequently, we can expect the active participation of fall weevils in host selection.

Our results demonstrate that feeding preference tests can be done with adults of both sexes without any significant interaction. Both sexes react similarly to bark type, with both preferring bark from non-water-stressed plants. VanderSar and Borden (1977) and Alfaro et al. (1979) reported similar feeding preferences for both sexes, although they speculated that male weevils could be less sensitive in selecting hosts. The only difference we occasionally saw between

males and females was that females ate more than males. This was also observed by Alfaro et al. (1979).

Specific chemical compounds playing an important role in feeding stimulation of the white pine weevil still have not been identified. Alfaro et al. (1980) demonstrated that feeding is stimulated by nonvolatile substances and that volatile chemicals could act as synergists, but he did not identify any nonvolatile substance. We observed that sucrose (5 μM) was stimulating when added to white pine bark (R.L., unpublished data). In the present experiment, chemical analyses were performed to obtain general information on bark chemistry following the watering regimes and precise relations between bark chemistry and weevil preferences are difficult to establish. However, there was less nitrogen, phosphorus, and potassium in bark from non-water-stressed plants used in 1989 and 1990. Goyer and Benjamin (1972) also observed in weevil-infested plantations that roots from jack pine had significantly less total nitrogen than did the roots of trees in adjacent noninfected stands. Enhancement of the feeding response on damaged bark is also associated with a higher potassium content in this bark. Xydias and Leaf (1964) observed more attacks on white pine trees fertilized with potash. Furthermore, the insect's preference for bark with a low content in nitrogen, phosphorus, and potassium may indicate that these elements are too general as indicators of food quality and that specific products that play an important role in insect feeding preferences have to be identified. Some phenols, amino acids, and carbohydrates are known to act as phagostimulants for other weevil species (Blanc, 1972; Thomas and White, 1971; Hsiao, 1969; Doss, 1983) but this remains to be demonstrated for the white pine weevil.

Larsson (1989) mentioned that it is unlikely that a range of insect species with different feeding habits will respond consistently to stress-induced changes in plant tissues. Price (1989) considered that a dichotomy could help to explain that some herbivore species are keyed to stressed plants, while other species develop a specialization for the most vigorous plants or plant parts. The white pine weevil's preference for nonstressed plants may also explain the typical damage caused by this insect. On a vigorous tree, the leader will allow maximal oviposition by one female and support brood development. In conclusion, the white pine weevil, like a number of other insect species, prefers to feed on nonstressed plants and therefore we would expect its biological performance to be favored by vigorously growing plants rather than by stressed plants.

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ELEVATIONAL VARIATION OF QUINOLIZIDINE ALKALOID CONTENTS IN A LUPINE (*Lupinus argenteus*) OF THE ROCKY MOUNTAINS

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Abstract—Quinolizidine alkaloid contents of leaves and seeds of *Lupinus argenteus* (Fabaceae) collected from seven different localities near Gothic, Colorado were determined by capillary GLC. Differences in alkaloid levels between sites are substantial and alkaloid quantity decreases as elevation increases. Leaves at the lowest elevation, for example, contain six times the alkaloid levels of leaves at the highest elevation. Seeds from plants of low- and high-elevation sites were grown under identical conditions in the greenhouse. Alkaloid levels of leaves of seedlings were significantly higher in those seedlings derived from populations of low elevations than those of high elevations, indicating that the observed differences in the field are at least partly genetic and not environmental. To determine whether predation rates were responsible for these genetic differences, data on seed predation rates and observations on herbivory were collected.

Key Words—Seed predation, lupine alkaloids, flower production, elevational gradient, quinolizidine alkaloids, *Lupinus argenteus*.

INTRODUCTION

Much has been made of the observation that the quantity and quality of stored secondary chemicals vary tremendously between plant species (Ehrlich and Raven, 1964; Feeny, 1976; Rhoades and Cates, 1976; Coley et al., 1985). However, allelochemical variation within species and even between organs of

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the same plant seems also to be the rule (Bowers and Puttick, 1988; Dirzo and Harper, 1982a; Dolinger et al., 1973; Langenheim et al., 1986; Macedo and Langenheim, 1989; Gould, 1988; Wink, 1987a,b, 1990, 1992, 1993a,b), and much of our insight into the mechanistic causes and consequences of such variation comes from intraspecific studies (Berenbaum, 1981; Berenbaum et al., 1989; Cates and Redak, 1988; Dirzo and Harper, 1982b; Vrieling et al., 1991).

Species within the genus *Lupinus* are infamous for variation both in morphology and allelochemical quantity and quality (Dolinger et al., 1973; Meissner and Wink, 1992; Wink, 1984, 1993a). The allelochemicals characteristic of *Lupinus* are the quinolizidine alkaloids (QAs), a number of which are known to be toxic to a variety of herbivores (Bentley et al., 1984; Cantot and Papineau, 1983; Keeler, 1969, 1976; and, for review, see Wink, 1987a,b, 1988, 1992, 1993a,b; Wink and Witte, 1991; Szentesi and Wink, 1991).

Our goals with this research were twofold. First, we wanted to evaluate the relationship between elevation and quinolizidine alkaloid levels in *L. argenteus* growing in the Rocky Mountains. Second, if a relationship was found, we wanted to explore whether these differences were environmentally or genetically based.

Natural History of Lupinus argenteus. L. argenteus is an herbaceous perennial that occurs at high elevations throughout the western Rocky Mountains and the Great Basin and is considered to be toxic to livestock (Dunn, 1956; Keller and Zelenski, 1978). On the western slope of the Colorado Rockies, near the Rocky Mountain Biological Laboratory, *Lupinus argenteus* (Fabaceae) can be found from 2775 to 3665 m. The growing season (no snow) at the lower elevations is approximately double that at the higher elevations: approximately April–October and July–September, respectively; and at the higher elevations, snow and freezing temperatures may occur at any time during the summer.

METHODS AND MATERIALS

Plants. Seven sites were established in the summer of 1990. All sites were on west-facing slopes and were chosen for accessibility and elevational range, which was between 2775 and 3665 m. The Belleview site (3599 m) was on the western slope of Mt. Belleview between the peak and the 401 trail. Site 401 (3355 m) was along the 401 trail on a west-facing section of the southern slope of Mt. Belleview. Paradise (3416 m) was on the southern side of Paradise Pass. Site 403B (3294 m) was 0.5 km NE of the 403 trailhead just above the Washington Gulch road, and 403T (3477 m) was just north of the highest point on the 403 trail. The Gothic site (2867 m) was near the workshop in Gothic. Between these upper six sites, *L. argenteus* was common, and one could find patches of this lupine at least every 100 m. Two pairs of sites (Belleview and

401, 403B, and 403T) were at different elevations on the same hillside and part of huge, nearly contiguous patches of *L. argenteus*. The last site, Upper Loop (2776 m) was quite isolated, however. It was 1.5 km up the Upper Loop trail from Grant Lake and the only location where *L. argenteus* was found within several kilometers in any direction.

Patch Size. At each site, the number of *L. argenteus* in the immediate patch were counted or estimated. Ten meters was arbitrarily chosen as the distance necessary to separate two patches (i.e., clusters of lupine plants more than 10 m apart were considered separate patches).

Flower Production. In each patch, 20 plants were selected along a linear transect, and for each plant, the number of flowers produced for the year were counted. Where flowers had fallen off, peduncle scars were counted. Occasionally, inflorescence buds were too young to count individual flowers, and in these instances, flower numbers were a crude estimate.

Seed Predators. At the time that alkaloids were sampled in the summer of 1990, seed pods were also collected from the same plants as well as the ten most mature pods of 20 other plants in the immediate vicinity. These pods were all opened and examined for damage or seed predators (there are several species of dipteran and lepidopteran larvae that feed on lupine seeds; details in Carey, 1992). Any pod with damage or containing seed predators was counted as damaged. Healthy seed pods were classified as undamaged. Using the same methods and at the same localities, pods were again sampled for seed predators on August 26, 1991.

Herbivores. Other studies on lupines near Gothic (Carey, 1992) have found the most common predator of lupine herbaceous tissue to be gophers. All sites were examined for gopher activity and other evidence of herbivory both in 1990 and 1991.

Alkaloid Sampling. Plants at all seven localities were sampled within an hour of noon on August 10 and 12, 1990, in order to avoid variation due to diurnal rhythms (Wink and Witte, 1984). Both days were clear until noon. From each locality, three plants were systematically chosen at least 10 m apart, and each consisted of at least 10 stalks bearing both flowers (on secondary inflorescences) and nearly matured seeds (on primary inflorescences). Undamaged leaves attached to the fourth leaf node from the top of such stalks were snipped off, weighed in the field using a spring scale accurate to 0.1 g (all samples were about 5 g), and placed immediately in methanol. In addition, seed pods bearing full sized, but still green and moist, seeds were collected. Several hours later, pods were examined in the laboratory for seed predators (see below), and undamaged seeds were weighed and placed in methanol.

Alkaloid Analysis. All samples were analyzed with capillary GLC in Heidelberg, Germany. GLC-MS was carried out by L. Witte (Braunschweig). For extraction, samples were thoroughly ground, allowed to stand in MeOH, and

filtered with fresh MeOH. MeOH was removed with a rotavapor and the residue transferred into 15 ml 0.5 M HCl. Homogenate was then made alkaline with 6 M NaOH and subjected to solid-phase extraction using Chem elute columns (Analytichem, Frankfurt) and methylene chloride as a solvent. Solvent was evaporated with a rotavapor. For analysis, alkaloid extracts were separated on fused-silica columns (30 m × 0.32 mm, 1 μm film) with bonded methylsilicone phases (DB-1; J&W Scientific). A Varian gas chromatograph (3300), equipped with a nitrogen-specific detector and a Spectra Physics integrator, was employed. GLC conditions: carrier gas: helium 1.2 bar, split; injection 1:20; injector temperature: 250°C; detector: 300°C; oven: 150°C, 1 min isothermal, 150–300°C with 10°C/min, then 20 min isothermal. Sparteine was used as an external standard. GLC-MS consisted of a Carlo-Erba 5160 GC (equipped with a DB-1 30-m × 0.32-mm column), which was coupled to the quadrupole mass spectrometer Finnigan MAT 4515. Retention indices were calculated using cochromatographed standard hydrocarbons (Wink 1993a). Spectra were recorded at 70 eV and evaluated with the Incos Data System. Details on alkaloid identification have been reported in Meissner and Wink (1992) and Wink and Carey (1994).

Seedling Alkaloids. During the fall of 1991, dried seeds were collected from three sites: Upper Loop, 403T, and Belleview. Seeds of equivalent weight were planted in potting soil in planters and grown intermixed in a greenhouse in Tucson, Arizona. On January 12, when surviving plants were 4 months old and approximately 21 cm high, leaves were clipped, weighed immediately on a spring scale (as above), and placed in methanol. There was no obvious difference in size or appearance between seedlings from different locations. Samples were mailed to Germany and analyzed by capillary GLC.

RESULTS

A Spearman rank correlation between elevation and mean alkaloid contents at each site shows a significant decrease in alkaloid level with increasing elevation for both leaves ($R_s = 0.857$, two-tailed $P < 0.05$) and seeds ($R_s = 0.929$, two-tailed $P < 0.01$) (Figure 1). Although the elevational effect is strong, individual variation in alkaloid contents is large at most sites (note the large standard errors, Figure 1). To give one extreme example, although leaf alkaloids at the highest site, Belleview, averaged one fourth of those at a much lower site, Gothic; one Belleview individual had a greater leaf alkaloid concentration than one Gothic individual. Very little of this variation comes from the alkaloid analysis itself. Samples analyzed repeatedly always varied by less than 5%.

Pod predation was not consistent between years and bore no relationship

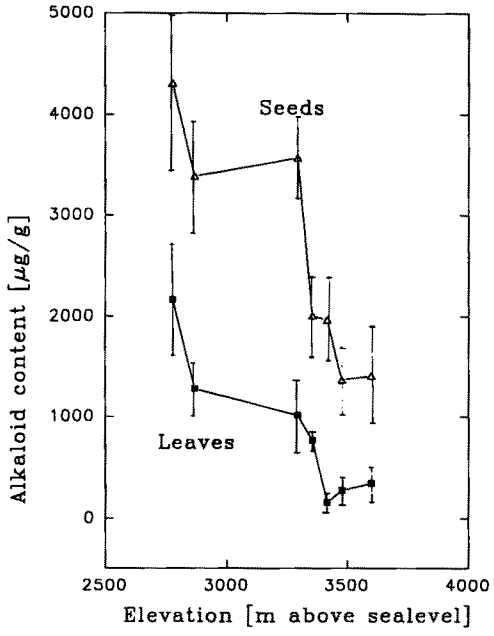

FIG. 1. Correlation between elevation and alkaloid contents of leaves and seeds. Three plants were sampled at each site. Given is the average (±SE) micrograms per gram wet weight.

to elevation, patch size, or seed alkaloid levels (Table 1). The only seemingly consistent trend was low levels of pod predation at Belleview and Upper Loop, the highest and lowest sites, respectively. Gopher activity was common at all but the Upper Loop site, but there was no other evidence of grazing at any site.

There was a consistent relationship between elevation and the number of flowers produced per plant. Plants at lower elevations averaged more flowers (Table 1). A Spearman rank correlation shows the relationship between elevation and mean flower number to be statistically significant in 1990 ($R_s = 0.89$, two-tailed $P < 0.05$) and almost so in 1991 ($R_s = 0.73$, two-tailed $P < 0.10$).

Due to experimental problems, the number of seedlings produced from seeds of wild lupines (grown in a greenhouse under identical conditions) was comparably small. The corresponding data are therefore preliminary. Plants derived from seeds originating from high elevations (403T and Belleview) produced a significantly lower concentration of leaf alkaloids than did those from lower elevations (Upper Loop): 105.4 ± 38 ($N = 7$) and 512.1 ± 86 ($N = 6$) μg alkaloid per gram wet weight, respectively (t test; $P < 0.001$). Although

TABLE 1. CHARACTERISTICS OF SEVEN *L. argenteus* PATCHES[a]

Site	Elevation (m)	Patch size (No. of plants)	Flowers/Plant		Pods Predated (%)	
			1990	1991	1990	1991
Belleview	3,599	19,000	212	134	0	4
403T	3,477	28,000	330	324	1	28
Paradise	3,416	1,410	347	330	0.1	14
401	3,355	77,000	266	318	15	26
403B	3,294	1,990	410	312	9	32
Gothic	2,867	42	520	400	18.5	8
Upper Loop	2,776	124	683	704	2.5	1

[a] Elevation was taken from a topographical map. Patch size was determined by counting the number of plants in the patch in 1990. Both the mean number of flowers produced per plant and the mean percentage of seed pods with predators were determined for both 1990 and 1991. For determination of the latter, more than 200 pods were sampled for each year at each patch.

the absolute alkaloid contents are lower in these greenhouse plants than in corresponding lupines grown in the wild (Figure 1), the general trend is similar.

DISCUSSION

The relationship between elevation and alkaloid level is statistically significant: lupines from higher elevations accumulate lower alkaloid contents than those growing at lower elevations (Figure 1). This phenomenon persists even when seedlings from the highest and lowest elevations are grown under identical conditions in a greenhouse. This latter result indicates a genetic basis for the elevational pattern, which could explain at least some of the effects observed.

The lower alkaloid contents of greenhouse plants could be due to several reasons: Since alkaloid accumulation can be induced by herbivory (Wink, 1983; Johnson et al., 1989), it could be argued that lupines in the wild are already induced by herbivore damage, whereas greenhouse plants were unmolested and therefore not induced. Quinolizidine alkaloid biosynthesis is light-dependent (Wink, 1987a,b, 1990). Since light is a problematic factor in most greenhouse experiments, lower alkaloid accumulation may be due to insufficient light.

What could be the selection pressures leading to the genetically based elevational gradient of alkaloid accumulation? It has been shown for other lupine species that quinolizidine alkaloids provide the plants with chemical defense against herbivores (both mammals and insects) and, to some degree, against microorganisms (Wink, 1983, 1984, 1987a,b, 1988, 1992, 1993b). A preliminary field experiment at the study site (near Upper Loop) in the Rocky mountains

indicates that herbivores exert selection pressure on lupines that is correlated with alkaloid content. Alkaloid-rich seeds of *Lupinus albus* (bitter lupines) and nearly alkaloid-free seeds (sweet lupins) were grown in the field unprotected by any fence. Whereas seeds and seedlings of sweet lupines disappeared within several weeks, about 50% of the alkaloid-rich counterparts survived. Thus, alkaloid accumulation and predation are highly correlated. We expect that predation should lead to a selection of plants genetically tuned for high alkaloid accumulation.

Despite a sixfold difference in alkaloid contents between sites 1 and 7 (Figure 1), actual herbivory was almost equal (Table 1). This seems to be strong evidence that lower elevation plants have been naturally selected for higher QA levels because predation pressures at low elevations are greater, i.e., much higher alkaloid concentrations are required to maintain the same level of damage.

Alternatively, we suggest that the longer growing season at lower elevations could make rapid growth less critical—plants can afford to allocate a greater amount of resource to protection. Moreover, genetically based physiological responses to light quality or temperatures (both factors vary with altitude) on alkaloid production cannot be ruled out.

Our results clearly implicate a strong genetic component for altitude-dependent alkaloid production. Because of limited sample size, our data do not explain unequivocally which forces have been responsible for the natural selection that must have taken place, but an impact of herbivory seems most likely.

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CHEMOTACTIC TUBE-FOOT RESPONSES OF A SPONGIVOROUS SEA STAR *Perknaster fuscus* TO ORGANIC EXTRACTS FROM ANTARCTIC SPONGES

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Abstract—Hexane, chloroform, and methanol extracts of 18 species of antarctic sponges were tested for their ability to induce sustained tube-foot retraction in the antarctic spongivorous sea star *Perknaster fuscus*. Extracts were imbedded in silicone and used to coat the tip of a glass rod, which was allowed to contact an extended tube-foot. Retraction times were measured and compared with three controls: contact with a glass rod coated with a hexane extract of fish (feeding stimulant), contact with the glass rod alone (mechanical control), and contact with the glass rod coated with silicone alone (silicone control). Only extracts of the sponge *Mycale acerata* did not elicit significantly longer tube-foot retraction times than controls for at least one of the three organic extracts. Hexane sponge extracts elicited the lowest levels of significant tube-foot responses, with only 39% of the sponge species tested showing activity in this fraction. In contrast, chloroform and methanol extracts elicited a significant tube-foot retraction response in 73% and 78% of the species tested, respectively. This indicates that in this assay repellent metabolites are generally more polar substances. It remains to be determined that secondary metabolites are responsible for all of the tube-foot retraction responses detected in sea stars exposed to sponge extracts; bioactive secondary metabolites have been isolated from a number of these antarctic sponges. It may be of ecological significance that the two rapidly growing sponges, *Homaxinella balfourensis*

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and *Mycale acerata*, were either not repellent or had low repellency, and that *M. acerata* is the primary dietary item of *Perknaster fuscus*.

Key Words—Antarctica, sponge, sea star, *Perknaster fuscus*, chemotactic, repellent, chemical defense, polar, marine benthos.

INTRODUCTION

Numerous studies have examined aspects of the natural product chemistry and chemical ecology of temperate and, particularly, tropical marine sponges (reviewed by Faulkner, 1978, 1984; Bakus et al., 1986; Scheuer, 1990). These studies document a high incidence of biological activity and an immense array of natural products produced by sponges. The numbers of studies focusing on the chemistry and chemical ecology of antarctic sponges, of which more than 300 species have been described to date (Koltun, 1970), pale by comparison with studies from other biogeographic areas.

The antarctic benthos is relatively old when compared with other marine ecosystems (Dayton, 1990; Eastman, 1993), and it supports a surprisingly diverse assemblage of marine invertebrates (Crame, 1993) with sponges dominating benthic biomass at depths greater than 33 m (Dayton et al., 1974). At these depths, the environment is stable and the benthic community is apparently structured primarily by biological factors such as predation and competition (Dayton et al., 1974). Although there is a lack of browsing spongivorous fish, as seen in warmer seas (Bakus and Green, 1974; Vermeij, 1978; Targett, 1981; Eastman, 1993), many antarctic sea stars are conspicuous spongivores, and, to a lesser extent, nudibranchs may also feed on antarctic sponges (Dayton et al., 1974; McClintock, 1987). Antarctic sponges generally have extremely low growth rates and are likely to have remarkable longevities (Dayton et al., 1974; Dayton, 1979). These conditions, a geologically old and stable environment, prolonged longevity, and the prevalence of predation and competition as community structuring agents, are all factors that may select for the evolution of chemical defense.

Until recently, there have been few studies to evaluate whether antarctic marine invertebrates produce compounds that may defend them from predation, fouling organisms or that may mediate allelochemical interactions. Nonetheless, chemical bioactivity appears to be widespread across phyla and has been detected in the tissues of at least one or more species of antarctic sponges (Blunt et al., 1990; McClintock and Gauthier, 1992; also see Barthel and Gutt, 1992), bryozoans (Winston and Bernheimer, 1986), nemerteans (Heine et al., 1991), soft corals (Slattery et al., 1990), nudibranchs (McClintock et al., 1992), pteropods (McClintock and Janssen, 1990), brachiopods (McClintock et al., 1993), echinoderms (McClintock, 1989; Blunt et al., 1990; McClintock and Vernon, 1990),

and ascidians (McClintock et al., 1991). Only a few attempts have been made to isolate and describe natural products from antarctic marine invertebrates (Seldes et al., 1986; Molinski and Faulkner, 1987, 1988; Blunt et al., 1990; Davies-Coleman and Faulkner, 1991; Kong et al., 1992; Hamann et al., 1994).

McClintock (1987) employed a goldfish assay to test for toxicity in crude whole tissue homogenates of antarctic sponges. This technique is problematic, as whole tissue homogenates are not desalted and inorganic salts may cause mortality in fish. Moreover, goldfish assays do not provide ecological information relevant to predator and prey. The present study improves on these techniques by employing sequential extractions of sponges using a series of organic solvents of increasing polarity. The tube-foot retraction response of the spongivorous antarctic sea star *Perknaster fuscus* is examined, thereby facilitating a more ecologically relevant interpretation of patterns of biological activity. Sloan (1980a,b) indicates that dermal chemosensitivity in the tube-feet of asteroids is fundamentally a defensive response and probably evolved as a mechanism to protect the organism as it encounters significant alterations in its sensory environment. Contact chemoreception has been reported in all asteroids investigated to date (at least 25 species; reviewed by Sloan and Campbell, 1982), and it is generally accepted that echinoderm tube-feet are one of the primary sites for perception of chemical stimuli (Lawrence, 1975; Sloan and Campbell, 1982; McClintock et al., 1984). Tube-foot retraction responses may assist natural product chemistry by providing a bioassay-guided approach in the search for bioactive compounds.

METHODS AND MATERIALS

Sponges were collected using SCUBA between 25 and 40 m depth from McMurdo Sound, Antarctica. All species we examined were from Hut Point with the exceptions of *Mycale acerata* and *Isodictya erinacea*, which were collected from Danger Slopes, and *Gellius tenella*, which was collected from Cape Evans (Figure 1). The spongivorous sea star *Perknaster fuscus* (radius = 8–12 cm) was collected from Hut Point at depths ranging from 20 to 30 m.

Each sponge was subsampled and a 100-g wet wt tissue sample was lyophilized until dry. Twenty grams of this dry material was broken up into small pieces and then extracted sequentially with hexane, chloroform, methanol, and 70% aqueous methanol, respectively. In order to optimize yield, each step of the extraction was repeated three times. The respective solvents were then evaporated with a rotovap, and the resultant dry extract was used for bioassays.

Tube-foot assays were conducted in the laboratory by placing individual *P. fuscus* onto their aboral side in a 25-cm-diameter finger bowl containing 1500 ml of sea water at 0°C. Sponge extracts were prepared in a 1:1 ratio (w/w)

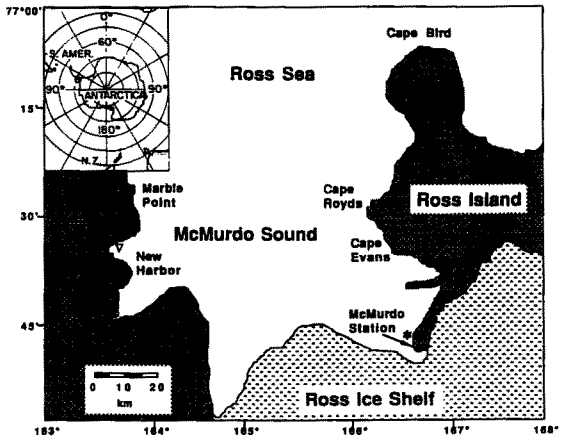

FIG. 1. Map of McMurdo Sound, Antarctica. Sponges were collected at Cape Evans, Hut Point (directly in front of McMurdo Station) and Danger Slopes (2 km north of the station).

with an inert silicone grease. The tip of a glass rod thinly coated with the silicone–extract mixture was presented to an extended tube-foot such that the motion of the tube-foot created contact with the rod. An attempt was made to employ tube-feet located towards the tip of the arm, which are known to function primarily in chemosensory activities (Sloan, 1980a,b). The response of the tube-foot to contact with the tip of the glass rod was recorded. Tube-foot retractions were timed up to a period of 60 sec, at which time the experimental treatment was terminated.

Three different controls were conducted simultaneously with experimental treatments: presenting a tube-foot to a glass rod coated with a 1 : 1 ratio (w/w) of fish muscle (*Trematomus bernacchii*) extract and silicone (positive control); to a glass rod alone (mechanical control); and to a glass rod coated with silicone alone (silicone control). Ten trials of each of the four treatments (sponge extract and three controls) were conducted with the sequence of presentation randomized. After each 10 randomized trials, the sea star being tested was replaced with a naive individual.

A Kruskal-Wallis analysis of variance of ranks test was conducted to compare experimental and control tube-foot retraction time for each of the sponge extracts tested. When significant differences were found, pairwise comparisons were conducted using a Mann-Whitney U test.

RESULTS

Perknaster fuscus showed strong and consistent tube-foot retraction responses to the aqueous methanol extracts. However, due to the potential problem of the presence of inorganic salts in this fraction, we chose not to include these data in our analysis. Tube-foot retraction responses to hexane, chloroform, and methanol extracts are presented in Figure 2. In all trials, sea stars showed very little or no retraction response to either the glass rod or the glass rod coated

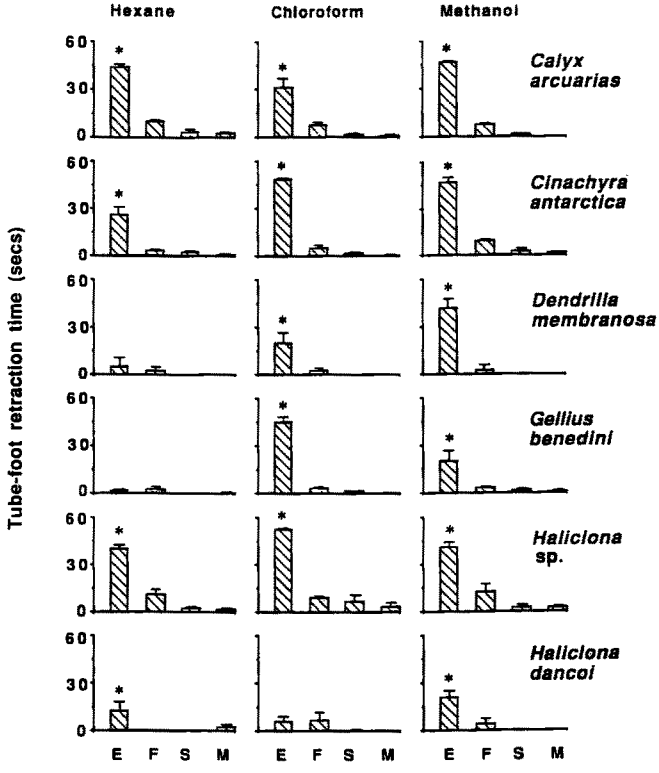

FIG. 2. Chemotactic tube-foot responses of the antarctic sea star *Perknaster fuscus* to organic extracts (E) of 18 species of antarctic sponges. Each bar shows the mean ± 1 SE tube-foot retraction time (sec) for 10 trials. Asterisks indicate statistical significance ($P < 0.05$; Kruskal-Wallis analysis of variance of ranks followed by pairwise comparison with a Mann-Whitney U test) when compared with the three controls (F = fish extract; S = silicone; M = mechanical). ND = No data. See Methods and Materials for details of experimental protocol.

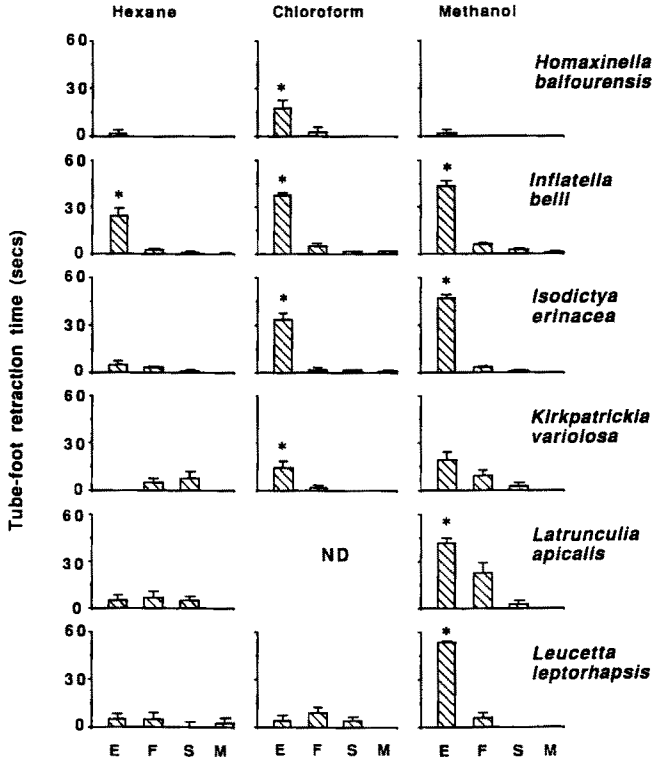

FIG. 2. Continued

with silicone. In many cases, when the tube-foot contacted a glass rod, it attached to the rod with the sucker. Tube-feet that were allowed to contact silicone-treated rods attached much less frequently but did not retract in most cases. There was a higher incidence of retraction of tube-feet in response to extracts containing fish. Nonetheless, these retractions were brief (< 10 sec) and were followed by protraction of the tube-foot towards the point of stimulus.

A relatively small percentage of the sponges tested (39%) showed significant tube-foot retractions in response to hexane extracts of sponges (Figure 2). Moreover, only the sponges *Calyx acuarias*, *Haliclona* sp., and *Polymastia invaginata* had "moderately strong" retraction responses (i.e., >30 sec) to hexane extracts. In contrast, 73% of the chloroform extracts tested (11 of 15 sponges) elicited significant tube-foot retractions when compared with controls (Figure 2). Seven of these chloroform extracts caused tube-foot retraction responses that lasted longer than 30 sec. The highest incidence of significant

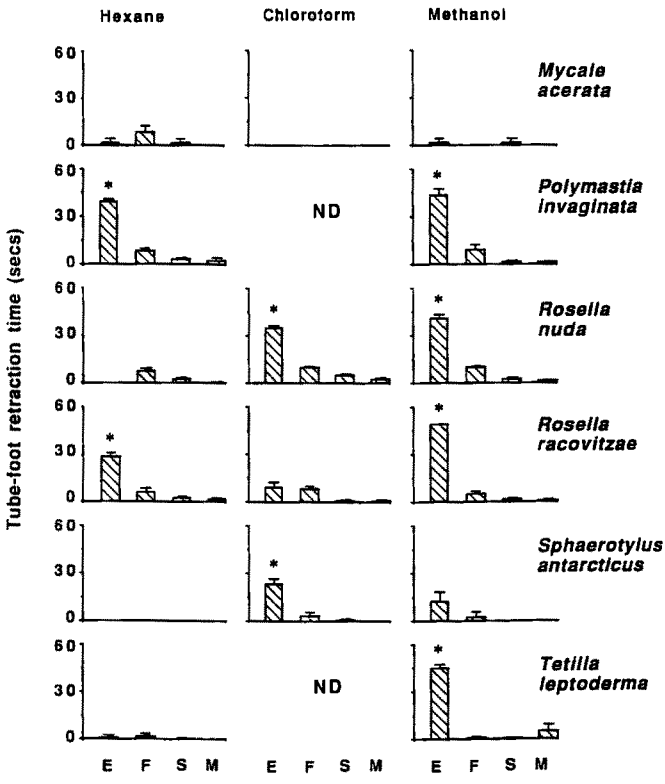

FIG. 2. Continued

tube-foot retractions were elicited by methanol sponge extracts (78%). Twelve of the methanol sponge extracts caused tube-foot retractions lasting more than 30 sec. The only sponge that did not elicit any significant tube-foot responses from any of the three extracts was *Mycale acerata*.

DISCUSSION

A detailed analysis of the arm-curling and tube-foot responses of the asteroid *Crossaster papposus* in the north Pacific (Sloan, 1980b) indicated that feeding stimulants may cause a short term (5- to 11-sec) retraction response in tube-feet. This results from an innate defensive response when the animal encounters a gross sensory change in its environment, followed immediately by sustained protraction of tube-feet towards the feeding stimulant. In the present study, similar behavior was observed in the tube-feet of the antarctic sea star *P. fuscus*

exposed to a feeding stimulant (extract of fish tissue), with sustained tube-foot protraction immediately following an initial retraction response generally lasting less than 10 sec. In contrast, exposure to some organic extracts of antarctic sponges caused a more sustained defensive response, with tube-foot retractions lasting a minimum of 30–60 sec. Such sustained retraction responses, which exceed those commonly caused by feeding stimulants, are indicative of the presence of compounds that are irritants to individual tube-feet and, ultimately, may be the basis of avoidance of a given sponge as a potential food item. Similarly, Targett (1979) found that gastropod tentacle retractions were indicative of biological activity in marine microalgae.

The incidence of significant tube-foot retractions in *P. fuscus* exposed to antarctic sponge extracts varied both qualitatively and quantitatively. Hexane sponge tissue extracts were the least likely to induce a sustained tube-foot retraction (39% of the extracts caused significant retractions). This is likely related to the less polar nature of this solvent when compared with both chloroform and methanol. Both of the latter solvent systems yielded extracts which, when tested against the tube-feet of *P. fuscus*, induced tube-foot retractions at levels twofold that seen in hexane (73% and 78% of the extracts tested, respectively). Quantitatively, among those experimental trials revealing significant tube-foot retraction responses, the duration of the responses was generally shorter for tube-feet exposed to hexane sponge extracts. This suggests that for this assay few nonpolar metabolites are repellents.

The antarctic slimy sponge *Mycale acerata* did not elicit tube-foot repellency. This supports the observations of Barthel and Gutt (1992), who examined *M. acerata* from the Weddell Sea and cited recent unpublished work by Emrich that revealed no antimicrobial activity in this sponge. McClintock (1987) detected icthyotoxicity in *M. acerata*, but used crude whole tissue assays that did not control for the effects of inorganic salts. All of the remaining sponges caused significant tube-foot repellency in *P. fuscus*, although the strength of the response was variable. The nature of the secondary metabolites responsible for the observed responses is currently unknown, and, in the case of methanol extracts, tube-foot repellency could also be the result of salts, large polysaccharides, or peptides. Nonetheless, for a number of these antarctic sponges, bioactive secondary metabolites have been identified.

Two biologically active compounds, 9,11-dihydrogracilin A and the diterpene membranolide, have been extracted from the tissues of the antarctic cactus sponge *Dendrilla membranosa* (Molinski and Faulkner, 1987, 1988) and were implicated as defensive chemicals. We detected significant *P. fuscus* tube-foot retraction responses in both the chloroform and methanol extracts of *D. membranosa*. However, the hexane fraction, containing a significant proportion of 9,11-dihydrogracilin and related terpenes, displayed no significant tube-foot

repellency. Several potent cytotoxic alkaloids (variolins) have been reported in the tissues of the red sponge *Kirkpatrickia variolosa* (M. Munro and D.J. Faulkner, personal communication). Nonetheless, these compounds do not appear to have much activity against the tube-feet of *P. fuscus*; no significant tube-foot retraction was detected in response to either the hexane or methanol extracts and only weak activity was detected in the chloroform extract. The green sponge *Latrunculia aplicalis* contains a number of methanol-soluble discorhabdin pigments (Baker et al., 1993) that are cytotoxic (Blunt et al., 1990). These compounds are likely repellents in *P. fuscus*, as significant tube-foot retraction occurred in response to the methanol fraction. Moreover, recent work with the antarctic rubber sponge *Leucetta leptorhapsis* has indicated the presence of strongly cytotoxic, highly polar, lipids (Hamann et al., 1994) that are notably more potent than structurally similar antimicrobial lipids discovered in the tropical congener *Leucetta microraphis* (Kong and Faulkner, 1993). The strong tube-foot retraction activity detected in the methanol fraction of the rubber sponge indicates that these bioactive lipids may be sea star feeding deterrents. Our preliminary HPLC and NMR analysis indicates that the chloroform extract of the antarctic polychaete sponge *Isodictya erinacea* has secondary metabolite chemistry. Significant tube-foot retractions occurred in response to this chloroform fraction. Finally, antarctic sponges from other geographic areas have recently been found to possess toxic compounds. Emrich (unpublished data, cited in Barthel and Gutt, 1992) found *Tedania vanhoeffeni* and *T. triraphis* from the Weddell Sea to have strong antibacterial activity. No information is given on the type of compound(s) responsible for this activity or whether bioactive compounds deter potential sponge predators.

Although some sponges in McMurdo Sound may protect themselves to varying degrees from predators with their long spicules (e.g., the polychaete sponge *Isodictya erinacea* and the spiky sponge *Cinachyra antarctica*), this mode of defense may be relatively ineffectual against sea stars (Dayton et al., 1974), the primary sponge predators in this ecosystem. Many sea stars are capable of everting their cardiac stomachs to digest prey (Hyman, 1955) and therefore do not have to cope with spicules within the cardiac or pyloric stomachs. McClintock (1987) found no significant correlation between spicule contents of 17 species of antarctic sponges and electivity of sponge-eating predators. Therefore, antarctic sponges may rely on either fast growth or secondary metabolites to prevent or decrease rates of predation.

Adult *P. fuscus* are sponge specialists, feeding almost exclusively on the slimy sponge *Mycale acerata* (Dayton et al., 1974). We were unable to detect tube-foot bioactivity in extracts from this sponge, suggesting that secondary metabolites do not mediate this predator–prey relationship. As *Mycale acerata* is a rapidly growing sponge that is capable of dominating epibenthic space, the ability of *P. fuscus* to keep this species in check is ecologically significant

(Dayton et al., 1974). Although *M. acerata* is the primary food of adult *P. fuscus*, young and occasionally adult *P. fuscus* feed on another rapidly growing antarctic sponge, the bushy sponge *Homaxinella balfourensis*, which is particularly common at shallower depths (15–30 m) at Hut Point, McMurdo Sound (Dayton, 1989; McClintock et al., personal observation). No tube-foot repellency in response to hexane or methanol extracts of *H. balfourensis* was observed, and only weak activity was seen in the chloroform fraction, suggesting that this species is also poorly defended against *P. fuscus*.

With the exceptions of *Mycale acerata* and *Homaxenella balfourensis*, which have rapid growth, the remaining antarctic sponges examined in the present study have extremely low growth rates (see Dayton et al., 1974; Dayton, 1979). Some have shown no appreciable growth over periods of years (Dayton, 1979). The fact that the only two rapidly growing species are apparently poorly defended, suggests some evolutionary trade-off between growth and defense (optimal defense theory; Rhoads, 1979). However, to test this hypothesis adequately, it will be necessary to compare defense and growth rates of both sponges in habitats similar in resources but differing in levels of spongivory (Herms and Mattson, 1992). Such studies may be difficult to conduct as it is hard to control for differences in abiotic environments. The static nature of the antarctic marine environment (Dayton, 1990; Pearse et al., 1991; Eastman, 1993) should provide a superior medium for such intraspecific comparisons, in contrast to more environmentally variable temperate or tropical systems.

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MATING DISRUPTION OF PEA MOTH *Cydia nigricana* F. (LEPIDOPTERA: TORTRICIDAE) BY A REPELLENT BLEND OF SEX PHEROMONE AND ATTRACTION INHIBITORS

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Abstract—Synthetic sex pheromone of the pea moth *Cydia nigricana*, (*E*,*E*)-8,10-dodecadien-1-yl acetate (*E*8,*E*10–12:Ac), was applied in polyethylene dispensers at a rate of 30 g/ha and 600 dispensers/ha in a 0.6-ha pea field. The release rate of *E*8,*E*10–12:Ac was 140 mg/ha/day after six days, and 82 mg/ha/day after 20 days. Aerial concentrations of *E*8,*E*10–12:Ac, as measured by a portable EAG apparatus, ranged from 2 ± 2 to 7 ± 3 ng/m^3. The antennal signal was high and rather constant within pea canopy, but was lower and fluctuated strongly above canopy. Initially, >99% isomerically pure *E*8,*E*10–12:Ac was released, and male moths were attracted to dispensers. After nine days, isomeric blend composition had equilibrated to approx. 92% *E*8,*E*10–12:Ac and 8% of the inhibitory isomers *E*,*Z*-, *Z*,*E*-, and *Z*8,*Z*10–12:Ac. Males were then repelled from the pheromone-permeated field. Traps baited with 100 μg *E*8,*E*10–12:Ac caught 258 ± 133 *C. nigricana* males/trap in the control, but no males at all in the disruption field.

Key Words—Sex pheromone, attraction inhibitor, behavioral antagonist, mating disruption, air permeation, field EAG, *Cydia nigricana*, Tortricidae, Lepidoptera, pea moth, (*E*,*E*)-8,10-dodecadien-1-yl acetate.

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INTRODUCTION

Disruption of sexual communication and mating by atmospheric permeation with synthetic pheromones is employed worldwide as a specific, environmentally safe, and economical technique for the management of Lepidopteran pest insects (Jutsum and Gordon, 1989; Ridgway et al., 1990).

In the pea moth, *Cydia nigricana*, a number of elements should facilitate mating disruption. *C. nigricana* uses only one sex pheromone component, (*E*,*E*)-8,10-dodecadien-1-yl acetate (*E*8,*E*10–12:Ac) (Greenway, 1984; Witzgall et al., 1993). Its alcohol analog, *E*8,*E*10–12:OH is used for control of codling moth *C. pomonella*, and economic synthesis is already available (Yamamoto and Ogawa, 1989). *C. nigricana* has one flight period and green peas grown for canning or deep-freezing have to be protected only during three to four weeks; the crop is sprayed with insecticides until shortly before harvest. The canopy of pea fields is low and homogenous, and *C. nigricana* is day-active; both factors facilitate maintenance of pheromone concentrations and behavioral observations.

Complex pheromone blends comprising several or unstable compounds, crops susceptible to attack by more than one insect generation or species, and unhomogeneous or high crop canopies can be serious obstacles for the successful and efficient use of pheromonal methods.

Most of all, for the advancement of both new and established applications of the mating disruption technique, experimental field data on the behavior of moths and molecules is imperative (Arn, 1992; Kirsch, 1992). A portable electroantennogram apparatus, using the insect antenna as pheromone detector, has been developed by Sauer et al. (1992) to cover a most important methodological gap: the rapid and sensitive measurement of disruptant chemical under field conditions.

We have measured, during a first mating disruption trial against *C. nigricana:* (1) the amount and isomeric purity of sex pheromone *E*8,*E*10–12:Ac released from the dispenser material, (2) its aerial distribution and concentration in a pea field, and (3) visually observed male and female behaviors.

METHODS AND MATERIALS

Chemicals and Dispensers. (*E*,*E*)-8,10-Dodecadien-1-yl acetate (*E*8,*E*10–12:Ac) was formulated in polyethylene tube dispensers (Shin-Etsu Chemical Co., Tokyo) at ca. 50 mg *E*8,*E*10–12:Ac/dispenser. Isomeric purity of *E*8,*E*10–12:Ac before formulation was 99.1% (0.6% *E*,*Z*; 0.3% *Z*,*E*; <0.05% *Z*8,*Z*10–12Ac) and chemical purity was 99.7%, by capillary gas chromatography (GC).

Measurement of Release Rates. Dispensers collected from the field were suspended in stoppered, round-bottom glass flasks (250 ml) at 22°C for 2 hr

($N = 3$). The atmospheric concentration of *E*8,*E*10–12:Ac in these flasks was saturated after >5 hr (>50 μg recovered from the walls). Flasks were washed three times with 2 ml redistilled hexane, and 50 μg dodecyl acetate was added as internal standard to the first solvent portion. The combined extract was condensed under N_2 to 1 ml, and 3 μl was analyzed by GC. Data were corrected for 69% recovery of *E*8,*E*10–12:Ac from the glass surface.

GC analysis was done on a Hewlett Packard 5890 instrument with flame ionization detection (FID), on a nonpolar SE-54 column (splitless injection, 25 m, 0.32 mm ID, Kupper & Co., Bonaduz, Switzerland), programmed from 60°C (hold 2 min) at 10°/min to 100°C, and 1.5°/min to 230°C.

Field Facilities and Dispenser Placement. Dispensers (360) were placed in an unsprayed pea field (0.6 ha) near Höör (Skåne, Sweden) on June 23, 1992 (600 dispensers/ha; 30 g *E*8,*E*10–12:Ac/ha). This test field was partitioned into unit squares of 100 m^2, and their corners were flagged. On the corners, on the middle of the sides, and in the centers of these squares, dispensers were tied to top shoots of pea plants. Each square along the field border received four additional dispensers at 5 m around the center. Dispenser density was accordingly 9 and 13 per unit square; overall density, due to shared corners and sides was 3.3 and 10.2 dispensers/100 m^2, within the field and along the border. Dispensers in the centers of 10 squares were removed to install traps or the EAG apparatus.

The test field was surrounded by trees and bushes and was situated 50 m downwind from an unsprayed control pea field (8 ha). Within this control field, 300 m away from the pheromone-treated test field, three single test plots of 100 m^2, spaced at 30 m, received 4, 8, and 12 dispensers, respectively.

Field Trapping. Tetra traps (Arn et al., 1979) were baited with 100 μg *E*8,*E*10–12:Ac (99.8% isomeric purity by GC; Witzgall et al., 1993) on red rubber septa (Thomas Scientific, Illinois). Traps were placed ca. 10 cm below canopy, within the 0.6-ha test field (five traps), along its border (five traps), in the control field (10 traps), and in the centers of the three isolated 100-m^2 test plots in the control field (three traps). Traps in the pheromone-treated field were set in the centers of unit squares (see above), instead of a dispenser. Traps were checked and replaced every two to five days.

Larval Counts. After the annual flight period of *C. nigricana*, lots of 1 m^2 were chosen at random in the pheromone-treated ($N = 50$) and the control field ($N = 50$), and all pea pods were checked for *C. nigricana* larvae.

Behavioral Observations. Counts of adult female and male *C. nigricana* were made on 15 days, between June 22 and July 17, during their period of sexual activity, lasting 2 to 3 hr between 5 and 9 PM. At distances of <5 m, females and males are easily distinguished by their flight behavior; they were caught with a sweepnet in case of doubt. The observers were stationary in 100-m^2 squares for 15 min.

Field Electroantennogram Recordings. The field EAG device was developed by Sauer et al. (1992). It consists of an antenna holder, protected by a glass chamber from ambient air, a suction pump, a charcoal filter, a motor-driven syringe to deliver defined pulses of synthetic pheromone, and hardware/software peripherals to amplify and record the EAG signal. The antenna holder is a Plexiglas disk (20 × 3 mm), with two wells (3.5 mm diam.) adjacent to a scission (1.2 mm) across the center. The antenna bridges this slit, its cut ends are locked into the wells filled with Ringer solution (Beadle-Ephrussi).

Ambient air was pulled through the glass chamber and the slit in the antenna holder at 42 ml/sec. For measurement of the resting potential of the antenna, the airstream was filtered with active charcoal (100 g); for measurements of ambient pheromone, the charcoal filter was removed. Data were sampled at 18.2 Hz on a portable computer; peak heights were averaged off-line for the first 2 sec of each recording.

To compare recordings from different antennae, EAG amplitudes were calibrated to a standard stimulus, to obtain relative EAG amplitudes: a 2-ml pulse from a 20-ml syringe, holding a rubber septum loaded with 10 μg *E*8,*E*10–12:Ac (3–5 days old, stored at −20°C), was injected into the charcoal-filtered airstream within the holding chamber. Three such standard stimuli preceded and followed three measurements of ambient air, lasting up to 5 sec.

Field EAG recordings were done with antennae of *C. nigricana* males, during their diel flight period between 5 and 9 PM. Males were collected in the control field and stored at 8°C for one day; each antenna was used during ca. 30 min. Measurements were made at 16 selected locations in the pheromone-permeated test field and the control field, at ca. 20 cm above and below pea canopy. The apparatus was set in the center of 100-m^2 squares, where a monitoring trap had been placed instead of a dispenser (see above). The EAG apparatus thus had a maximal distance to dispensers; the trap was removed during recordings.

Calculation of Absolute Pheromone Concentrations. Pheromone concentrations were calculated from: (1) relative EAG amplitudes in response to ambient air from the test field, and an exponential correlation between (2) relative EAG amplitudes in response to stimuli from syringes containing filter papers loaded with different amounts of *E*8,*E*10–12:Ac and (3) the extrapolated concentrations of *E*8,*E*10–12:Ac in these syringes (Figure 1).

From 5-ml syringes, holding filter papers with 10:Ac (100, 300, 500, and 1000 μg) or *E*8,*E*10–12:Ac (500 and 1000 μg), 2 ml of the headspace volatiles was injected on the GC. Concentrations of *E*8,*E*10–12:Ac in these syringes, at filter paper loads used for EAG recordings (0.1–100 μg), were extrapolated under the assumptions that *E*8,*E*10–12:Ac is approx. 16 times less volatile than 10:Ac (a factor of 4 for each methylene group) and that the release rate is directly proportional to the filter paper load (see Bengtsson et al., 1990). From

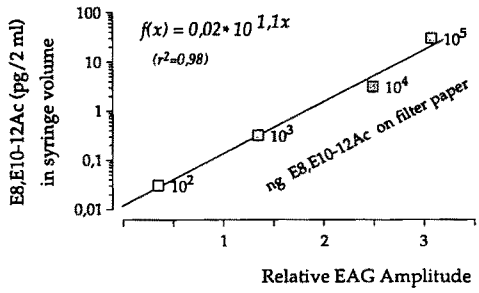

FIG. 1. Correlation between concentrations of *E*8,*E*10–12 : Ac (pg/2 ml) in 5-ml syringes holding filter papers loaded with 10^2–10^5 ng *E*8,*E*10–12 : Ac and relative EAG amplitudes in response to 2-ml pulses from these syringes ($N = 5$). EAG amplitudes were calibrated to the standard stimulus from a syringe holding a rubber septum loaded with 10 μg *E*8,*E*10–12 : Ac.

5-ml syringes, holding filter papers with 0.1–100 μg *E*8,*E*10–12 : Ac, 2 ml was injected into the holding chamber of the EAG apparatus within 0.5 sec. The relative EAG amplitudes to these stimuli were then correlated to the extrapolated *E*8,*E*10–12 : Ac concentrations in these syringes (Figure 1).

On injection into the EAG device, 2 ml of the syringe atmosphere was added within 0.5 sec to an air flow of 42 ml/sec and was therefore diluted by 1 : 23. Absolute concentrations of *E*8,*E*10–12 : Ac within the holding chamber of the EAG apparatus, from air sampled in the field, can thus be calculated from the exponential correlation shown in Figure 1.

All EAG recordings of ambient air in the field and of filter paper stimuli in the laboratory were calibrated to the standard stimulus (relative EAG amplitudes; see above). Both filter papers and rubber septa were kept in the syringes for 1 hr at ambient temperature before use. Error intervals were determined from the estimated experimental inaccuracies (air flow in EAG apparatus, *E*8,*E*10–12 : Ac concentrations in syringe, EAG amplitudes). Calibration of the EAG system is hampered by low sensitivity of the FID, compared to the male antenna: filter paper loads that allow concentration measurements by GC are beyond the response range of the antenna. This method, therefore, gives only an estimate of aerial pheromone concentrations.

RESULTS

The principal parameters of this first mating disruption trial against the pea moth *C. nigricana* are summarized in Table 1.

Release of Disruptant Chemicals. The mean release rate of *E*8,*E*10–12 : Ac

TABLE 1. PRINCIPAL PARAMETERS OF *C. nigricana* MATING DISRUPTION (HÖÖR, 1992)

Insect species	*Cydia nigricana* F. (Lep., Tortricidae)	
Crop treated	0.6-ha pea field (*Pisum sativum* L.)	
Sex pheromone	*E*8,*E*10–12:Ac	Greenway (1984)
Disruptant chemical	*E*8,*E*10–12:Ac	
Impurities		
After 6 days	3% *E*,*Z*-, 2% *Z*,*E*-, 1% *Z*8,*Z*10–12:Ac	Figure 2
After 20 days	4% *E*,*Z*-, 3% *Z*,*E*-, 2% *Z*8,*Z*10–12:Ac	
Activity	Attraction inhibitors (antagonists)	Witzgall et al. (1993)
Dispenser material	Polyethylene tube (Shin-Etsu)	
Dispenser placement	600 disp/ha	
Amount applied	50 mg/dispenser; 30 g/ha	
Release rate		
After 6 days	10 μg/disp/hr; 140 mg/ha/day	Figure 2
After 20 days	6 μg/disp/hr; 82 mg/ha/day	
Aerial concentration	~2 to ~7 ng/m^3 (within canopy)	Figures 1, 4
Communication disruption		
Traps (100 μg *E*8,*E*10–12:Ac)	100% reduction	Figure 5
Male behavior	Repelled from treated field	Table 2
Female behavior	Immigration of mated females	
Infestation		
Control field	67 larvae/m^2	
Treated field	52 larvae/m^2	

from the polyethylene tube dispensers, as measured by glass adsorption in a static atmosphere, was 7.6 ± 2.0 μg/hr between day 6 and day 20 (Figure 2). Isomeric purity of *E*8,*E*10–12:Ac was >99% before and after (day 0) formulation. Six and 20 days after dispenser placement, the nonpheromonal isomers *E*,*Z*; *Z*,*E*; and *Z*8,*Z*10–12:Ac (Witzgall et al., 1993) were released at 5.7% and 8.3% of *E*8,*E*10–12:Ac (Figure 2). This compares to isomerization of *E*8,*E*10–12:OH on polyethylene dispensers (Brown et al., 1992), but isomerization of *E*8,*E*10–12:Ac is faster on rubber septa (Davis et al., 1984; Witzgall et al., 1993).

Pheromone glands of calling *C. nigricana* females contain 0.8 ng *E*8,*E*10–12:Ac (Witzgall et al., 1993) and the release rate from the female gland is 1–5 ng/hr (Witzgall, unpublished). The dispensers used in this experiment thus emitted a 1000- to 10,000-fold amount of *E*8,*E*10–12:Ac.

Field EAG Measurements. A portable EAG apparatus was used to monitor the distribution and concentration of ambient pheromone under field conditions. Figure 3 shows recordings from *C. nigricana* antennae in the control pea field and the pheromone-treated field. The baseline is the resting potential of the antenna in filtered air; removal of the charcoal filter immediately changes the antennal potential, as ambient air is measured. Above the canopy, the antennal

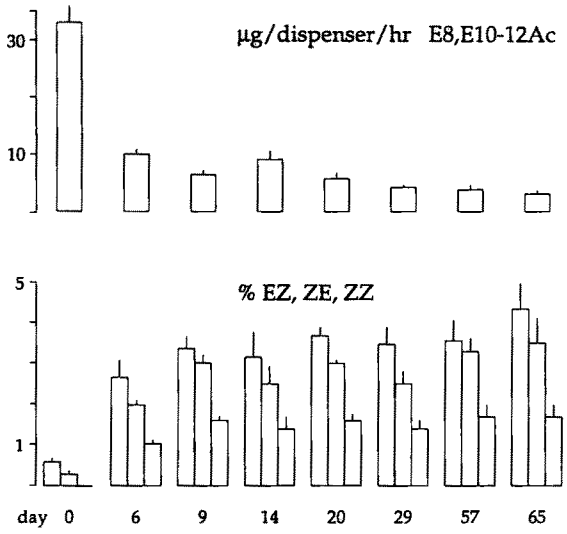

FIG. 2. Release of sex pheromone *E*8,*E*10–12:Ac (µg/hr) and antagonistic *E*,*Z*; *Z*,*E*; *Z*,*Z* isomers (relative to *E*,*E*) from polyethylene dispensers, 0–65 days after field application, as measured by glass adsorption in static atmosphere at 22°C (mean values ± SD; $N = 3$).

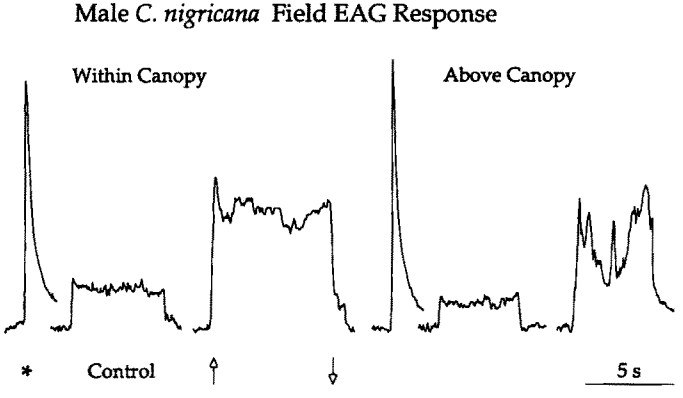

FIG. 3. Male *C. nigricana* field EAG response within the pea canopy and 20 cm above, nine days after dispenser application. Standard stimulus (asterisk: 10 µg *E*8,*E*10–12:Ac on rubber septum), control recording in untreated pea field, recording in test field (arrows: charcoal filter off/on).

signal fluctuated strongly, and overall response was always lower than within the canopy. Perception of plant volatiles probably accounted for the response in the control field.

Top pheromone concentrations built up along the border of the 0.6-ha test field (Figure 4), where 10.2 dispensers/100 m^2 had been placed, compared to 3.3 dispensers/100 m^2 in the middle. Trees protected the field border from wind and sun, peas were >60 cm high, with lush foliage; peas in the middle of the test field were <40 cm high, with sparse foliage.

In the 8-ha control pea field, upwind from the field permeated with pheromone, 4, 8, and 12 dispensers were placed in three isolated plots of 100 m^2. The average EAG response within the canopy of the 12-dispenser-plot was significantly higher than in the two other plots. Measurements above the canopy were not different (Figure 4; $N = 6$, Tukey test, $P = 0.05$).

Homogeneous distribution of pheromone within the pea canopy was probably due to less turbulent and slower air movement and desorption of pheromone from the pea foliage (Wall et al., 1981; Karg et al., 1990): 30 min after removal of the dispensers, relative EAG amplitudes within canopy of the 12-dispenser-plot were 0.35 ± 0.04 ($N = 3$), compared to 0.52 ± 0.08 immediately before removal.

Calculation of Pheromone Concentrations. Correlation of *E*8,*E*10–12:Ac concentrations in syringes and EAG responses to stimuli from these syringes was used to estimate absolute concentrations of ambient pheromone from the field EAG recordings (Figure 1). The average concentration within the pea

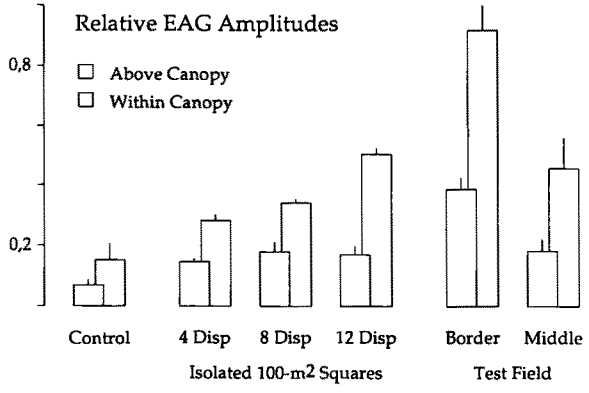

FIG. 4. Relative EAG amplitudes recorded in the centers of 100-m^2 squares, 6–25 days after dispenser application: in the pheromone-permeated test field, 5 m from the border (squares with 12 dispensers) and in the middle of this field (8 dispensers; $N = 18$), and in isolated squares within the control field (4, 8, 12 dispensers; $N = 6$). EAG amplitudes calibrated to standard stimulus (10 μg *E*8,*E*10–12:Ac on rubber septum).

canopy was 7 ± 3 ng *E*8,*E*10–12 : Ac/m^3 on the border, and 2 ± 2 ng *E*8,*E*10–12 : Ac/m^3 in the middle of the disruption field (data from Figure 4).

EAG Response to Nonpheromonal Isomers. The other isomers were tested in the laboratory at 3 to 100 ng on filter paper (half-decadic steps, $N = 4$). The EAG response to the *E*,*Z* isomer was 0.55 ± 0.09, to *Z*,*E* 0.27 ± 0.14 and to *Z*,*Z* 0.35 ± 0.08, relative to equal amounts of *E*8,*E*10–12 : Ac. The contribution of these isomers to the overall field response was estimated to ca. 2% on day 6 and ca. 3% on day 20, according to the proportions at which they were released from the dispensers (Figure 2); tests with binary blends showed no synergistic effect at the antennal level.

Field Trapping. Disruption of pheromonal communication in *C. nigricana* was monitored with 100 μg *E*8,*E*10–12 : Ac on red rubber septa. The dispensers were applied after 28 and 23 males had been trapped in the control and the test field. Trap catch on the border and within the pheromone-permeated field was thereafter completely suppressed throughout the whole flight period (Figure 5). Traps in the centers of the three isolated 100-m^2 plots within the control field, treated with 4, 8, and 12 dispensers, caught one, two, and one males on the first two days after dispensers were applied, but no males at all during the following 23 days.

Male Behavior. In untreated pea fields, male pea moths fly actively over the canopy in sunshine during late afternoons (Bradley et al., 1973; this study).

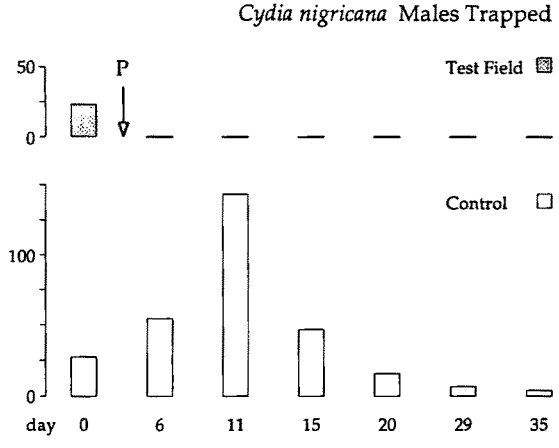

FIG. 5. Field attraction of *C. nigricana* males to traps baited with 100 μg of synthetic sex pheromone *E*8,*E*10–12 : Ac, in the control pea field ($N = 10$) and the pheromone-permeated field ($N = 10$), 0–35 days after dispenser application. Dispenser application (P).

They switch to slower, upwind-oriented flight as they encounter sources of pheromone.

On the first two days after dispenser placement, male moths were attracted to and even landed and wing-fanned on the dispensers or on surrounding pea plants (Table 2). From the untreated field, males flew upwind over several meters to fresh dispensers placed in three isolated 100-m^2 test plots; four males were also attracted to traps within these plots (see above).

In the permeated field (600 dispensers/ha), males were flying among pea plants, frequently alighting and wing-fanning on leaves, also in the vicinity of the dispensers (Table 2). However, directed orientation flights over more than 1 m towards dispensers were not observed. Males terminated searching by flying out of the plot or by resting on leaves. During this period, males may have found calling females, but traps in the test field were not attractive (Figure 5).

Male behavior was greatly changed a few days after dispenser placement. This was probably due to an increasing release of the inhibitory isomers *E,Z*-, *Z,E*-, and Z8,Z10–12:Ac, together with *E*8,*E*10–12:Ac (Figure 2) (Witzgall et al., 1993). Males were then no longer observed within the test field, and the few males arriving to the field border were obviously repelled and flew out rapidly, often 2 to 3 m above the ground. In the control field, *C. nigricana* males were observed throughout the whole flight period (Table 2).

Female Behavior. Mated females are attracted to peas, especially to the flowering parts, by upwind-oriented flight. They deposit single eggs on or near flowers and young pods (Bovey, 1972; this study). This behavior was unchanged in the presence of disruptant. Calling females were not seen in the control or test field.

Larval Counts. To facilitate behavioral observations, we chose field sites

TABLE 2. COUNTS OF ADULT *C. nigricana* IN MATING DISRUPTION AND CONTROL PEA FIELD

	Control field	4-dispenser plot	Test Field		
			Border	Border	Middle
Observation period[a]	6–17	0–1	0–2	6–17	6–17
Duration (hr)	9	5	6	11	5
Males/hour (±SD)	6.6 ± 3.5	14.4 ± 6.8	4.3 ± 4.0	0.8 ± 0.9	0
Males < 30 cm from dispenser	—	10.3 ± 8.2	3.0 ± 3.2	0	0
Females/hour	4.9 ± 2.7	[b]	[b]	7.2 ± 3.1	3.6 ± 2.1

[a] Days after dispenser placement.
[b] Not registered.

with high population densities and installed dispensers only one week after the onset of the flight period. The test field (0.6 ha) was 50 m downwind from the control pea field (8 ha), where 44 ± 14% of the pods were infested after the end of the flight period (67 larvae/m^2). Larval attack in the test field was not reduced significantly (34 ± 8%; 52 larvae/m^2) and we assume that this was due to immigration of gravid females.

DISCUSSION

The sexual communication of the pea moth, *C. nigricana*, was suppressed over the entire flight period by permeating a pea field with a blend of synthetic sex pheromone, *E*8,*E*10–12:Ac, and the antagonistic geometric isomers *E*,*Z*-, *Z*,*E*-, and *Z*8,*Z*10–12:Ac. Our results denote the potential of the mating disruption technique for the control of *C. nigricana*, as long as immigration of mated females is prevented, for example, in isolated fields.

A portable EAG system (Sauer et al., 1992), using the male antenna as detector, made it possible to monitor the fine-scale distribution and aerial concentration of disruptant chemical in the field. EAG recordings immediately "visualize dispersal of the disruptant chemical" (Arn, 1990), and greatly facilitate optimization of dispenser placement and interpretation of male behaviors. A disadvantage of the EAG method is the difficulty of calibrating the antennal response. Absolute concentrations of airborne disruptant may be more accurately determined by sampling several cubic meters of air over a few hours for subsequent chemical analysis (Caro et al., 1980, 1981; Wiesner et al., 1980).

Male *C. nigricana* behaviors changed as *E*8,*E*10–12:Ac isomerized within a few days in the field. Release of rather pure *E*,*E* isomer attracted males to dispensers and stimulated close-range search behavior, but release of *E*8,*E*10–12:Ac plus >5% of the inhibitory isomers *E*,*Z*-, *Z*,*E*-, and *Z*8,*Z*10–12:Ac (Figure 2) (Witzgall et al., 1993) repelled males from the treated plots. In *C. nigricana*, a blend of pheromone plus attraction inhibitors is thus a more efficient disruptant than pheromone alone.

A potent attraction inhibitor in the larch casebearer, *Coleophora laricella*, did not induce behavioral responses by itself, but acted as a strong repellent even to resting males when blended with sex attractant (Priesner and Witzgall, 1984). Upwind orientation of male *C. laricella*, *Trichoplusia ni*, and *Epiphyas postvittana* was suppressed by a blend and not by separate sources of attractant and inhibitor (Priesner and Witzgall, 1984; Witzgall and Priesner, 1991; Liu and Haynes, 1992; Rumbo et al., 1993). In the antennal lobes of the corn earworm, *Helicoverpa zea*, a distinct class of synergist neurons responded to a pheromone/inhibitor blend, but not to separate compounds (Christensen et al., 1991).

Control of lesser peachtree borer, *Synanthedon pictipes*, by mating disruption with sex pheromone *E*3,*Z*13–18:Ac was superior to insecticide treatment (Pfeiffer et al., 1991). The formulation contained 30% of *Z*3,*Z*13–18:Ac, a strong attraction inhibitor (Karandinos et al., 1977). Reliable control has been accomplished in the European grape berry moth, *Eupoecilia ambiguella* (Neumann et al., 1988; Neumann, 1990). Composition of the commercial formulation is not available from literature; a blend of sex pheromone *Z*9–12:Ac and at least 2% of inhibitory *E*9–12:Ac (Arn et al., 1986) was used by Rauscher and Arn (1979) and Vogt (1987). Racemic disparlure is being applied against the gypsy moth *Lymantria dispar* (Schwalbe and Mastro, 1988; Webb et al., 1988; Kolodny-Hirsch and Schwalbe, 1990); the (−)-enantiomer of disparlure is a weak behavioral antagonist (Miller and Roelofs, 1978; Preiss and Kramer, 1983). Male gypsy moths have been observed to terminate searching in arrays treated with racemic disparlure by rapidly flying high up into the tree canopy (Cardé et al., 1975).

In analogy to inhibitors, synergistic pheromone components do not initiate specific behaviors by themselves, but enhance male response when blended with the main component (Linn et al., 1986). In a number of lepidopteran species, including the Oriental fruit moth, *Grapholita molesta*, and codling moth, *C. pomonella*, suppression of mating is to date best achieved with a complete pheromone blend (e.g., Charlton and Cardé, 1981; Sanders, 1982; Audemard et al., 1989; Miller et al., 1990; Rice and Kirsch, 1990; Suckling and Clearwater, 1990; Tatsuki, 1990; Barnes et al., 1992; Howell et al., 1992; Pfeiffer et al., 1993).

There are also reports on the successful use of incomplete or imbalanced blends in *Vitacea polistiformis* (Johnson et al., 1991) and *Adoxophyes orana* (Charmillot and Pasquier, 1992). Male pink bollworms, *Pectinophora gossypiella*, intensified searching under permeation with two-component gossyplure, while they appeared inactive in response to the single components (Flint and Merkle, 1983). Similar observations were made for red-backed cutworm, *Euxoa ochrogaster* (Palaniswamy and Underhill, 1988). Application of the minor, but not of the major component, shifted the response of male *P. scutigera* to synthetic two-component blend ratios (Flint and Stone, 1985). Inhibitors alone were used against *Diparopsis castanea* (Marks, 1976) and *C. pomonella* (Hathaway et al., 1985).

Camouflage of natural plumes, competition between natural and synthetic plumes, imbalance of sensory input, and sensory overload have been proposed as the behavioral and physiological mechanisms of mating disruption (Bartell, 1982; Cardé, 1990). Sensory adaptation or central habituation alone cannot explain communication disruption (Charlton and Cardé, 1981; Novak and Roelofs, 1985; Miller et al., 1990), because the males may still use visual and

tactile stimuli (Richerson, 1977; Palaniswamy et al., 1986). False trail following or camouflage of calling females depends on assumptions that are hardly met in the field, i.e., sensorially largely unaffected insects, and unstructured synthetic plumes in the latter case (Bartell, 1982). Plume characteristics and stimulus concentrations are also expected to vary greatly within the crop. Quite obviously, the observed behavioral modifications cannot easily be attributed to distinct mechanisms; several mechanisms are assumed to synergize (Cardé, 1990).

The mechanisms of mating disruption derive from the disruptant chemicals—in relation to the pheromone composition of each species. Different chemicals, release rates, and dispenser densities result in different behaviors (e.g., Charlton and Cardé, 1981; Flint and Merkle, 1983; Palaniswamy et al., 1983; Flint and Stone, 1985; Palaniswamy and Underhill, 1988; Schwalbe and Mastro, 1988). Other variables are the insect's age and the duration of exposure to disruptant.

"If the proposition is accepted that a better understanding of the underlying mechanisms is desirable in order to design more robust systems of pest control through communication disruption, then it is clear that much work remains to be done at a fundamental level" (Bartell, 1982). This is still true in 1994. Such fundamental research depends most of all on the assessment and interpretation of behaviors. Field studies are difficult to achieve with night-active insects, and the natural milieu cannot be simulated in the laboratory; tools to identify the combined effects of adaptation, habituation, and disorientation are not yet available.

Pheromones are successfully applied against a number of pest species, and such mating disruption systems can be described by a few basic parameters. These must be assessed in order to interpret and optimize experiments, but also because they underlie the behavioral modifications to be studied. They can be measured with current techniques but are most often incompletely available from literature: (1) chemical composition of the disruptant and its behavioral effects at low doses, compared to natural pheromone; (2) dispenser placement, release rate, aerial concentration and distribution of disruptant; and (3) degree of communication disruption, as measured by pheromone-baited traps or live females, in relation to population density. Comparative analysis of these parameters from different applications and species may provide immediate input for further development.

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ETHYL PROPIONATE: SYNERGISTIC KAIROMONE FOR AFRICAN PALM WEEVIL, *Rhynchophorus phoenicis* L. (COLEOPTERA: CURCULIONIDAE)

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Abstract—Small trunk pieces of a freshly felled 10-year-old oil palm, *Elaeis quineensis* (Jacq.), were placed in a modified Nalgene desiccator, and volatiles captured for six days on Porapak Q. Gas chromatographic (GC) analysis of Porapak-Q-trapped volatiles with both flame ionization (FID) and electroantennographic detection (EAD) using male or female *R. phoenicis* antennae revealed several EAD-active compounds. They were identified as: ethyl acetate, ethyl propionate, isobutyl propionate, ethyl butyrate, and ethyl isobutyrate. In field experiments in the La Me Research Station, Côte d'Ivoire, ethyl propionate (50 mg/24 hr) but not all esters combined (50 mg/24 hr each) significantly increased capture of *R. phoenicis* in pheromone-baited (3 mg/24 hr) traps. One kilogram of 1- to 3-day-old palm tissue was significantly more effective than ethyl propionate in enhancing pheromone attraction. Superior attraction of palm tissue may be attributed to additional as yet unknown semiochemicals. Alternatively, release rates and/or ratios of synthetic volatiles differed from those of palm tissue at peak attraction.

Key Words—Coleoptera, *Rhynchophorus phoenicis*, *Elaeis quineensis*, kairomone, synergism, oil palm, palm weevil, palm volatiles, host selection,

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primary attraction, aggregation pheromone, 3-methyl-octan-4-ol, ethyl acetate, ethyl propionate, isobutyl propionate, ethyl butyrate, ethyl isobutyrate.

INTRODUCTION

Rynchophorus palm weevils are destructive pests of commercial and ornamental palms in the tropics (Hill, 1983). Weevils are attracted to wounded or stressed palms or fermenting palm sap (Kalshoven, 1950; Lever, 1969; Leong, 1987; Giblin-Davis and Howard, 1989; Sadakathulla, 1991). After oviposition on soft palm tissue, larvae tunnel into the terminal bud or trunk of the tree, leading to its death. In recent laboratory and field studies, methyl-branched, secondary alcohols have been identified as aggregation pheromones in the American palm weevil, *R. palmarum* L. (Rochat et al., 1991; Oehlschlager et al., 1992a); the African palm weevil, *R. phoenicis* L. (Gries et al., 1993; Rochat et al., 1993); the Asian palm weevils, *R. vulneratus* (Panz.), *R. ferrugineus* (Oliv.) (Hallett et al., 1993a,b), *R. bilineatus* (Montr.) (Oehlschlager et al., 1993b); and the sabal palmetto weevil, *R. cruentatus* (F.) (Weissling et al., 1994). As in the maize weevil, *Sitophilus zeamais* Motschulsky (Walgenbach et al., 1987), attraction of *Rhynchophorus* weevils to aggregation pheromones invariably required the presence of synergistic plant tissue (Oehlschlager et al., 1992a,b, 1993a,b,c; Gries et al., 1993; Hallett et al., 1993a,b; Rochat et al., 1993; Weissling et al., 1994). We report the identification and field testing of oil palm constituent ethyl propionate, which enhances attraction of the *R. phoenicis* aggregation pheromone "phoenicol" (3-methyl-octan-4-ol).

METHODS AND MATERIALS

Volatile Collection. In the La Me Research Station, Côte d'Ivoire, lower trunk sections of a freshly felled 10-year-old oil palm were cut into small cubes and placed in a modified Nalgene desiccator. An aspirator-driven, charcoal-filtered airstream was maintained through the desiccator for five days, collecting palm volatiles on Porapak Q. Volatiles were eluted from Porapak Q with pentane and concentrated by distillation.

Instrumental Methods. Porapak Q extracts were analyzed by gas chromatographic–electroantennographic detection (GC-EAD) (Arn et al., 1975), employing a Hewlett Packard (HP) 5885B gas chromatograph equipped with a SP-1000-coated, fused silica column (30 m × 0.25 mm ID) (Supelco, Inc. Bellefonte, Pennsylvania). A HP 5985B coupled GC–mass spectrometer (GC-MS) fitted with the same column was used for GC-MS analyses of plant volatiles in both electron impact (EI) and chemical ionization (CI) modes.

Field Bioassay. Weevil response to synthetic palm volatiles was tested in

6- to 10-year-old oil palm stands of the La Me Research Station. Fifteen-liter bucket traps (Oehlschlager et al., 1992a) were attached at breast height to palm trees in complete randomized blocks with traps at 27-m intervals and blocks 27–100 m apart. Release devices suspended 5 cm below the trap lid dispensed test chemicals. Synthetic, stereoisomeric phoenicol (98% purity) (Gries et al., 1993), was released at 3 mg/day (at 25°C), whereas each of the palm volatiles was released at 50 mg/day (at 25°C). A 5-cm-wide ring of petrolatum (Anachemia, Rouses Pount, New York 12979) on the inner upper surface of each trap, and a wet yellow sponge on the trap bottom, treated with insecticidal (biodegradable) Evisect-"S" (0.3% thiocyclam-hydrogenoxalate in water), retained captured weevils.

The first prescreening seven-treatment experiment tested phoenicol alone and in combination with either one or all of the following compounds: ethyl acetate (EA), ethyl propionate (EP), isobutyl propionate (IBP), ethyl butyrate (EB), or ethyl isobutyrate (EIB). The second four-treatment experiment tested phoenicol alone and in combination with either EA, EP, or both. A final three-treatment experiment tested phoenicol alone and combined with either EP or 1 kg of freshly cut oil palm tissue. All compounds were purchased (Aldrich Chem. Company, Inc., Milwaukee, Wisconsin 53233) and were >98% chemically pure.

RESULTS

Four palm volatiles elicited good antennal responses in GC-EAD analyses (Figure 1). EI and CI mass spectra of these compounds indicated that they were, respectively, EA, EP, EIB, and EB (Figure 1). Palm volatiles and authentic standards had comparable antennal activity and identical retention and mass spectrometric characteristics. Additional esters, such as isobutyl propionate, were identified in palm volatiles but were only weakly EAD active. In the prescreening field experiment, EA or EP but not all synthetic esters combined enhanced attraction to phoenicol. Ethyl propionate alone more than in combination with EA enhanced pheromone attraction (Experiment 2, Figure 2), but palm tissue was two to three times more synergistic than EP (Experiment 3, Figure 3).

DISCUSSION

Selection of specific plants for feeding and reproduction has been well documented in the Coleoptera, particularly for scolytid and curculionid beetles (Wattanapongsiri, 1966; Moeck et al., 1981 and references therein; Ryker and Oester, 1982; Gara et al., 1984; Miller et al., 1986; Moeck and Simmons, 1991; Byers, 1992 and references therein; Tunset et al., 1993). Short-chain

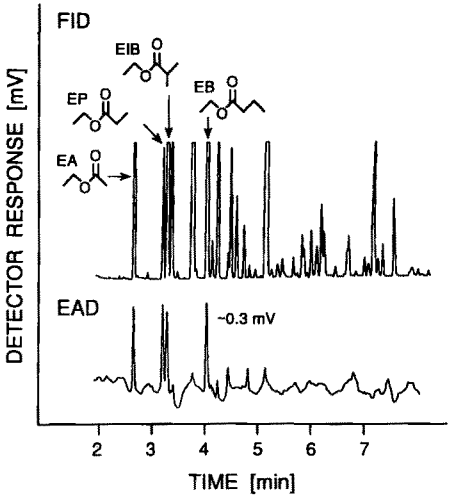

FIG. 1. Flame ionization detector (FID) and male *R. phoenicis* antenna (EAD) responses to African oil palm volatiles chromatographed on a SP-1000-coated, fused silica column (1 min at 50°C, 10°C/min to 180°C).

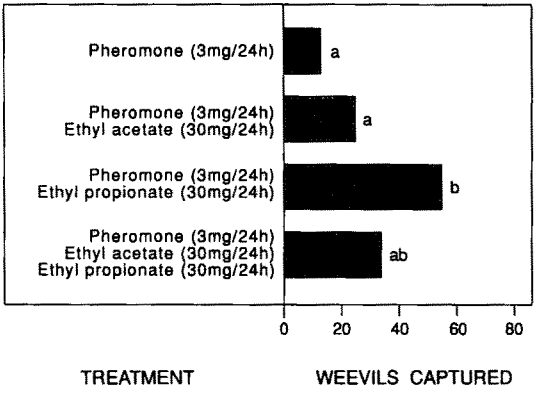

FIG. 2. Total capture of *R. phoenicis* in traps baited with the aggregation pheromone, 3-methyl-4-octanol, alone or in combination with either ethyl acetate, ethyl propionate, or both. La Me Research Station, Côte d'Ivoire, May 11–17, 1993, $N = 7$. Bars superscripted by the same letter are not significantly different; $P < 0.05$, ANOVA followed by Scheffé test.

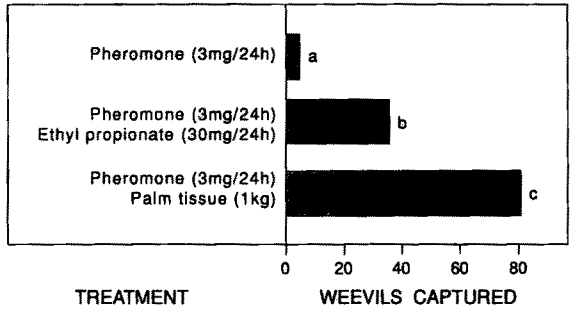

FIG. 3. Total capture of *R. phoenicis* in traps baited with pheromone alone or in combination with either ethyl propionate or 1 kg of 1- to 3-day-old palm tissue. La Me Research Station, Côte d'Ivoire, May 14–17, 1993, $N = 10$. Bars superscripted by the same letter are not significantly different; $P < 0.05$, ANOVA followed by Scheffé test.

alcohols, oleoresin, monoterpenes, and sesquiterpenes alone or in combination have been associated with host selection behavior (Byers, 1992 and references therein).

Plant-derived volatiles and beetle-produced pheromones have often been shown to mediate synergistically host selection behavior. Although aggregation pheromones of many beetles are known (Borden, 1985; Byers, 1989), few synergistic host plant kairomones have been identified. Ethanol, myrcene, α-pinene, β-phellandrene, and camphene enhance response to aggregation pheromone in various bark and ambrosia beetles (Bedard et al., 1969; Moeck, 1970; Vité and Bakke, 1979; Kohnle, 1985; Paiva and Kiesel, 1985; Byers et al., 1988; Miller and Borden, 1990). α-Cubebene, typically released from moribund elm trees, enhances pheromone attraction in the elm bark beetle, *Scolytus multistriatus* (Marsham) Peacock et al., 1984). Propanoic and butanoic acids, methanol, 2-propanol, 1-heptanol, methyl butanoate, and propanal were most effective in synergizing pheromone attraction of the dried fruit beetle, *Carpophilus hemipterus* (L.) (Dowd and Bartelt, 1991). In mimicking whole-wheat bread dough odor, a blend of synthetic acetaldehyde, ethyl acetate, ethanol, 1-propanol, 2-methylpropanol, 2-methylbutanol, and 3-methylbutanol attracted the nitidulids *Carpophilus lugubris* Murray, *Glischrochilus quadrisignatus* (Say), and *G. fasciatus* (Olivier) (Lin and Phelan, 1991a,b) and synergistically enhanced attraction of *C. lugubris* pheromone (Lin et al., 1992). We report EP as a synergistic host plant kairomone for *R. phoenicis* (Figure 2 and 3).

EP significantly increased capture of *R. phoenicis* in phoenicol-baited traps (Figure 2) but was still less effective than freshly cut palm tissue (Figure 3). *R. phoenicis* preference for palm- over EP-baited traps (Figure 3) was unlikely due to different microclimates within traps. The weevil-preferred humid environment

(Weissling and Giblin-Davis, 1993; Oehlschlager et al., 1993c), as provided by palm tissue, was prevailing in *all* traps due to wet sponges on trap bottoms. Superior synergism of palm tissue may therefore be attributed to additional, as yet unknown palm volatiles.

Increase, culmination, and decrease of palm tissue attraction within three to five days after cutting (Weissling et al., 1992; Gries et al., 1993; Hallett et al., 1993a; Oehlschlager et al., 1993a) indicate proportional changes of semiochemicals that may be critical for optimal attraction of weevils. Potato leaf volatiles, e.g., *cis*-3-hexen-1-ol, *cis*-3-hexenyl acetate, *trans*-2-hexenal, and *trans*-2-hexen-1-ol only induce positive anemotaxis in the Colorado beetle, *Leptinotarsa decemlineata* (Say), when released in natural ratios (Visser, 1979). Antennally active palm volatiles should therefore be field tested at ratios and release rates equivalent to palm tissue at peak attraction. Abundance and ratio of palm volatiles in volatile extracts may not accurately reflect natural release rates of palm tissue and may in part depend on the volatile collection technique. Aside from Porapak Q, other absorbents, such as charcoal, Tenax, glasswool, or cryogenic traps (Golub and Weatherston, 1984) need to be evaluated, particularly for trapping low-boiling-palm volatiles.

Ethyl propionate, EA, EB, and EIB are common volatile constituents in (fermenting) African oil palm, coconut palm, *Cocos nucifera* L., and cabbage palmetto, *Sabal palmetto* (Walter) (Gries et al., unpublished). As all these esters elicit antennal responses in *R. phoenicis* and congeneric *R. palmarum*, *R. cruentatus*, *R. ferrugineus*, *R. vulneratus*, and *R. bilineatus* (Gries et al., unpublished), EP or any other "palm ester" alone or in combination with other palm volatiles may be a primary attractant for *Rynchophorus* palm weevils. In a recent empirical study, EA has been shown to be a semiochemical for *R. cruentatus* (Giblin-Davis et al., 1993) and for *R. palmarum* (Jaffé et al., 1993). Synthetic palm volatiles in pheromone-baited traps could replace currently used insecticide-treated sugar cane or palm tissue, thereby promoting the further development of commercial, semiochemical-based management of *Rynchophorus* weevils in oil, coconut, and date palm (Oehlschlager et al., 1992b; Chinchilla et al., 1993).

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REPELLENT, TOXIC, AND FOOD PROTECTANT EFFECTS OF PITHRAJ, *Aphanamixis polystachya* EXTRACTS AGAINST PULSE BEETLE, *Callosobruchus chinensis* IN STORAGE

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Abstract—Ground leaves, bark, seeds, and four seed extracts of pithraj, *Aphanamixis polystachya* (family Meliaceae), a locally grown plant in Bangladesh, were evaluated for their repellency, contact toxicity, and food protectant efficacy against adult pulse beetle (*Callosobruchus chinensis* L.). The seed extracts showed poor repellent effects, but high contact toxicity to adults at 72 hr after application. The ground leaves, bark, and seeds provided good protection for mung beans against pulse beetles, and the seed powder greatly reduced the F1 progeny and seed damage rates.

Key Words—*Aphanamixis polystachya*, pithraj, repellent, contact toxicity, food protectant, *Callosobruchus chinensis*, Coleoptera, Bruchidae.

INTRODUCTION

Pithraj (*Aphanamixis polystachya* Wall & Parker, also known as *Amoora ruhituka* Wright & Arn.) is a perennial tree that grows in tropical climates (Islam, 1984, 1985). This plant, which belongs to the family Meliaceae, is used traditionally for insect control in Bangladesh. Farmers use the seed-oil to avoid insect bites during the early morning and evening field works. Ground leaves, bark, and seeds are used to protect grain in storage. This plant is also a source of pharmaceutical compounds (Srivastava and Agnihotri, 1984). Few studies have been conducted on the antiinsect properties of this plant (Islam, 1984, 1985; Khanam et al., 1990). Experiments were carried out to determine the

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repellent, contact-toxic effects of pithraj seed extracts and the food protectant value of ground leaves, bark, and seeds against the pulse beetle, *Callosobruchus chinensis* (L.), a major storage pest of pulse seeds.

METHODS AND MATERIALS

Insects

The pulse beetle, *Callosobruchus chinensis* (L.) (Coleoptera: Bruchidae), a major stored-product insect pest, was collected from the Agrochemical Evaluation Unit, University of Southampton, England, and reared on a diet of 100 g mung bean (*Phaseolus mungo*) per jar (size 14 × 10.5 × 30 cm), in the laboratory at 27 ± 2°C with alternating 12-hr light and dark periods. The relative humidity was kept constant at 70 ± 5% by using a standard solution of sodium chloride (Greenspan, 1977).

Preparation of Plant Extracts

Pithraj seeds, leaves, and bark were collected from Bangladesh in 1993. Seeds were air-dried and ground in an electric grinding machine. In a Soxhlet apparatus 75 g of ground seeds were extracted with redistilled petroleum ether (bp 40–60°C) for 6 hr. The marc was then extracted successively with acetone, 95% ethanol, and methyl alcohol, for 6-hr periods in each case. Solvents from each extract were evaporated in a rotary vacuum evaporator under reduced pressure and yielded the petroleum ether, acetone, ethyl alcohol, and methyl alcohol extracts, respectively. The diagrammatic presentation of the whole extraction process is given in Figure 1.

Bioassays

Repellency Tests by Filter Papers. Repellency was tested according to the McDonald's standard method number 3 with some modifications (McDonald et al., 1970). Substrates were prepared by cutting filter-paper circles (Whatman No. 40), 9 cm in diameter, in half. Pithraj extracts were redissolved in known amounts of solvents to provide a concentration of 10 mg/ml. One milliliter of solution of each extract was applied to half-filter papers as uniformly as possible with a pipet, so that the treated substrate contained 0.16 mg/cm^2 of extract. The treated half circles were then air-dried to evaporate the solvent completely. One full circle was then remade by attaching a treated half to an untreated (control) half circle of the same dimension by cellotape. Precautions were taken so that the attachment did not interfere with the free movement of insects from one half to another, but a small gap was left between the filter paper halves to prevent seepage of test samples from one half of circle to another. The filter paper

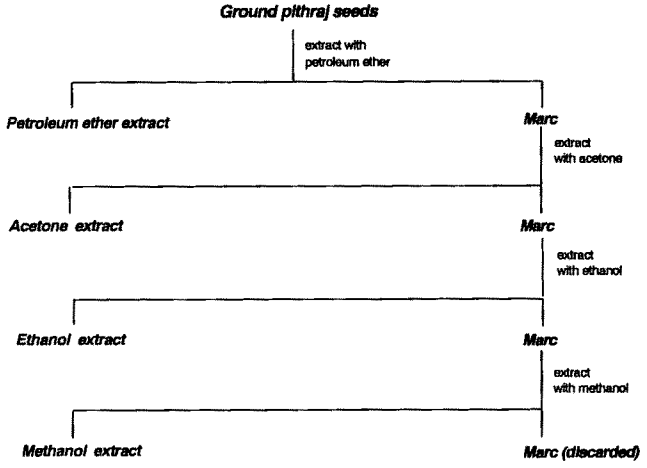

FIG. 1. Schematic presentation of pithraj seed extraction.

circles were then placed in Petri dishes (one circle in one Petri dish) with the seams oriented in four different directions to avoid the influence of any external stimulus on the distribution of insects. Ten insects were released in the middle of each filter-paper circle and the cover was replaced on the Petri dish. There were four replications for each concentration of plant extract. Insects present on each strip were counted at 1-hr intervals up to 5 hr. The average of counts was converted to express percentage repellency (PR) by the following formula:

$$PR = (N_c - 5) \times 20$$

where N_c is the number of insects present in the control half.

Positive values (+) expressed repellency and negative values (−) attractancy. Data were analyzed by the analysis of variance (ANOVA) after transforming them into arcsin $\sqrt{}$percentage values. The average values were then categorized according to the following scale:

Class	Repellency rate (%)
0	>0.01-<0.1
I	0.1-20
II	20.1-40
III	40.1-60
IV	60.1-80
V	80.1-100

Contact Toxicity. For contact toxicity by topical treatment, laboratory tests were conducted according to the standard method number 1 with slight modi-

fications (McDonald et al., 1970). Stock solutions were prepared by dissolving 100 mg of the petroleum ether extract, acetone extract, ethanol extract, and methanol extract in 1 ml of the respective solvent. Lower concentrations (60, 40, 20, and 10 mg/ml) were obtained by dilution of the stock solution with solvent. Insects were chilled for a period of 10 min, and the immobilized insects were picked up individually using a small suction tube. Using a capillary tube, 1 μl of solution (100, 60, 40, 20, or 10 μg/insect) was applied to the dorsum of the insect. Fifty unsexed insects, in five replicates of 10 insects each, were treated with each dose. Fifty insects also were treated with solvents only as controls. After treatment, insects were transferred into 9-cm-diameter Petri dishes, placing 10 insects/Petri dish, each containing 5 g of mung beans. Insects were examined daily, and those that did not move or respond to a gentle touch were considered to be dead. Insect mortalities were recorded at 24, 48, and 72 hr after treatment, corrected by Abbott's (1987) formula and then analyzed by ANOVA; the mean values were compared with Duncan's (1951) multiple range test. Concentration–mortality lines were calculated using probit analysis (Finney, 1971) with a $\log_{10}$ transformation of concentrations of pithraj seed extracts. Results were expressed as micrograms per insect. Two LD_{50}s were considered to be significantly different ($P < 0.05$) if their 95% fiducial limits did not overlap; slopes were similarly considered to be significantly different if their standard errors did not overlap. For time–mortality probit lines (LT_{50}), mortality data were analyzed using a probit model (Finney, 1971) that produced a straight-line fit to the probit of mortality response to the logarithm of the treatment times.

Food Preference Methods. Pithraj leaf, bark, and seeds were air-dried and ground in an electric grinding machine. The leaf, bark, and seed powders were applied to mung beans at the rate of 2.5% by weight. The food preference apparatus consisted of a plastic Petri dish of 13.5 cm diameter. The Petri dish had eight holes, 2.5 cm in diameter, equally spaced along the periphery to accommodate smaller Petri dishes, which were filled to the rim with treated or untreated mung beans (about 5 g each). Eighty adults (24–48 hr old) were released in the middle of each test arena and allowed to oviposit for seven days. At the end of the exposure period, the beetles were sieved from the mung beans. The treated and untreated Petri dishes were arranged in different orders for each experiment. On the subsequent days (up to day 33) the following were recorded: (1) number of adults in each small Petri dish (on day 7); (2) number of eggs deposited per 50 mung seeds; (3) number of hatched eggs; (4) number of F_1 adults emerging from each dish (from day 21 to day 33); (5) seed damage rate (random sample of 50 mung seeds at the end of the experiment); and (6) oviposition preferences measured by means of a discrimination quotient (*DQ*), where

$$DQ = \frac{\text{eggs } (N) \text{ on control} - \text{eggs } (N) \text{ on treated}}{\text{eggs } (N) \text{ on control} + \text{eggs } (N) \text{ on treated}}$$

The data were transformed using a square root ($Y = \sqrt{X} + 1$) and arcsin values and then analyzed by ANOVA; the mean values were compared with Duncan's (1951) multiple range test.

RESULTS

Repellency Effects. The pithraj seed extracts were weakly repellent to pulse beetles (Table 1). Among the four extracts, the methanol extract had the maximum repellency (44%, class III), followed by the ethanol extract (30%, class II), the acetone extract (26%, class II), and the petroleum ether extract (19%, class I); however, the means were not significantly different ($P > 0.05$).

Contact Toxicity. Mortality data were obtained for pulse beetles 24, 48, and 72 hr after topical application (Table 2). The toxicity of pithraj seed extracts to pulse beetles showed that the ethanol extract possessed highly toxic activity at 72 hr after application, followed by the acetone extract, the methanol extract and the petroleum ether extract. All of the ethanol extracts caused >50% mortality. Lower doses of the extracts were not very effective.

The probit statistics, estimates of LD_{50} and their 95% fiducial limits, and the slopes of regression lines are presented in Table 3. All the extracts caused significant mortality of the pulse beetle at lower LD_{50} levels (Table 3). From the probit analyses, comparison of LD_{50}s among the extracts showed that the ethanol extract was highly toxic (LD_{50} 10 μg/insect), followed by the acetone extract (12 μg/insect), the methanol extract (40 μg/insect), and the petroleum ether extract (49 μg/insect). Comparison of lethal mean times (LT_{50}) among the insects and treatment times showed that pulse beetle was highly susceptible to

TABLE 1. AVERAGE REPELLENCY OF PITHRAJ SEED EXTRACT TO PULSE BEETLE ADULTS USING TREATED FILTER PAPER TEST[a]

Extract	Concn. (mg/cm²)	Average % repellency rate at hours after insect release					Mean rate	Repellency class
		1	2	3	4	5		
Petroleum ether	0.16	25	20	10	20	20	19	I
Acetone	0.16	25	20	20	35	30	26	II
Ethanol	0.16	65	20	20	15	30	30	II
Methanol	0.16	40	35	40	45	60	44	III
F value		NS	NS	NS	NS	NS	NS[b]	

[a]Original data were transformed into arcsin √percentage values during ANOVA test.
[b]NS = not significant.

TABLE 2. TOXICITY OF PITHRAJ SEED EXTRACTS APPLIED TOPICALLY TO PULSE BEETLE ADULTS[a]

Extract	Dose (μg/insect)	Average % mortality ± SE[b] (at hours after treatment)		
		24[c]	48	72
Petroleum ether	10	6 ± 0.8 b	17 ± 1.4 b	18 ± 1.7 c
	20	8 ± 1.2 b	24 ± 1.7 b	31 ± 1.5 bc
	40	14 ± 1.3 ab	31 ± 1.7 b	48 ± 2.1 ab
	60	23 ± 1.9 a	33 ± 2.1 ab	56 ± 2.5 a
	100	25 ± 1.8 a	49 ± 1.3 a	67 ± 0.9 a
Acetone	10	4 ± 0.8 d	19 ± 1.9 d	38 ± 1.4 b
	20	6 ± 0.8 cd	30 ± 0.6 c	63 ± 2.6 a
	40	12 ± 1.6 bc	40 ± 0.6 bc	66 ± 2.2 a
	60	16 ± 1.3 ab	49 ± 1.9 ab	74 ± 1.6 a
	100	28 ± 1.2 a	62 ± 0.7 a	81 ± 1.0 a
Ethanol	10	12 ± 1.2 c	20 ± 2.0 d	58 ± 1.7 c
	20	18 ± 1.2 bc	31 ± 1.9 cd	60 ± 2.1 c
	40	20 ± 1.0 bc	55 ± 1.2 bc	75 ± 1.5 bc
	60	26 ± 0.7 ab	59 ± 2.3 b	83 ± 2.1 b
	100	39 ± 1.6 a	86 ± 2.1 a	97 ± 0.9 a
Methanol	10	6 ± 0.8 c	78 ± 0.6 c	16 ± 1.4 c
	20	10 ± 1.0 bc	20 ± 0.2 b	34 ± 1.5 b
	40	14 ± 0.8 b	23 ± 0.8 b	48 ± 1.3 b
	60	20 ± 1.0 ab	30 ± 1.3 b	54 ± 1.9 b
	100	30 ± 1.0 a	45 ± 1.1 a	80 ± 1.3 a

[a]Original data corrected by Abbott's formula and then transformed into arcsin √percentage values before ANOVA and DMRT test.
[b]SE = standard error of mean.
[c]Values followed by the same letter within a column are not significantly different at the 0.05 level by DMRT.

the duration of exposure to extracts and had lowest LT_{50} values (50 hr/insect for the ethanol extract, 62 hr/insect for the acetone extract, 76 hr/insect for the methanol extract, and 83 hr/insect for the petroleum ether extract).

The probit regression lines for the effects of extracts on the pulse beetle showed a clear linear relationship between percentage mortality and extract concentrations (Figure 2). Because the adults were treated with more toxin for the same period at higher concentrations, the slopes of the probit lines were steeper as concentration increased. For the pulse beetle, the regression lines $Y = 2.63 + 1.40X$ for the petroleum ether extract, $Y = 3.90 + 1.03X$ for the acetone extract, $Y = 3.66 + 1.34X$ for the ethanol extract, and $Y = 2.33 + 1.67X$ for the methanol extract were calculated. Comparing all four regression lines, the highest probit mortality was found with the ethanol extract.

TABLE 3. PROBIT ANALYSIS FOR CONTACT TOXICITY AT 72 HOURS AFTER TOPICAL APPLICATION OF PITHRAJ EXTRACTS TO PULSE BEETLE ADULTS[a]

Extract	Insects (*N*)	LD_{50} (μg/insect[b])	95% fiducial limit	Slope ± SE
Petroleum ether	250	49.0 b	34.0–70.0	1.40 ± 0.36
Acetone	250	12.0 a	6.0–22.0	1.03 ± 0.26
Ethanol	250	10.0 a	5.0–19.0	1.34 ± 0.32
Methanol	250	40.0 b	31.0–53.0	1.66 ± 0.31

[a]Values were based on five concentrations and five replicates of 10 insects each.
[b]Values followed by the same letter within a column are not significantly different at the 0.05 level by DMRT.

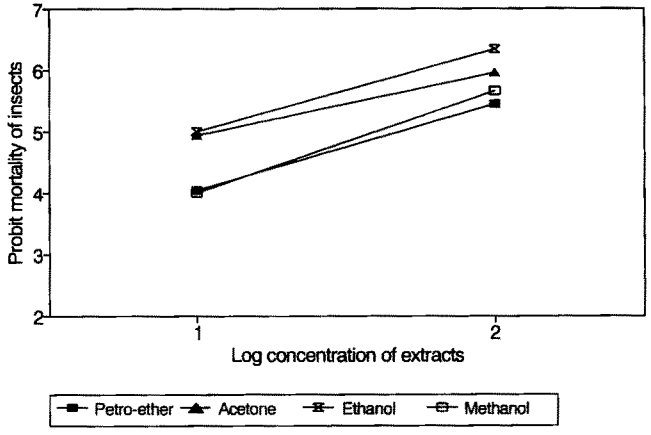

FIG. 2. Log concentration–probit mortality graph for four seed extracts against the pulse beetle.

A linear relationship between the duration of exposure and mortality percentage of treated insects also was observed in all cases (Figure 3). The steepness of the slopes indicates the time effectiveness on pulse beetle mortality. The calculated regression line was $Y = -9.41 + 7.51X$ for the petroleum ether extract, $Y = -5.06 + 7.51X$ for acetone extract, $Y = -2.75 + 4.56X$ for the ethanol extract, and $Y = -4.52 + 5.06X$ for the methanol extract. Comparing all four regression lines, the highest mortality was found with the ethanol extract.

Food Preference Methods. At a concentration of 2.5% (w/w), the leaf, bark, and seed powders deterred oviposition by the pulse beetle to some extent

(Table 4). Only a few beetles were found in treated mung bean at seven days after treatment. Mung beans treated with leaf powder had the least number of beetles. Beans treated with seed powder had the smallest number of eggs. It was noted that although 60% of the eggs laid hatched in the dishes treated with seed powder, no F_1 adults emerged from those dishes. The highest number of F_1 adults (81.25) emerged from the control mung beans. There was no damage to mung beans treated with seed powder. The discrimination quotient (*DQ*)

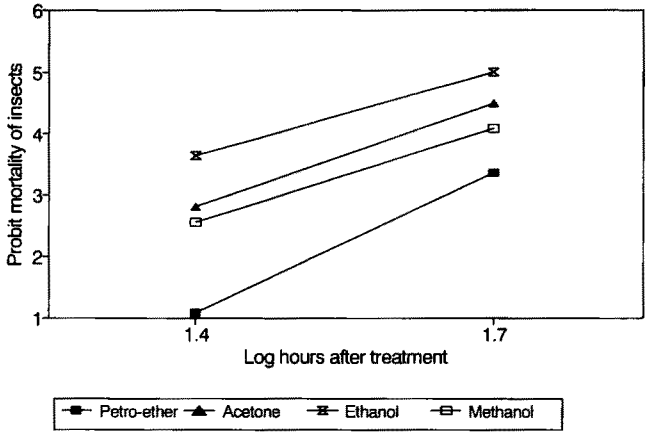

FIG. 3. Log hour–probit mortality graph for four pithraj seed extracts against the pulse beetle.

TABLE 4. AVERAGE VALUES OF FOOD PREFERENCE TESTS FOR PULSE BEETLES[a]

Pithraj powder	Insects (*N*)/ dish[b] (7 DAT)	Total eggs/dish (7 DAT)	Eggs hatched (%) (7 DAT)	F_1 adults emerged (*N*) (33 DAT)	Seed damaged (%) (33 DAT)	Discrimination quotient
Leaves	4 a	74 a	85 a	53 b	47.50 b	0.24 a
Bark	4 a	79 ab	89 a	61 bc	51.25 b	0.19 a
Seeds	6 ab	23 a	60 a	0 a	0.00 a	0.64 a
Control	11 b	115 b	88 a	81 c	63.75 b	—

[a] Data of insects and egg number, F_1 and DQ were transformed into square root ($Y = \sqrt{x} + 1$) values and hatching rate and damage rate data into arcsin $\sqrt{}$percentage values before ANOVA and DMRT tests were applied.

[b] Values followed by the same letter within a column are not significantly different at the 0.05 level by DMRT.

values indicated that oviposition deterrency was highest in mung beans treated with pithraj seed powder, followed by leaf and bark powder.

DISCUSSION

When evaluated by the filter paper repellency method, pithraj seed extracts showed poor repellent effects on pulse beetles at a dose of 0.16 mg/cm^2. Islam (1984) reported repellent effects of pithraj on Angoumois moths (*Sitotroga cerealella*), rice green leafhopper (*Nephotettix nigropictus* Stal.), and brown planthopper (*Nilparvata lugens* Stal.). The present result was in agreement with his findings. However, in the present experiment, the repellent effects of all four extracts (petroleum ether, acetone, ethanol, and methanol) were not statistically different from one another ($P < 0.05$). The weak repellent effects of seed extracts on the pulse beetle might be related to the fact that pulse beetle adults do not feed as adults. The pithraj seed extracts showed higher contact toxicity rates to the pulse beetle. Mortality percentages were directly proportional to the concentration and to the duration of exposure. Khanam et al. (1990) reported toxic effects of pithraj seed coat extracts on *Tribolium confusum* Duval. Among the tested extracts, the ethanol extract showed the highest toxic effects and the lowest LD_{50} and LT_{50} values. On the basis of the present results, higher concentrations contributed significantly more to the efficacy of extracts on the mortality of insects, and they appeared to be the most important factor in the degree of control obtained with pithraj seed extracts. The efficiency of pithraj powders as protectants for mung beans was evaluated by comparing the number of F_1 progeny of the insects from the treated dishes and control dishes. Data for food preference tests (Table 4) showed that the leaf, bark, and seed powders of pithraj deterred insect oviposition to some extent. All three powders were effective in reducing the ovipositional preference and survivorship of eggs. Significantly fewer adults were found in powder-treated Petri dishes compared with control dishes. Seed damage rates were also low in treated dishes. The results suggest that seed powder would be a good protectant for mung beans. Islam (1987) reported that pithraj seed extracts reduced ovipositional preference and inhibited adult emergence of the rice hispa, *Dicladispa armigera* Oliv. and the pulse beetle, *Callosobruchus chinensis* L. The present laboratory studies reveal the poor repellency, high contact toxicity, and high food protectant capability of pithraj seed extracts on tested pulse beetles. Finally, the initial efficacy tests described here indicate a potential use of pithraj extracts in storage pest management systems against pulse beetles.

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QUANTIFICATION OF ELECTROANTENNOGRAM RESPONSES OF THE PRIMARY RHINARIA OF *Acyrthosiphon pisum* (HARRIS) TO C_4–C_8 PRIMARY ALCOHOLS AND ALDEHYDES

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Abstract—Electroantennograms (EAGs) of the distal and proximal primary rhinaria (DPR and PPR, respectively) were recorded from excised antennae of alate virginoparous pea aphids, *Acyrthosiphon pisum* (Harris) (Homoptera: Aphididae). Primary unsaturated alcohols and aldehydes with varying carbon length (C_4–C_8) were used as volatile stimuli. EAGs were recorded for a series of source concentrations from the DPR and PPR separately through the use of sectional electroantennography. A logistic equation was fitted to the source concentration–response data. Differences in relative EAG response of the DPR and PPR to the alcohols and aldehydes were analyzed by deriving five parameters from this logistic equation. These parameters relate to particular characteristics of sigmoid curves: the saturation (maximum) EAG response (R_s), the concentration for which the relative EAG response is $\frac{1}{2}R_s$ (CR_{50}), the stimulus response range ($SR_{0.9}$), the threshold concentration (CR_1), and the EAG response area (A_R). Of these parameters, the EAG response area showed the largest separation between EAG responses of the DPR and PPR to the

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two homologous groups and between compounds with varying carbon chain lengths. The DPR was significantly more responsive to alcohols than to aldehydes, while the reverse was true for the PPR, indicating a basic difference between the two primary rhinaria. The highest overall responses were elicited by 1-hexanol, hexanal, and heptanal.

Key Words—Homoptera, Aphididae, pea aphid, *Acyrthosiphon pisum*, EAG, sectional electroantennography, antenna, alcohols, aldehydes, structure–activity, placoid sensilla, chemoreception, olfaction.

INTRODUCTION

Several studies have shown that, in addition to vision (Kennedy et al., 1961; Burrows et al., 1983; David and Hardie, 1988; Pelletier, 1990; Nottingham et al., 1991), olfactory cues are used by aphids to detect and orient towards host plants. These studies include behavioral responses to host-plant volatiles in several types of olfactometers (Jones, 1944; Alikhan, 1960; Pettersson, 1970, 1973; Pospisil, 1976; Pravdina, 1979; Dilawari and Atwal, 1989; Birch et al., 1990; Nottingham et al., 1991), in wind tunnels (Visser and Taanman, 1987), and in the field (Profft, 1939; Chapman et al., 1981; Campbell et al., 1990). Electrophysiological studies have implicated that the primary rhinaria on the antenna contain the olfactory receptors that perceive plant volatiles (Bromley and Anderson, 1982; Yan and Visser, 1982).

Placoid sensilla are the only type of olfactory sensilla found on the six-segmented antenna of aphids (Bromley et al., 1979, 1980). Segments five and six each possess a primary rhinarium. These rhinaria are labeled primary because they are present in all life stages and forms (Flögel, 1905). The proximal primary rhinarium (PPR) consists of a single placoid sensillum found on segment 5. The distal primary rhinarium (DPR) is found on segment 6 and consists of two small and one large placoid sensilla and four coeloconic pegs. Secondary rhinaria are found on the third to fifth antennal segments of adults. The number and distribution of secondary rhinaria, unlike primary rhinaria, varies among morphs and aphid species (Shambaugh et al., 1978). Secondary rhinaria were found to be sex pheromone receptors in both behavioral (Pettersson, 1971; Marsh, 1975) and electrophysiological studies (Dawson et al., 1987, 1988; Campbell et al., 1990).

Bromley and Anderson (1982) recorded single-cell activity from the primary rhinaria of the lettuce aphid, *Nasanovia ribis-nigri* Mosley. They tested 70 volatile, host plant-related chemicals, and reported the observed activity in qualitative terms, i.e., neutral, inhibitory, or excitatory. No apparent receptor specificity for any chemical group was found. Yan and Visser (1982) used plant volatiles to elicit electroantennogram (EAG) responses from the antennae of the cereal aphid, *Sitobion avenae* (F.). The highest relative EAG responses were

elicited by the six- and 7-carbon alcohols and other green leaf volatiles such as (*E*)-2-hexenal and hexanal. The primary rhinaria are also receptors of alarm pheromones (Nault et al., 1973; Wohlers and Tjallingii, 1983), the main component of which was identified as (*E*)-β-farnesene for a number of species (Bowers et al., 1972; Edwards et al., 1973; Wientjes et al., 1973).

Although Hodgson (1991) recently argued that the importance of apterous aphids in population dispersal and their contribution to virus spread is underestimated, it is the alate form that is most important in long-distance migration as well as the spread of viruses. We hypothesize that if orientation to plant volatiles does play a role in aphid host selection, it is likely to be more important in alate than in apterous aphids. Alatae, therefore, are expected to be more sensitive to plant volatiles than apterous aphids. Thus, this study focused primarily on the alate form.

The objective of this investigation was to come to a more quantitative method for determining EAG responses and to use this method to determine possible differences in the sensitivity and selectivity of the primary rhinaria to primary alcohols and aldehydes of four to eight carbons of which several have been identified as naturally occurring plant odors, e.g., green leaf volatiles.

METHODS AND MATERIALS

Insect Colony. Acyrthosiphon pisum (Harris) (Homoptera: Aphididae) was collected from pea (*Pisum sativum* L.) in May 1991. In the laboratory, *A. pisum* was maintained parthenogenetically on sugar pea (*Pisum arvense*, var. Oregon Sugar Pod II), under a 16:8 hr light–dark regime and 23 ± 4°C. Plants with aphids were held in clear acrylic plastic cages (53 cm H × 33 cm W × 53 cm D) that were ventilated and lighted according to Forbes et al. (1985). Aphid colonies were supplied regularly with fresh plants to maintain production of apterous virginoparae. Alate virginoparae were obtained by crowding a separate colony.

Olfactory Stimuli. The stimuli consisted of the following volatile compounds (listed purities are according to the manufacturer): hexanal (99%), 1-butanol (99.9%), and 1-pentanol (>99%) were obtained from Aldrich Chemical Co. (Milwaukee, Wisconsin); butanal (>99%), 1-hexanol (>99%), 1-heptanol (>99.5%), 1-octanol (>99.5), pentanal (>98%), heptanal (>97%), and octanal (>98) from Fluka Chemical Co. (Ronkonkoma, New York). Dilutions of each stimulus were prepared in paraffin oil and stored at 4°.

Odor Delivery. A stimulus applicator model CS-05b (Syntech, Hilversum, The Netherlands) was used for air and odor delivery. A constant flow (1 liter/min) of charcoal-filtered and humidified compressed air was passed over the antennae through a stainless steel tube. The tube was positioned 5 mm from the

antenna. During stimulation, part of the constant flow was diverted for 0.4 sec (flow rate = 11.2 ml/sec) through a Pasteur pipet containing the stimulus. The stimulus duration of 0.4 sec was long enough to inject the complete volume of air in the pipet into the constant flow. The air was exhausted to a fume hood after passing over the preparation.

Stimulation Procedure. A Pasteur pipet was loaded with a V-shaped strip of filter paper (Whatman No. 1, 6 cm long, 0.5 cm wide, and folded lengthwise) containing a particular concentration (v/v) of stimulus compound in paraffin oil (Merck Uvasol, EM Science, Gibbstown, New Jersey). A 30-sec time interval was maintained between stimulations to prevent adaptation of the antennal sensilla. Although it is the amount of compound evaporating from the paraffin oil that stimulates the antenna, in later discussions we will refer to the concentration of the compound in paraffin oil, or source concentration.

Target Concentration. Headspace samples (5 μl) were taken to determine the equilibrium concentration of (*Z*)-3-hexen-1-ol inside the Pasteur pipet. Samples were immediately injected into a gas chromatograph (GC) (Hewlett Packard model Sigma 300) set at the lowest attenuation (=1), and fitted with a DB-1 column (15 m in length, 0.32 mm inner diameter, coating 1.0 μm, oven temperature 120°C, injection temperature 200°C, detector temperature 200°C, no splitting). Concentrations tested were $10^{-0.3}$, 10^{-1}, 10^{-2}, and 10^{-3} (25 μl, v/v). Peak areas were measured and correlated with the concentration in paraffin oil.

Electroantennogram Recording. Antennae were excised by cutting through the first segment (scape). The extreme tip of the flagellum was removed to enhance conductivity. First, the proximal and the distal ends of the excised antenna were brought into contact with the opening of the indifferent and recording electrode. The antenna adhered to the Ringer's solution (Barbosa, 1974) in the electrodes via surface tension and hydrophilic attraction. The wider opening at the tip of the recording electrode enabled it to sleeve over the distal end of the antenna for use with sectional electroantennography (described below). A silver wire in the Ringer's solution connected the recording and indifferent electrodes with a guarded probe (model PB-01/S, Syntech) which was connected to an amplifier via a shielded cable. Recording, storing, printing, and quantifying EAG amplitudes was performed using an IBM-AT (80386) equipped with an interface card and software (PC-EAG version 2.4) from Syntech. The interface card contained a software-controlled amplifier, zero return circuit, and A/D conversion circuit with a 12-bit resolution and a sampling rate of 100 per second. EAGs were measured as the maximum amplitude of depolarization elicited by a stimulus.

Experiments and Data Analysis. To verify that the proximal and distal primary rhinaria are responsible for the perception of plant volatiles, the antenna was divided into four sections labeled A–D, with sections B and C containing

the PPR and DPR, respectively (Figure 1). The recording electrode was sleeved over the distal end of the antenna, thereby selectively exposing a decreasing number of sections. Sections of the antenna were stimulated with (Z)-3-hexen-1-ol (10^{-2} v/v in paraffin oil).

Additivity of EAGs recorded from different antennal sections was examined by comparing the EAG response of a totally exposed antenna (sections A–D exposed, Figure 1) to the separate EAGs from the DPR (only section C exposed) and PPR (only section B exposed) stimulated by (Z)-3-hexen-1-ol (10^{-2} concentration). This experiment was performed because DPR and PPR responses to alcohols and aldehydes were not determined by exclusively exposing the sections of the antenna in which the DPR (section C) and PPR (section B) are located. Instead, the PPR response to a stimulus was considered to be the response recorded when only sections A and B were exposed to the stimulus,

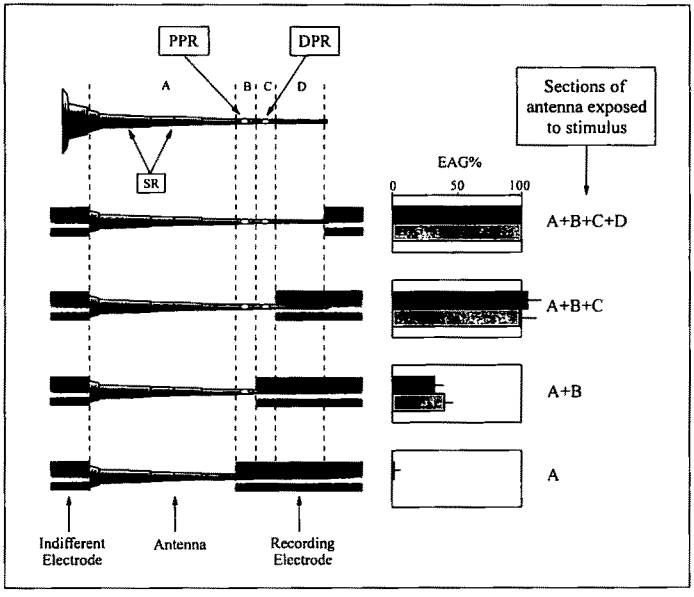

FIG. 1. Sectional electroantennography. The recording electrode was sleeved over the distal end of the antenna to selectively expose sections A–D. Histograms represent relative EAG responses of different sections of the antenna to hexanal from alate (dark grey bars) and apterous (light grey bars) virginoparous *A. pisum*. The control stimulus was (Z)-3-hexen-1-ol. Stimuli were dissolved in paraffin oil (25 μl of a 10^{-2} concentration, v/v). Bars represent means $\pm$95% confidence intervals for six antennae from different individuals (DPR = distal primary rhinarium; PPR = proximal primary rhinarium, SR = secondary rhinaria).

while the DPR response was the response of the complete antenna (section A–D exposed, Figure 1) minus the PPR response.

Absolute EAG responses to test compounds were corrected for decrease in amplitude over time by converting them to relative EAG responses. This was done by expressing responses to test compounds as percentages of expected responses to the standard (*Z*)-3-hexen-1-ol (10^{-2}, v/v) at the time of stimulation with these test compounds. Expected standard responses were calculated via linear interpolation between two successive EAGs elicited by the standard. The first recording elicited by the standard was made 5 min after positioning the antennae between the electrodes. The first recording to a test stimulus was made between 6 and 8 min after antennal positioning. To examine the decay in absolute EAG response over time and validate linear interpolation between expected standard responses, EAG responses to a 10^{-2} concentration of the standard, (*Z*)-3-hexen-1-ol, were measured for 30 min at 30-sec intervals after the antenna was positioned between the electrodes. If detected, the relative EAG responses to the control (pure paraffin oil) recorded before and after the stimulus compound were subtracted from the relative EAG response to that stimulus compound.

The relationship between log source concentration and relative EAG response was examined by stimulating an antenna of five different individuals to increasing concentrations (10^{-7}–10^{-1}, and $10^{-0.3}$, all v/v) of test stimuli dissolved in paraffin oil. For all volatile stimuli tested, EAG responses were observed to approach saturation within the range of source concentrations. A logistic function was found to adequately describe the observed relationship between log source concentration and relative EAG response:

$$R(C) = \frac{\alpha}{1 + e^{-\beta(C-\gamma)}} \tag{1}$$

In this equation, $R(C)$ is the relative EAG response to the log source concentration (C) of the test compound in paraffin oil and α, β, and γ are constants. Five parameters describing a number of characteristics of sigmoid curves were derived from equation 1. Visual representations of these parameters are given in Figure 2.

A commonly used parameter is the saturation (or maximum) response (R_s). This parameter was calculated through

$$R_s = \lim_{C \to \infty} R(C) = \alpha \tag{2}$$

Equation 1 was rewritten, expressing C as a function of R, so that parameters describing characteristics that are related to log source concentrations could be determined:

$$C(R) = \gamma - \frac{1}{\beta} \ln \left[\frac{\alpha}{R} - 1 \right] \tag{3}$$

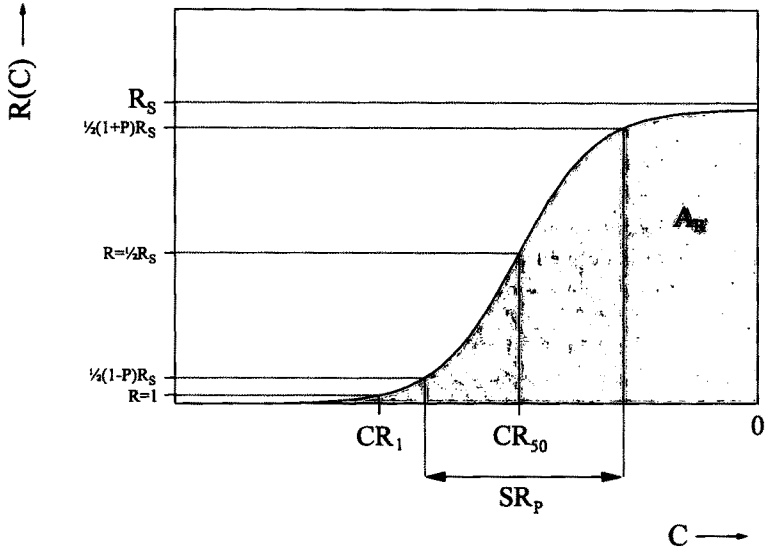

FIG. 2. Parameters derived from the logistic function (see equation 1). Explanation of parameters: R_s = saturation (or maximum) response; CR_{50} = log source concentration for which the relative EAG response is 50% of the saturation response, R_s; SR_P = stimulus–response range, i.e., the range of log stimulus concentrations over which most of the increase in relative EAG response is observed. P in SR_P is the fraction over which the relative EAG response ranges between $\frac{1}{2}(1 - P)R_s$ and $\frac{1}{2}(1 + P)R_s$, symmetrically around $R = \frac{1}{2}R_s$; CR_1 = threshold concentration, here defined as the log source concentration for which the relative EAG response is 1 EAG%; A_R = EAG response area, i.e., the surface area under the sigmoid curve between minus infinity and zero.

A second useful parameter, the stimulus log source concentration for which the relative EAG response R is equal to $\frac{1}{2}R_s$ ($=\frac{1}{2}\alpha$), was derived from this equation. This parameter, CR_{50}, is equal to γ.

The range of log source concentrations over which the relative EAG response to a given compound increases from near zero to near R_s was labeled as the stimulus–response range (SR_p). This third parameter was calculated as:

$$SR_P = \frac{2}{\beta} \ln \left[\frac{1 + P}{1 - P} \right] \tag{4}$$

where P is the fraction over which the relative EAG response ranges between $\frac{1}{2}(1 - P)R_s$ and $\frac{1}{2}(1 + P)R_s$, symmetrically around $R = \frac{1}{2}R_s$. In the present analysis, P was arbitrarily set to 0.9, resulting in

$$SR_{0.9} = \frac{2}{\beta} \ln (19) \tag{5}$$

A fourth useful parameter is the threshold log source concentration, here arbitrarily defined as the log source concentration for which $R = 1$. This commonly used parameter (CR_1) was calculated using equation 3:

$$CR_1 = \gamma - \frac{1}{\beta} \ln (\alpha - 1) \tag{6}$$

The fifth and last parameter is the area under the fitted curve between a log source concentration of minus infinity and a log source concentration of zero. This area is defined by the integral of equation 1:

$$A_R = \int_{-\infty}^{0} R(C)\, dC = \frac{\alpha}{\beta} \ln (1 + e^{-\beta\gamma}) \tag{7}$$

A_R is labeled as the EAG response area and combines the collective effect of α, β, and γ.

RESULTS

Sectional Electroantennography. Electroantennogram responses to the standard compound (Z)-3-hexen-1-ol, could only be recorded when the antennal sections containing the PPR (section B), DPR (section C), or both were exposed (Figure 1). No difference in relative EAG response was observed between the sections that do not contain primary rhinaria. No response was elicited in alatae when only the section containing secondary rhinaria (section A) was exposed to the stimulus (bottom antenna in Figure 1).

EAG Additivity. The total EAG response recorded from a completely exposed antenna equaled the sum of the EAGs recorded separately from the PPR (section B) and DPR (section C) (Figure 3). Therefore, the response recorded when only sections A and B were exposed to the stimulus is considered in the results discussed below to be the PPR response. The DPR response is the total response (section A–D exposed) minus the PPR response.

EAG Decay. After positioning the antenna between the two electrodes, the absolute EAG response to the standard decreased exponentially over time (Figure 4). However, decay in absolute EAG response was virtually linear during each 5-min interval.

Target Concentrations. Two sets of headspace samples were obtained from the air inside Pasteur pipets containing stimuli dissolved in paraffin oil. Regression of the log peak area on the log source concentration revealed a linear relationship (Figure 5).

Analysis/Quantification of Source Concentration-Dependent EAG Responses. The logistic equation 1 was used to fit a curve to the raw data (relative EAGs). Examples of such sigmoid shaped curves are given in Figure 6. The

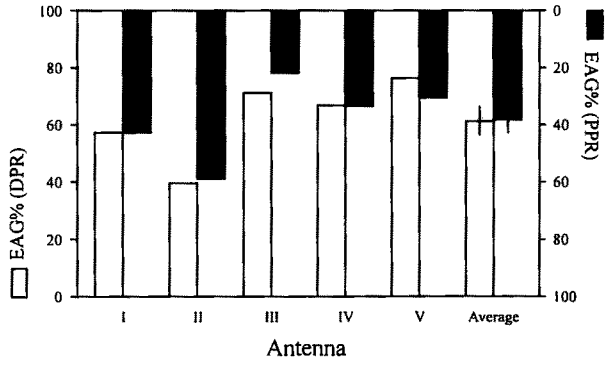

FIG. 3. Additivity of relative electroantennogram responses of the DPR and PPR of five different antennae of alate virginoparous *A. pisum*. The stimulus used was (*Z*)-3-hexen-1-ol (25 μl of 10^{-2} v/v in paraffin oil). Columns on the far right show the average of all five DPR and PPR responses $\pm$95% confidence intervals.

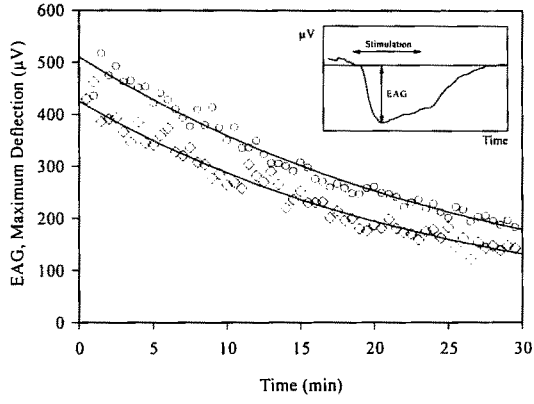

FIG. 4. Absolute EAG amplitude–time curves for antennae of alate virginoparous *A. pisum*, responding to (*Z*)-3-hexen-1-ol (25 μl of 10^{-2} v/v in paraffin oil). Antennae were stimulated every 30 sec for 30 min. Two separate tests with two different antennae were done: (○) $EAG(t) = 515.96e^{-0.036t}$; (◇) $EAG(t) = 431.09e^{-0.040t}$.

different parameters derived from the fitted logistic equation were plotted with the DPR on the x axis and the PPR on the y axis. Absolute values for CR_1 and CR_{50} were used, so that high values for these two parameters denote high sensitivity.

To both alcohols and aldehydes, the saturation responses (R_s) are at least twice as high for the DPR as for the PPR (Figure 7a). In addition, the DPR

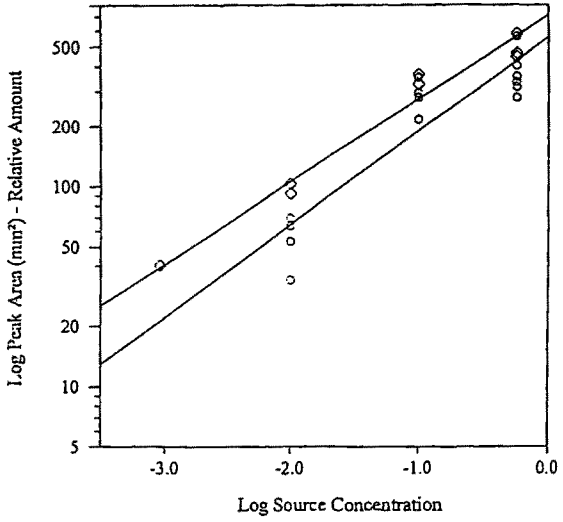

FIG. 5. Linear regression of the log GC peak area (or relative amount) on the log source concentration of (*Z*)-3-hexen-1-ol in paraffin oil. Two separate tests were done: (○) $y = 2.747 + 0.468x$; (◇) $y = 2.855 + 0.415x$.

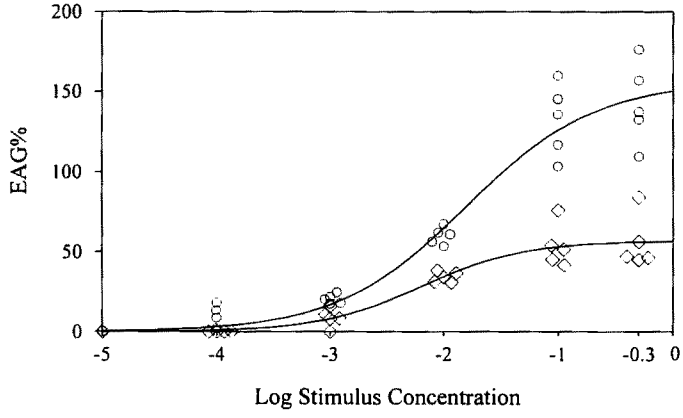

FIG. 6. Log source concentration–response curves for EAGs recorded separately from the distal primary rhinarium (DPR, ○, $R(C) = 156.420[1 + e^{-1.769(C+1.805)}]^{-1}$) and proximal primary rhinarium (PPR, ◇, $R(C) = 56.920[1 + e^{-2.217(C+2.190)}]^{-1}$) of alate virginoparous *A. pisum*. (*Z*)-3-hexen-1-ol was used as test stimulus.

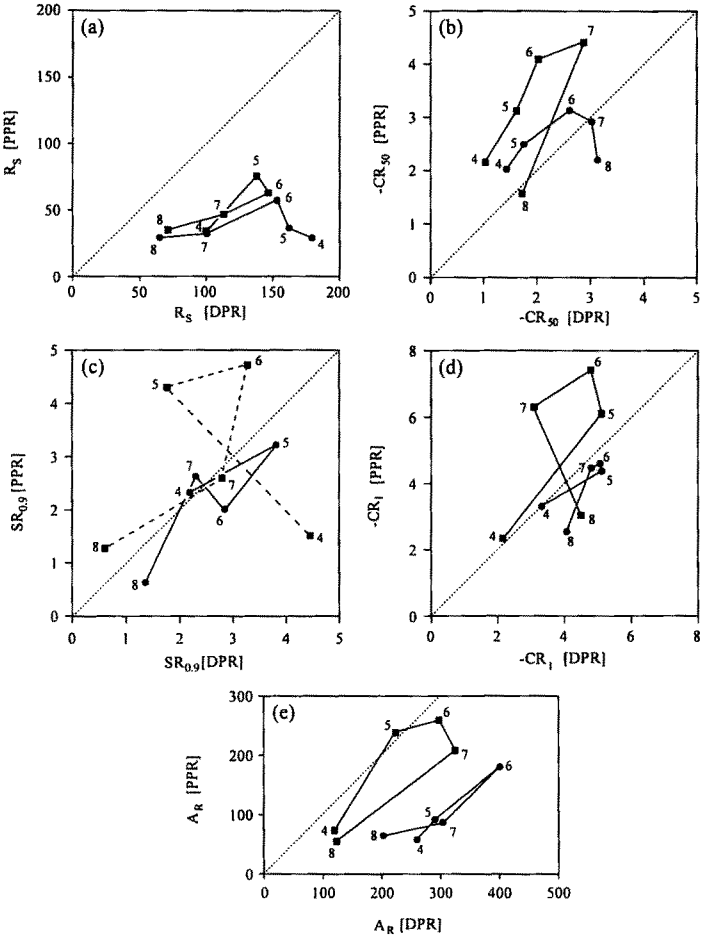

FIG. 7. DPR versus PPR plots of responses to primary monounsaturated alcohols (•) and aldehydes (■), characterized by five different parameters: (a) R_S, (b) $-CR_{50}$, (c) $SR_{0.9}$, (d) $-CR_1$, (e) A_R. Numbers adjacent to data points denote carbon chain length.

and PPR differ in their responses to the two compound groups, i.e., they differ in their relative positions in the two-dimensional DPR/PPR space. Compared with the DPR, the PPR has higher R_s values for aldehydes. For the aldehydes, there is an optimum DPR and PPR saturation response to pentanal (C_5) and hexanal (C_6). For the alcohols, an optimum is found for the PPR, but not for the DPR, where R_s response increases with decreasing carbon length.

Although the R_s for the DPR showed higher responses than for the PPR,

it is the PPR that shows higher overall CR_{50} responses, i.e., most data points are above the PPR = DPR line (Figure 7b). As for R_s, there is a difference between the CR_{50} responses to the two functional groups. Aldehydes elicit relatively higher CR_{50} responses in the PPR, while CR_{50} responses to alcohols are much closer to the PPR = DPR line (Figure 7a). The path of the lines going through the data points is similar for DPR and PPR, i.e., a steady increase in overall CR_{50} response with a maximum for C_6 and C_7, followed by lower responses for C_8.

No clear trend among primary rhinaria or functional group was found for $SR_{0.9}$ (Figure 7c).

Threshold log source concentrations, CR_1, for either DPR and/or PPR decreased towards the right upper corner of Figure 7d. As was found for the parameters R_s and CR_{50}, a clear separation was observed between threshold responses of the primary rhinaria to alcohols and aldehydes. The concentrations eliciting threshold responses of the PPR were lowest for aldehydes, i.e., all data points are left of the PPR = DPR line, except the response to octanal, while the DPR appears to be more sensitive to alcohols (Figure 7d). The C_5, C_6, and C_7 compounds separate out as being detected by DPR and PPR at the lowest concentrations.

An even larger separation between the DPR and PPR responses to the two functional groups is obtained by estimating the parameter A_R, i.e., the EAG response area (Figure 7e). In the DPR/PPR space, A_R values for the aldehydes were found much closer to the PPR = DPR line than those for the alcohols. In addition, a clear separation was found between pentanal (C_5), hexanal (C_6), heptanal (C_7), and hexanol (C_6) and the rest of the compounds.

DISCUSSION

An electroantennogram is assumed to reflect the summation of receptor potentials of all the individual olfactory neurons in the antenna that respond to a particular stimulus (Schneider, 1957; Nagai, 1983; Roelofs, 1984; Mayer et al., 1984; Boeckh et al., 1965). This definition implies that EAG responses would be additive. Indeed, EAG responses have been found to be linearly dependent on either the number of sensilla or the length of the antenna exposed to the stimulus for a number of species; *Argyrotaenia velutinana* (Roelofs and Comeau, 1971), *Heliothis virescens* (Almaas and Mustaparta, 1991), *Trichoplusia ni* (Mayer et al., 1984), *Ostrinia nubilalis* (Nagai, 1981), *Oryzaephilus surinamensis* (White, 1991), and *Dacus oleae* (Crnjar et al., 1989). In the present study, the EAG response of a totally exposed antenna is equal to the sum of the EAGs for the DPR and the PPR. Because the two primary rhinaria are spatially separated, individual EAGs from the distal and proximal primary

rhinaria could be obtained by selectively exposing antennal sections through sleeving the recording electrode over the distal end of the antenna. This technique was first used by Wohlers and Tjallingii (1983), who demonstrated that the primary rhinaria detect alarm pheromone. Sleeving the recording electrode over the antenna does not affect subsequent antennal response (Wohlers and Tjallingii, 1983). Not having to sleeve the indifferent electrode over the proximal end to selectively expose the DPR greatly enhances the speed with which recordings can be made. This speed is important because aphid antennae are relatively small in size and deteriorate rapidly.

After positioning an antenna between the two electrodes, the absolute EAG response to a stimulus, such as the standard, decreased exponentially over 30 min (Figure 4). Taken over intervals of 5 min, however, EAG decline was virtually linear. Thus, linear interpolation between expected EAG values was a legitimate method of calculating relative EAG responses.

A strong linear relationship was observed between the relative amount (log GC-peak area) of compound in the headspace sample and the log source concentration (Figure 5). Therefore, serial dilutions of a volatile compound in paraffin oil produce equivalent serial concentrations of the compound in air. The stimuli that were tested do not have identical volatility and solubility characteristics in mineral oil. However, such differences among stimuli are not random. Punter and Menco (1981), using data from Dreisbach (1952), found a linear relationship between carbon length (n) and the log saturated vapor pressure (log SVP) for n-aliphatic alcohols (at $t = 25°C$: $\log(SVP) = -0.39n - 1.82$, $r = 0.99$). If differences between responses of the DPR and PPR to alcohols and aldehydes of varying carbon length could be explained solely by differences in volatility, then no clear optimum around C_6 would have been observed.

Characterization of relative EAG responses through nonlinear curve fitting is a relatively uncommon approach in EAG data analysis. A review of the literature on analysis of dose–response data in insect chemoreception revealed that in only a small percentage of the papers was regression of EAG or SCR (single cell recording) data on (log) stimulus dose performed. The years of publication of the 103 papers that were surveyed ranged from 1963 to 1993. Although a logistic (sigmoid) relationship between log stimulus concentration and antennal response could be detected visually in 74% of the papers, in only 5.8% of the papers was nonlinear regression used to qualify electrophysiological responses (Dickens and Moorman, 1990; Hanamori et al., 1972; Light, 1983; Light et al., 1992; Morita and Shiraishi, 1968; Patte et al., 1989). In all six papers, maximum responses were calculated, while parameters such as CR_{50} (four papers), CR_1 (three papers), SR_P (three papers), and A_R (no papers) were calculated less often.

What do R_s, CR_{50}, $SR_{0.9}$, CR_1, and A_R mean in biological terms? What do low or high values for a certain parameter imply? The parameters CR_{50} (the

stimulus source concentration eliciting a relative EAG response that is 50% of the saturation response, R_s) and CR_1 (threshold concentration) are both measures of receptor sensitivity. A relatively low CR_{50} or CR_1 means that the stimulus can be detected at relatively low concentrations. CR_{50} is solely dependent on γ (Figure 8), which means that a reduction in CR_{50} results in a shift of the whole sigmoid curve to the left, while the overall shape is retained. Unlike CR_{50}, CR_1 is dependent on all three constants (although mostly on β and γ, Figure 8) and therefore reduction of CR_1 may or may not be accompanied by a change in overall shape. Due to this difference between CR_{50} and CR_1, the paths for the two parameters of the alcohols and aldehydes through the DPR/PPR space are different (Figure 7b and d). For parameter CR_{50}, most compounds are found on the PPR side of the PPR = DPR line, while CR_1 values are clustered around the PPR = DPR line. Taking both parameter paths through the DPR/PPR space into account, it becomes clear that both DPR and PPR are most sensitive to C_{5-7} alcohols and aldehydes. In addition, the PPR appears to be more sensitive to aldehydes than to alcohols, while no differences in response to alcohols and aldehydes were observed for the DPR.

The saturation (or maximum) response, R_s, is a measure for the selectivity of the olfactory receptors for certain volatile stimuli. In most structure–activity studies (see below) only one subsaturation stimulus concentration is used. Although it is impossible to increase the log source concentration (C) in paraffin oil beyond zero, it is mathematically appropriate to use a limit to infinity for C

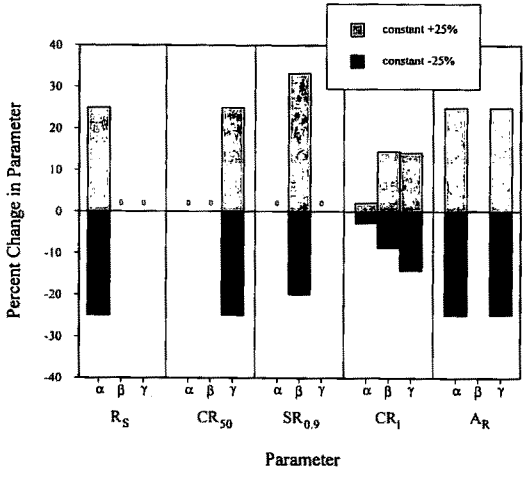

FIG. 8. Effects of a 25% change in the constants α, β, and γ on the parameters R_s, CR_{50}, $SR_{0.9}$, CR_1, and A_R. Default values are α (R_s) = 100, β = 2, γ (CR_{50}) = −3, $SR_{0.9}$ = 2.94, CR_1 = −5.3, A_R = 300.1.

to estimate the saturation response, which is only dependent on α (Figure 8). While the concentration in paraffin oil can not be higher than zero, it would technically be possible to further increase the concentration of the volatile compound in the air flowing across the antenna by, for instance, pipetting more of the $10^{-0.3}$ solution onto the filter paper or by using more filter paper strips to increase the surface area from which the volatile can evaporate. Light et al. (1992) designated the relative EAG response to a 100-μg dose as the maximum response for *Bruchophagus roddi* (Gussakovsky) (Hymenoptera: Eurytomidae). Contrary to this rather arbitrary choice, estimating R_s from a logistic equation reduces the influence of varying stimulus volatilities and unequal affinities with the paraffin oil when comparing different compounds.

When comparing saturation responses (R_s) of the DPR with those of the PPR, one should consider their comparative morphology. The DPR typically consists of one large and two smaller placoid sensilla, containing 15 neurons, while the PPR consists of just one large placoid sensillum, containing 10 neurons (Bromley et al., 1979). Apart from the difference in the number of neurons, there are two minor morphological differences between the larger and smaller types of placoid sensilla. The lamellae of the tormogen cells are not as well developed in the small placoid sensilla, and there is a difference in position of the inner cuticle. In all other respects, the two types of placoid sensilla are similar in morphology (Bromley et al., 1979). Since the DPR has more sensilla and more neurons that the PPR, it may also possess more receptor sites than the PPR. This hypothesis is consistent with the observed saturation EAG responses (R_s), which were approximately twice as high for the DPR when compared to the PPR for all compounds tested (Figure 7a). In addition, the PPR appears to be slightly more selective for aldehydes than for alcohols.

A broad stimulus–response range (SR_P) allows an insect to perceive both gross and subtle changes in concentration of a volatile compound as it is carried by the wind from its source and aids in the orientation of the insect to the odor source over distance (Seabrook, 1978). No clear trend was observed in $SR_{0.9}$ values with increasing carbon length (Figure 7c), suggesting that long-distance orientation to a kairomone source may be less important for (at least) this aphid species.

The EAG response area (A_R) showed the best separation between DPR and PPR responses to alcohols and aldehydes and between compounds with different carbon chain lengths (Figure 7e). This may be attributed to the fact that A_R is dependent on both constants α and γ (Figure 8), acting like a measure for both sensitivity (such as CR_{50}) and selectivity (such as R_s). Therefore, A_R may be the best indicator for overall responsiveness of the primary rhinaria to volatile stimuli.

Having taken all parameters into consideration, the conclusion is that the PPR is more responsive to aldehydes than the DPR, while the reverse is true

for responsiveness to alcohols. In addition, the highest parameter values were found for hexanol, pentanal, hexanal, and heptanal.

Structure–activity studies with alcohols and aldehydes of varying carbon lengths have been done for a number of insect species. In both DPR and PPR, the highest parameter values were observed for C_{5-7} compounds, which is typical for many herbivorous insect species in different orders (Visser, 1983). Examples are found in Coleoptera (Visser, 1979; Dickens and Boldt, 1985), Diptera (Light et al., 1988; Honda et al., 1987), Lepidoptera (Ramachandran et al., 1990; Ramachandran and Norris, 1991), and Hymenoptera (Ramachandran and Norris, 1991; Li et al., 1992). However, only one stimulus concentration is tested in most cases. Therefore, these studies could be considered two-dimensional, where dimension 1 is carbon chain length and dimension 2 is electrophysiological response (relative or absolute). The present study adds a third dimension, stimulus concentration. The only example of a structure–activity study in Homoptera has been reported by Yan and Visser (1982), who recorded electroantennogram responses from the whole antennae of alate and apterous cereal aphids, *Sitobion avenae* (F.), stimulated by monounsaturated alcohols. They found identical relative EAG optimum curves for alatae and apterae, although higher absolute EAG responses were found for alatae. In preliminary experiments, we observed relatively higher PPR values for the parameters CR_{50}, CR_1, $SR_{0.9}$, and A_R for alatae than for apterae when stimulated by hexanal and (*Z*)-3-hexen-1-ol. No such differences were found for the DPR. Single-cell recordings from the DPR and PPR of *N. ribis-nigri* revealed that terpenes consistently excited the PPR receptors and inhibited DPR receptors (Bromley and Anderson, 1982). These two apparent differences between DPR and PPR may indicate that there is some level of specialization between the two primary rhinaria.

The question whether specialization exists between the primary rhinaria and whether alate and apterous aphids differ in electrophysiological responsiveness remains largely open. The more mathematical approach of quantifying relative EAG responses through curve fitting will be helpful in answering these questions and in identifying potential kairomones used in host-plant selection, recognition, and acceptance.

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INFLUENCE OF CNICIN, A SESQUITERPENE LACTONE OF *Centaurea maculosa* (ASTERACEAE), ON SPECIALIST AND GENERALIST INSECT HERBIVORES

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Abstract—The sesquiterpene lactone cnicin was extracted from *Centaurea maculosa* and *Centaurea vallesiaca*. We examined its effects on the ovipositional response and larval development of generalist and specialist insect herbivores associated with *C. maculosa*. For the oviposition trials, three plant species (*C. maculosa*, *Achillea millefolium*, and *Cichorium intybus*), half of which were sprayed with 3% of cnicin, were exposed to the specialist moths *Stenodes straminea*, *Agapeta zoegana*, and *Pterolonche inspersa* in field cages. All three species significantly preferred *C. maculosa* to other plants and *P. inspersa* significantly preferred cnicin-sprayed plants to untreated plants for oviposition. Tested over all species, cnicin significantly increased the number of eggs laid on a given plant. A larval diet test examined the toxicity of cnicin for larvae of the generalist noctuid moth *Spodoptera littoralis*. Cnicin concentrations of 3% and 6% were lethal and 1% and 0.5% seriously inhibited growth and development. The larvae of the *C. maculosa* specialist *Stenodes straminea* survived at 6% cnicin, but none of the pupae hatched. *Agapeta zoegana* was able to survive at 1% and 3% cnicin. Both specialists had difficulties with the artificial diet, but weight increase and survival was not further reduced when cnicin was present compared with on the control diet. In conclusion, cnicin influenced host recognition by the specialist species, and larvae of the generalist did not survive on natural levels of cnicin. Growth and survival of the specialist were not influenced by cnicin but were considerably hampered on artificial diet.

Key Words—*Centaurea maculosa*, sesquiterpene lactone, cnicin, host-plant

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selection, oviposition behavior, antifeedant, attractant, *Spodoptera littoralis*, *Agapeta zoegana*, *Stenodes straminea*, *Pterolonche inspersa*, *Lepidoptera*, *Noctuidae*, *Cochylidae*, *Pterolonchidae*.

INTRODUCTION

Sesquiterpene lactones are secondary compounds characteristic of many Asteraceae. The great majority of the sesquiterpene lactones known by 1977 (Fischer et al., 1979) are from this plant family. Later, Seaman (1982) listed over 1300 sesquiterpene lactones only from Asteraceae, and they occur only infrequently in other families such as Lauraceae, Magnoliaceae, and Apiacea (Kery et al., 1987). Most of these terpenoids have, in addition to other biological activities (Rodriguez et al., 1976), antifeedant properties. They have been found to reduce growth and survival of insects (Jones et al., 1979; Picman et al., 1978; Picman and Picman, 1984) and to be feeding deterrents for herbivore vertebrates and insects (Burnett et al., 1974; Mabry et al., 1977; Nawrot et al., 1983; Harmatha and Nawrot, 1984). The antifeedant properties of sesquiterpene lactones may play a major role in specific plant-animal coevolution (Burnett et al., 1987).

Sesquiterpene lactones have been isolated from many plants, usually from the aerial parts (e.g., Geppert et al., Bloszyk and Drozdz, 1978; Jones et al., 1979). The sesquiterpene lactone cnicin ($C_{20}H_{26}O_7$) was first isolated from *Cnicus benedictus* and is found in many *Centaurea* species (e.g., Nowak et al., 1984), among others in *C. maculosa* (see, e.g., Huneck et al., 1986).

Spotted knapweed, *C. maculosa*, was accidentally introduced to America from Eurasia at the end of the last century and has become one of the most important rangeland weeds in the Northwest of the United States and Canada. Together with *Centaurea diffusa*, it has reduced the grazing potential of many areas by over 80% (Müller-Schärer and Schroeder, 1993). *C. diffusa* extracts were tested for allelopathy to try to explain the rapid spread of this weed (Muir and Majak, 1983). Inhibitory activity was detected in laboratory seed germination tests, but field studies failed to show any allelopathic effects (Muir et al., 1985). Two years later, the phytotoxic compound of *C. maculosa* was recognized to be cnicin, located in glandular trichomes on the epidermal surface (Kelsey and Locken, 1987). Its concentration varies among tissues and by time: in living tissue, Locken and Kelsey (1987) found most cnicin in the small leaves of the stem. They measured 0.5% of dry weight in spring and about 1% in late summer. The highest concentration of 2.8% dry weight was measured in October in dry branches. No cnicin was found in the roots.

C. maculosa plants in America have practically no natural enemies, although they were introduced over a century ago. On the other hand, many herbivores feed on European plants. According to the plant apparency theory (Feeny, 1976), *C. maculosa* is rather "unapparent" and "unpredictable" and could be expected

to be eaten mostly by generalist insects. The root-feeding insects of *C. maculosa* are, however, mostly specialists, with only 22% of generalists (Müller, 1989). Leaf damage in the field is not very common and only a few species use the leaves as their food niche (Schroeder, 1985). American *C. maculosa* was found to contain generally low cnicin concentrations, compared with European *C. maculosa* (Müller-Schärer, unpublished data).

We examined the effects of cnicin on the ovipositional response and larval development of specialist and generalist herbivores and predicted different responses for specialists and generalists. We asked whether cnicin on the surface of the leaves is an attractant for female specialist moths looking for their host plant to lay their eggs and if they are attracted by other plants if these are coated with cnicin. We next asked if cnicin reduces the growth and fitness of larval specialist and generalist Lepidoptera.

METHODS AND MATERIALS

Cnicin. Cnicin was extracted from *C. maculosa* Lam. and *C. vallesiaca* Jordan, which is taxonomically closely related to *C. maculosa*. The dried small leaves of the stems were ground, extracted twice at room temperature with diethyl ether, and washed with pentane. The separation was performed with column chromatography using chloroform and ethyl acetate. The fractions containing cnicin were found by thin-layer chromatography. It was recrystallized from distilled H_2O (Landau, 1993). NMR showed that the extracted cnicin was over 95% pure (J. A. Robinson, personal communication), compared with a reference (see Huneck et al., 1986). The reference, tested with MS, was 99% pure cnicin (H.-R. Buser, personal communication). Additionally, some samples were analyzed using HPLC as described by Locken and Kelsey (1987). Since the extracted concentrations were very small (0.15–0.2%), we also used reference material isolated by Huneck (see Huneck et al., 1986). Cnicin is white, odorless, more or less crystalline, and has a melting point of 143°C (Merck Index). Compared to other extraction methods (Locken and Kelsey, 1987), the obtained amount of cnicin was very low and the method used is not satisfactory. Namely, diethyl ether is not ideal for extraction of cnicin, and large amounts had to be used for extraction because, according to the Merck Index, cnicin is practically insoluble in ether. The main reason for using this method was, however, its proven evidence of yielding cnicin of suitable quality (see Huneck et al., 1986). Based on the data available from spectroscopic methods (MS, NMR), HPLC, melting point determination, and thin-layer chromatography, a purity of >97% was ascertained.

Plants. Centaurea maculosa (L.) (Asteraceae) is a biennial or a short-lived perennial and is widely distributed in eastern Europe, where it grows at ruderal

sites. Seeds from *C. maculosa*, *Cichorium intybus* (Asteraceae), and *Achillea millefolium* (Asteraceae) originated from the International Institute for Biological Control in Delémont and were grown in pots (13 cm diameter, 17 cm depth) in a greenhouse.

Insects. The generalist *Spodoptera littoralis* (Boisduval) (Lep.: Noctuidae), the Egyptian cotton leafworm, is widely distributed in Africa, the Middle East, and the circum-Mediterranean region. The larvae feed on about 40 plant families (Navon, 1985). The larvae used in the experiments were reared at the Sandoz Agrobiological Station (Witterswil, Switzerland). The oligophagous moth *Agapeta zoegana* (L.) (Lep.: Cochylidae) is widely distributed in Europe and is associated with the roots of *C. maculosa* (Müller et al., 1988). *Pterolonche inspersa* (Stgr.) (Lep.: Pterolonchidae), is also closely associated with *C. maculosa* (Dunn et al., 1989). Both species were introduced into North America for the biological control of *C. maculosa* (Müller-Schärer and Schroeder, 1993). *Stenodes straminea* (Haw.) (Lep.: Cochylidae) feeds on *Artemisia*, *Scabiosa*, and *Centaurea* species and therefore have a wider host range than the other described specialists (Müller, 1983). All three species lay their eggs on rosette leaves, and the hatching larvae mine in the leaves before they tunnel into the roots (Müller et al., 1988). The larvae of *A. zoegana* and *P. inspersa* were collected in roots of *C. maculosa* in Hungary, 60 km east of Budapest. *Stenodes straminea* larvae were collected in Lalden (Wallis, Switzerland), where they live in the bases of the rosette leaves and the root collar of *Centaurea vallesiaca*. For the larval diet test, the hatching *A. zoegana* larvae were transferred to *C. maculosa* rosettes in pots and reared in a greenhouse.

Oviposition Test. The experiment was conducted in field cages (1 × 1 × 1 m). Four specimens of three plant species were placed in each cage and were presented at ground level by burying their pots. The plants *Cichorium intybus* and *Achillea millefolium* were used in addition to *C. maculosa* (control). *A. zoegana* had laid on average 7.6 (five replicates) and 0 (three replicates) eggs per female per test, respectively, on these plants in previous oviposition tests (Müller et al., 1988). The plants were cut to the same size and randomly assigned to two groups. The dry weight of each species was estimated, and the amount of cnicin, which would give 3% of the dry weight, was calculated. Using a Biomat spray, half the plants were carefully coated with cnicin, which had been dissolved in a methanol–water solution (2 : 1). Twelve pots were randomly placed within each of the three cages (blocks). The newly hatched moths were introduced into the cages, where they lived for about nine days. They were offered a honey solution and a bunch of flowers as food. A few days later and at the end of the experiment, i.e., after six to nine days, the eggs were counted. The results were tested with repeated-measures ANOVA with plant species and cnicin as a between- and time as a within-subject factor. This allows testing, averaged over time, for differences among plant species and effect of cnicin.

Since there were two measurements recorded on each individual plant, using a repeated-measures analysis of variance design is recommended (Sokal and Rohlf, 1981).

Larval Diet Tests. Small tubes with artificial diet (Sandoz Standard) were used for all experiments. For the different diet concentrations, cnicin was dissolved in acetone and added to the liquid diet. For the two specialists, *A. zoegana* and *S. straminea*, 3% of powdered *C. maculosa* root was added. Larvae were taken from *Centaurea* roots and immediately transferred into the tubes. The treatments were always randomly assigned within blocks. *Spodoptera littoralis* were weighed every second day. Because *A. zoegana* and *S. straminea* mine within their webs, they could not be weighed during the experiment.

The larvae of *S. littoralis* were put into small tubes containing 3 ml of artificial diet, enough to reach pupation. In the first test, 0% (control), 3%, and 6% cnicin were tested on second-instar larvae. They were kept at 25°C, 65% relative humidity, 16-hr photoperiod. Later, third-instar larvae were used to test cnicin concentrations of 0%, 0.5%, and 1%. They were kept at 22°C, 85% relative humidity, 15-hr photoperiod. The tubes for *S. straminea* (tested at the fourth to sixth instar) contained 2 ml diet plus 0% (control) and 6% cnicin. They were kept in the dark at 20°C, 60% relative humidity. *A. zoegana* (fifth and sixth instar) were tested with cnicin concentrations of 0%, 1%, and 3% in 2 ml of diet. They were kept in the dark at 22°C, 85% relative humidity 15-hr photoperiod. The results were tested with repeated-measures ANOVA. Block effects were not significant and are therefore omitted everywhere (Sokal and Rohlf, 1981, p. 350). Means are given with ± 1 SE.

RESULTS

Oviposition Test. For *Stenodes straminea*, a total of 917 eggs were found in the three cages. Although eggs were laid in all treatments, 96% of the eggs were found on *C. maculosa* (Figure 1a), being responsible for the significant effect of the plant species (Table 1). The fact that many larvae hatched indicates that cnicin was not toxic as a substrate.

For *Agapeta zoegana*, of a total of 82 eggs, only three were not laid on *C. maculosa*. The factors plant species, but also time and their interaction were significant (Table 2), as more eggs were laid in the second half of the experiment. Although the cnicin-sprayed plants were preferred (Figure 1b), this factor was not statistically significant (Table 2).

For *Pterolonche inspersa*, the factors plant species, cnicin, and their interaction were highly significant. The females clearly preferred the cnicin-sprayed *C. maculosa* plants (Table 3 and Figure 1c).

When tested with Fisher's Combining Test (Sokal and Rohlf, 1981), the

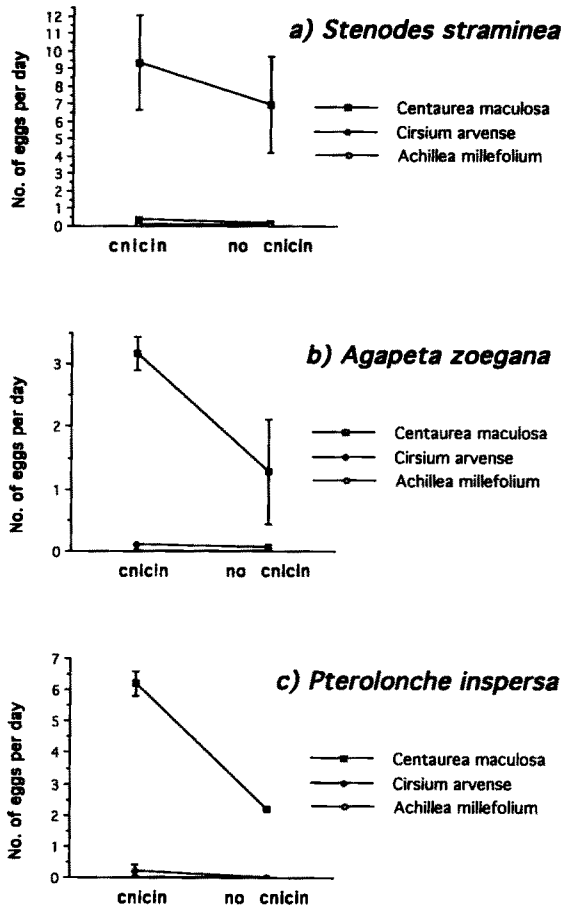

FIG. 1. The numbers of eggs (±SE) laid on the different plant species with and without cnicin.

specialists overall laid significantly more eggs on the cnicin-sprayed plants ($\chi^2_{df=6} = 24.94$, $P < 0.001$) (Figure 1).

Larval Diet Tests. For *Spodoptera littoralis*, the effect of cnicin, time, and their interaction had highly significant influences on the survival and weight of the larvae (Table 4 and 5). The concentrations of 3% and 6% were lethal; after five days all second-instar larvae had died, whereas the larvae in the control diet grew quickly (Figure 2). In the test with 0.5% and 1% cnicin, none of the larvae died during the first 30 days, but the larvae in the two cnicin diets showed reduced growth (Figure 3). The developmental rate was also reduced by cnicin:

TABLE 1. ANOVA FOR OVIPOSITION OF *Stenodes straminea*

	df	SS	*F* value	*P* value
Plant (A)	2	20700.028	17.138	<0.001
Cnicin (B)	1	268.347	0.444	0.510
A × B	2	409.694	0.339	0.715
Error	30	18117.417		
Time (C)	1	183.681	1.133	0.296
A × C	2	412.028	1.271	0.295
B × C	1	0.681	0.004	0.949
A × B × C	2	5.028	0.016	0.985
Error	30	4863.083		

TABLE 2. ANOVA FOR OVIPOSITION OF *Agapeta zoegana*

	df	SS	*F* value	*P* value
Plant (A)	2	26.661	24.284	0.001
Cnicin (B)	1	2.535	4.618	0.075
A × B	2	4.598	4.188	0.073
Error	6	3.294		
Time (C)	1	1.042	21.277	0.004
A × C	2	1.286	13.138	0.006
B × C	1	0.002	0.034	0.860
A × B × C	2	0.006	0.066	0.937
Error	6	0.294		

TABLE 3. ANOVA FOR OVIPOSITION OF *Pterolonche inspersa*

	df	SS	*F* value	*P* value
Plant (A)	2	92.631	3100.058	<0.001
Cnicin (B)	1	12.756	853.806	<0.001
A × B	2	21.388	715.781	0.001
Error	6	0.090		
Time (C)	1	0.196	0.042	0.845
A × C	2	0.100	0.011	0.990
B × C	1	0.511	0.109	0.753
A × B × C	2	0.397	0.042	0.959
Error	6	28.272		

TABLE 4. ANOVA FOR ARCSIN-TRANSFORMED SURVIVAL RATE OF *Spodoptera littoralis* LARVAE WITH 3% AND 6% CNICIN AFTER 1, 2, AND 4 DAYS

	df	SS	*F* value	*P* value
Cnicin (A)	2	7.608	50.455	<0.001
Error	15	1.131		
Time (B)	2	7.608	61.667	<0.001
A × B	4	5.757	23.333	<0.001
Error	30	1.851		

TABLE 5. ANOVA FOR *Spodoptera littoralis* LARVAL WEIGHT WITH 0.5% AND 1% CNICIN DURING FIRST TEN DAYS

	df	SS	*F* value	*P* value
Cnicin (A)	2	6314438.8	384.0	<0.001
Error	33	271337.1		
Time (B)	7	5118781.8	441.0	<0.001
A × B	14	5609378.8	241.6	<0.001
Error	231	383066		

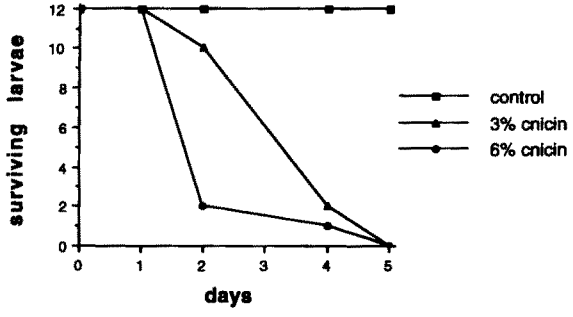

FIG. 2. *Spodoptera littoralis*: survival rate of second-instar larvae.

after 10 days, the larvae on the control diet started to pupate. Table 6 shows different larval stages on the 10th, 21th, 31th, and 38th day. At day 38, the experiment was stopped because the diet was very old. The pupae with cnicin were often malformed; pupation was incomplete for seven of 11 larvae in the 0.5% diet, and for four of seven larvae in the 1% diet; they were also very

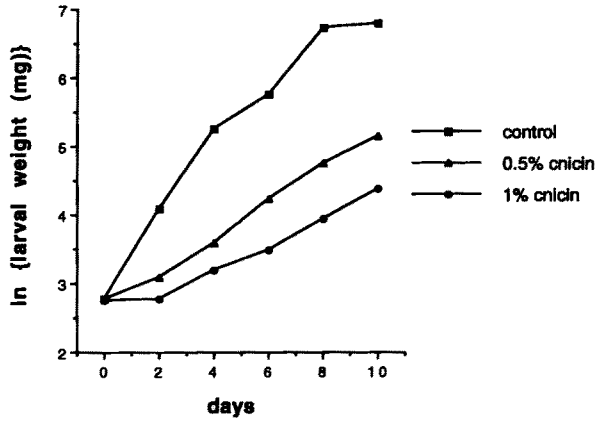

FIG. 3. Weight increase of *Spodoptera littoralis* larvae.

small or otherwise different from normal pupae. Pupal weight, which is generally correlated with fecundity of the adults, was lower with cnicin (Table 6).

For *Stenodes straminea*, rearing on artificial diet was difficult. Some larvae walked around the tubes for days without making a web. Within four days, they all lost about 65% of their weight. After that, they were left in their diet until they pupated or died. The results are given in Table 6. Although some larvae pupated at 6% cnicin, none of these emerged as adults. Pupal weight was also reduced ($df = 6$, $t = 6.5$, $P < 0.001$). Due to limited availability of cnicin, tests with lower concentrations could unfortunately not be made.

For *Agapeta zoegana*, after 43 days, the diet was very old and the experiment was stopped. None of the larvae, which were all in the fifth and sixth instar, when the experiment was started, pupated. Of the 27 larvae, 19 died. The reason for this was not cnicin, because they died in the control diet as well (Table 6). Cnicin influenced neither survival nor larval weight ($F_{5,7} = 2.1$, $P = 0.22$).

DISCUSSION

The widespread distribution of sesquiterpene lactones within the family Asteraceae implies that antifeedant properties of these compounds may have been an important factor in the evolution (Burnett et. al., 1987). The result of our larval diet tests showed that for generalist larvae of *S. littoralis* cnicin was noxious. In high concentrations (3% and 6%) the larvae died after four to five days, probably as a direct consequence of cnicin and not because of starvation. In a pilot experiment, starving third-instar larvae died only after seven to eight

TABLE 6. SUMMARY OF RESULTS OF LARVAL DIET TEST

	Cnicin treatment	N	Stage after 10 days	Stage after 21 days	Stage after 31 days	Stage after 38 days	Pupal weight (mg)
Spodoptera littoralis	Control	12	12 × 6[a]	12 × P	12 × A		332 ± 16.8
	0.5%	12	12 × 5	12 × 6	9 × P; 3 × 6	1 × A; 4 × P; 7 × dead	241 ± 41.9
	1%	12	11 × 4; 1 × 5	5 × 5; 7 × 6	1 × P; 10 × 6; 1 × 5	4 × P; 3 × 6; 1 × 5; 4 × dead	243 ± 41.9
			Days till pupation	Larvae pupated	Pupal weight (mg)	% pupae emerged	
Stenodes straminea	Control	10	18.6	5	19.4 ± 1.4	100	
	6%	10	28.6	3	6.9 ± 0.7	0	
			Larvae survived	Larval weight (mg) after 43 days			
Agapeta zoegana	Control	9	2	26			
	1%	9	2	11.2			
	3%	9	4	12.4			

[a] 12 × 6 = 12 larvae in the sixth instar; P = pupae; A = adult moth.

days. In addition, starving larvae lost 12% and 6% of their weight in the first and second days, respectively. During the same period, the larvae reared on 3% and 6% cnicin had already lost 35% after the first day and 13% and 26% after the second days, respectively. This strongly supports the hypothesis of a noxious effect of cnicin. Cnicin concentrations of 0.5% and 1% retarded the growth and larval developmental rate of *S. littoralis*. Almost half of the insects died either as larvae (sixth instar) or as pupae, and only one moth emerged. The experiment had to be stopped after 38 days because the diet was quite old and some larvae and pupae were diseased. All control larvae had emerged by day 31. As even 0.5% retarded and inhibited development, cnicin is probably a strong insect feeding deterrent. Harmatha and Nawrot (1984) found feeding by insects was deterred by a 1% solution. Mabry and Gill (1979), however, used only 0.125%, 0.25%, and 0.5% of glaucolide-A (a sequiterpene lactone from the genus *Vernonia*) for their larval diet tests and found reduction in growth for some species. Cnicin itself was only harmful to *Spodoptera littoralis* larvae when it was ingested. In a supplementary test, second-instar larvae were sprayed with a solution of cnicin. Even relatively high concentrations were not harmful (Gobeli, personal communication).

The specialist larvae of *Stenodes straminea* were able to develop and some to pupate, when reared in the diet containing 6% cnicin, but no emergence took place. Since none of the larvae in the cnicin diet metamorphosed, we can say that the tolerable concentration for complete development is under 6%. In nature, the larvae encounter rosette leaves, which contain about 0.3% to 1% cnicin (Locken and Kelsey, 1987).

Cnicin concentrations of 1% and 3% did not affect the survival rate of the other specialist *A. zoegana*, but overall survival was only 30%, and none of the larvae pupated. The temperature of 22°C might have been too high for these root specialists, or the consistency of the diet may not have been appropriate. In general, it very difficult to rear food specialists on artificial diets and more experiments with these species are needed. So far, *A. zoegana* have only been reared in *C. maculosa* roots (e.g., Müller and Steinger, 1990).

Preliminary results of a feeding test with adult beetles *Larinus obtusus* (Col. Curculionidae) (a *C. maculosa* specialist) showed that they, too, significantly preferred cnicin-sprayed leaves of the nonhost plant *Cirsium arvense* (Asteraceae) over control leaves sprayed only with methanol–water solution (same method as oviposition test) (Landau, 1993). This confirms the role of cnicin as a key substance for *C. maculosa* specialists.

The experiments made clear that specialists can tolerate a higher concentration of cnicin than generalists. Some *S. straminea* pupated with 6% cnicin, whereas 3% caused immediate death of *S. littoralis*. On the other hand, the specialists, too, had difficulties with high concentrations of cnicin and a tolerable level was not found, because of the difficulties of rearing the larvae in artificial

diet. It will probably be around the concentrations found in the plants. Mabry et al. (1977) tested specialists and generalists of *Vernonia* species with natural concentrations of a sesquiterpene lactone. Here, too, larval development was retarded for the generalists, but not for the specialists.

The impact of cnicin on the ovipositional behavior of *C. maculosa* specialists was clear; they preferred more cnicin on the plants. Cnicin may consequently not be a deterrent for them, but an attractant. It is thus not clear how they can distinguish different cnicin concentrations. Since not all eggs were laid on cnicin-treated plants, other factors such as leaf surface, plant and leaf form, odor, and taste may be important for host-plant selection. These could be eliminated by working with artificial leaves.

Discrimination of cnicin-sprayed plants was least pronounced in *S. straminea* (Tables 1–3, Figure 1), consistent with *S. straminea* not being specialized enough as a biological control agent to be introduced to America (Müller, 1983). This suggests that cnicin may be more important with increasing food specialization of insect herbivores.

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DIETARY SOURCE FOR SKIN ALKALOIDS OF POISON FROGS (DENDROBATIDAE)?

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Abstract—A wide range of alkaloids, many of which are unknown elsewhere in nature, occur in skin of frogs. Major classes of such alkaloids in dendrobatid frogs are the batrachotoxins, pumiliotoxins, histrionicotoxins, gephyrotoxins, and decahydroquinolines. Such alkaloids are absent in skin of frogs (*Dendrobates auratus*) raised in Panama on wingless fruit flies in indoor terraria. Raised on leaf-litter arthropods that were collected in a mainland site, such terraria-raised frogs contain tricyclic alkaloids including the beetle alkaloid precoccinelline, 1,4-disubstituted quinolizidines, pyrrolizidine oximes, the millipede alkaloid nitropolyzonamine, a decahydroquinoline, a gephyrotoxin, and histrionicotoxins. The profiles of these alkaloids in the captive-raised frogs are closer to the mainland population of *Dendrobates auratus* at the leaf-litter site than to the parent population of *Dendrobates auratus* from a nearby island site. Extracts of a seven-month sampling of leaf-litter insects contained precoccinelline, pyrrolizidine oxime **236** (major), and nitropolyzonamine (**238**). The results indicate a dietary origin for at least some "dendrobatid alkaloids," in particular the pyrrolizidine oximes, the tricyclic coccinellines, and perhaps the histrionicotoxins and gephyrotoxins.

Key Words—Alkaloids, indolizidines, pyrrolizidines, histrionicotoxins, coccinellines, dendrobatid frogs, insects, millipedes.

INTRODUCTION

A diverse range of alkaloids occur in the skin of poison frogs of the neotropical family Dendrobatidae, where they presumably serve in chemical defense against

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predators (Daly and Myers, 1967; Daly et al., 1987). Although presumed to be elaborated by poison frogs for storage in so-called granular skin glands (Neuwirth et al., 1979), the absence of such skin alkaloids in captive-raised frogs (Daly et al., 1980, 1992) suggests a possible dietary origin. 3,5-Disubstituted pyrrolizidines, 3,5-disubstituted indolizidines, 2,5-disubstituted pyrrolidines, and 2,6-disubstituted piperidines occur in ants (Jones and Blum, 1983), and tricyclic coccinellines occur in beetles (Ayer et al., 1976). Although the pyrrolizidine oximes have been found only in frog skin (Tokuyama et al., 1992), a related alkaloid, nitropolyzonamine, occurs in a millipede (Meinwald et al., 1975). However, many of the so-called "dendrobatid alkaloids" have never been detected in insects or other leaf-litter prey: These include the batrachotoxins, histrionicotoxins, gephyrotoxins, 5,8-disubstituted indolizidines, 2,5-disubstituted decahydroquinolines, 1,4-disubstituted quinolizidines, epibatidine, pyrrolizidine oximes, and the pumiliotoxin–allopumiliotoxin–homopumiliotoxin class. Thus, it is possible that dietary precursors rather than the alkaloids themselves might be supplied from leaf-litter prey.

Dendrobatid frogs (*Dendrobates auratus*) raised in Hawaii in outdoor terraria on a diet consisting mainly of termites and wild fruit flies had nearly the same profile of alkaloids as wild-caught Hawaiian frogs (Daly et al., 1992). However, levels were much lower, and an indolizidine **195B** that was absent in wild-caught frogs was present in the captive-raised frogs and the beetle alkaloid precoccinelline that was present in wild-caught frogs was absent in the captive-raised frogs. Recently, an uptake system, whereby dietary alkaloids are accumulated into skin of dendrobatid poison frogs of the genera *Dendrobates*, *Phyllobates*, and *Epipedobates* has been discovered (Daly et al., 1994). The system is absent in frogs of the non-alkaloid-containing dendrobatid genus *Colostethus*. The uptake system accumulated batrachotoxins, histrionicotoxins, pyrrolizidines, indolizidines, quinolizidines, decahydroquinolines, and pumiliotoxins. Pyrrolidines and piperidines did not appear to be accumulated. The presence of such a system strongly suggests that dietary alkaloids from insects or other small prey would accumulate in skin and could account for some or even all of alkaloids detected in skin of poison frogs. In order to explore this possibility, dendrobatid frogs (*Dendrobates auratus*) were raised in Panama in inside terraria either on wingless fruit flies or on leaf-litter arthropods collected from a site where a population of this dendrobatid frog occurs.

METHODS AND MATERIALS

Tadpoles of the poison frog *Dendrobates auratus* were collected on Isla Taboga in June 1992. Ten juvenile frogs were obtained. Eight were raised for seven months on leaf-litter arthropods in inside terraria and two were raised as controls on wingless fruit flies.

Frogs were housed in small glass terraria. Each of the terraria had a large aluminum Berless funnel mounted above it. Once or twice a week, about 18 liters of fresh moist leaf litter was placed in each of the funnels. As the 75-W light bulbs suspended above the funnels warmed and dried the leaf litter, the invertebrate inhabitants retreated down toward the cooler and moister litter near the bottom of the funnel, finally falling into the terrarium below. A total of about 29 leaf-litter collections were provided to each frog. During this period a total of 21 leaf-litter collections of arthropods were placed in alcohol for later analysis. The leaf litter was collected on the northern slope of Ancon Hill in Panama at a forest site in which a population of *Dendrobates auratus* occurred. Although collections of leaf-litter arthropods served as the primary food for the set of eight frogs, the diet was occasionally augmented with termites, ants, or fruit flies. Even with this supplemental nutrition, only three of the eight frogs fed on leaf-litter arthropods survived for the seven-month period. The set of two frogs served as controls and were fed only a strain of wingless fruit flies. The frogs had not attained full adult size in seven months. The snout–vent (s-v) lengths of the leaf-litter insect-fed frogs were 15 mm, 16 mm, and 19 mm, while the s-v lengths of the control frogs were 17 and 19 mm.

Two adult frogs from the leaf-litter site on Ancon Hill were collected for analysis of skin alkaloids. Their s-v lengths were 30 mm and 32 mm. Three adult frogs of the parent stock from Isla Taboga were collected for analysis of skin alkaloids. Their s-v lengths were 31 mm, 31 mm, and 27 mm.

Skins were extracted and alkaloid fractions were prepared as described (Daly et al., 1992). Analysis was by gas chromatography in conjunction with mass spectrometry and infrared spectroscopy, in order to identify the alkaloids present (see Daly et al., 1992, 1993, 1994). The gas chromatograms depicted in the figures were obtained with a 6-ft (2 mm ID) 1.5% OV-1-packed column with a flame ionization detector. A sample of 2 μl of a methanolic alkaloid fraction equivalent to 2 mg wet weight skin was injected at a column temperature of 150°C. After the solvent maximum had passed (0.3 min), the column was programmed to 280°C at 10°C/min.

RESULTS AND DISCUSSION

A number of alkaloids were found to be present in skin extracts from frogs raised on leaf-litter arthropods. Structures of these alkaloids or of representatives of a class of alkaloids are shown in Figure 1.

The two control frogs raised on wingless fruit flies exhibited virtually no alkaloids in skin extracts (Figure 2A). There was, however, a trace amount of the pyrrolizidine oxime **236**. Frogs of this species raised at the National Aquarium in Baltimore and in Minnesota under similar conditions had no skin alkaloids (Daly et al., 1992, 1994).

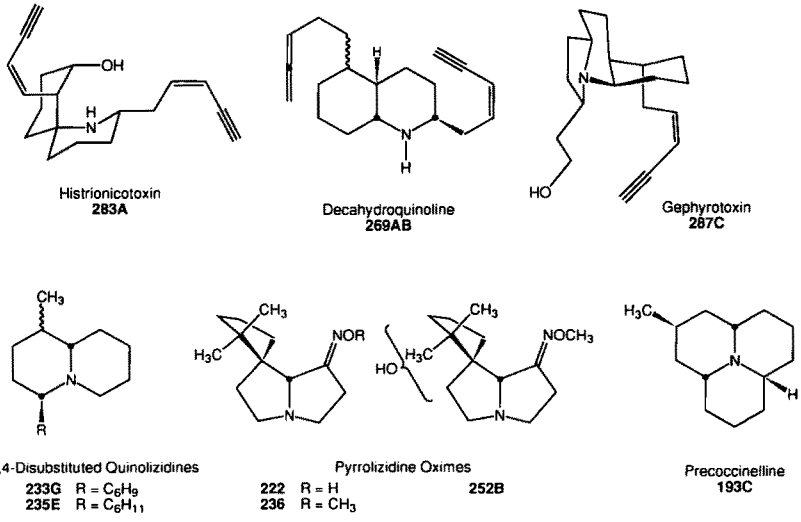

FIG. 1. Structure of alkaloids present in skin of frogs (*Dendrobates auratus*) raised on a diet of leaf-litter arthropods. Structures of decahydroquinoline **269AB**, pyrrolizidine oxime **252B**, and **233G** and **235E** have not yet been rigorously established by isolation and nuclear magnetic resonance spectral analysis. Structures of other apparent tricyclic alkaloids (**205D**, **219H**, **219I**, and **231H**) present in these frogs are unknown.

The three frogs raised on leaf-litter arthropods, by contrast, had substantial amounts of several alkaloids. The gas chromatographic profile for one of these is shown in Figure 2B. The two other frogs exhibited almost identical profiles. The major alkaloid for each frog was the pyrrolizidine oxime **236**. Minor alkaloids were the tricyclic precoccinelline (**193C**), a decahydroquinoline (**269AB**), and several histrionicotoxins (**283A**, **285A**, **285C**, **287A**). The amounts of the decahydroquinoline and histrionicotoxins were less in the other two frogs (data not shown). Other alkaloids consisted of additional tricyclic alkaloids of unknown structure (**205D**, **219H**, **219I**, **231H**), 1,4-disubstituted quinolizidines (**233G**, **235E**), a gephyrotoxin (**287C**), and pyrrolizidine oximes **222** and **252B**. A trace amount of the known millipede alkaloid nitropolyzonamine (**238**) was detected. The level of the major alkaloid (pyrrolizidine oxime **236**) in the captive-raised frogs was actually much greater than the levels in one of the wild-caught frogs from the leaf-litter site on Ancon Hill (Figure 3A and B) and in all three of the wild-caught frogs from the site of the parental stock on Isla Taboga (Figure 3C–E). Eighteen of the 21 alkaloids (86%) in the captive-raised frogs were shared with one or both of the wild-caught frogs of the leaf-litter site. Only ten alkaloids (48%) were shared with any of the three wild-caught frogs of the parental stock. The three pyrrolizidine oximes (**222**, **236**, **252B**) are shared in

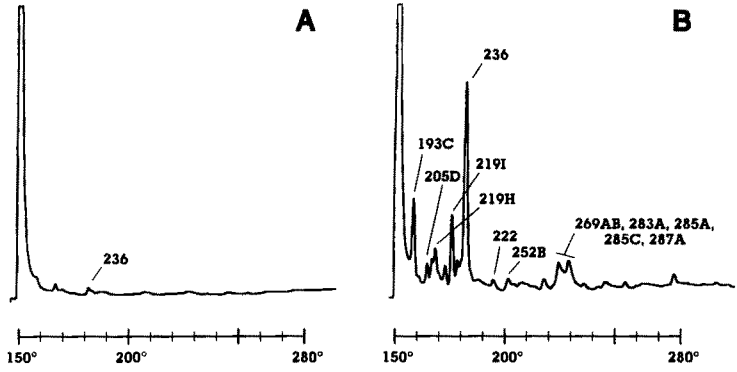

FIG. 2. Gas chromatographic profiles for alkaloids accumulated into skins of captive-raised poison frogs (*Dendrobates auratus*). (A) Alkaloid fraction from two *Dendrobates auratus* raised in terraria on wingless fruit flies. Snout-vent (s-v) length: 17 and 19 mm. (B) Alkaloid fraction from a *Dendobrates auratus* raised in a terrarium on leaf-litter arthropods, s-v: 19 mm. Similar profiles, but with smaller amounts of histrionicotoxins were obtained from two other frogs raised on leaf-litter arthropods. Gas chromatograms were obtained as described in Methods and Materials. The column is programmed from 150 to 280°C at 10°C/min. Emergent temperatures can differ somewhat with different columns and variations in flow rates. Code numbers for alkaloids are based on nominal molecular weight of each alkaloid and, if necessary, an identifying letter. For further details on gas chromatographic mass and infrared spectral identification of alkaloids, see Daly et al. (1987, 1992, 1993).

nearly all cases, as is the beetle alkaloid precoccinelline (**193C**). A comparison of alkaloids in all extracts is presented in Table 1 along with the results of previous analyses of skins obtained from frogs of the Isla Taboga site from 1968 to 1975 (see Daly et al., 1987). The variability seen in the two individual wild-caught frogs from Ancon Hill and the three individual wild-caught frogs from Isla Taboga is interesting. Earlier studies on individual variation in *Dendrobates auratus* from Isla Taboga and of *Dendrobates pumilio* from Isla Bastimentos, Bocas, Panama suggested that such individual variation was minimal. The present comparisons suggest that although the spectrum of alkaloids is similar for individuals from each population, the relative amounts (and hence the gas chromatographic profile) can vary considerably. Each population has a relatively distinctive spectrum of alkaloids, as has been the case for many prior studies on dendrobatid frogs (see Daly et al., 1987). If the two frogs from Ancon Hill are compared, only 23 of 47 alkaloids (49%) are shared, but if the trace alkaloids are excluded then 14 of 16 alkaloids (88%) are shared. Similarly, if the three frogs from Isla Taboga are compared, only 28 of 53 alkaloids (52%) are shared by all three frogs, but if the trace alkaloids are excluded then 13 of 15 alkaloids

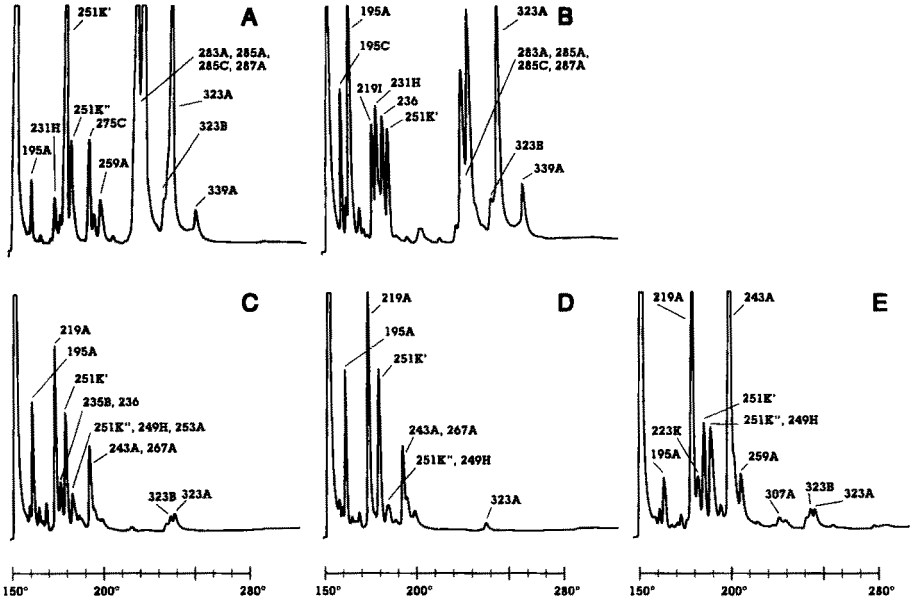

FIG. 3. Gas chromatographic profiles for alkaloids in skins of wild-caught poison frogs (*Dendrobates auratus*). (A) Alkaloid fraction from wild-caught *Dendrobates auratus*, Ancon Hill, Panama, s-v: 32 mm. (B) Alkaloid fraction from wild-caught *Dendrobates auratus*, Ancon Hill, Panama, s-v: 30 mm. (C) Alkaloid fraction from wild-caught *Dendrobates auratus*, Isla Taboga, Panama, s-v: 31 mm. (D) Alkaloid fraction from wild-caught *Dendrobates auratus*, Isla Taboga, Panama, s-v: 27 mm. (E) Alkaloid fraction from wild-caught *Dendrobates auratus*, Isla Taboga, Panama, s-v: 31 mm. Gas chromatographic parameters as in legend to Figure 1.

(87%) are shared. When the mainland (Ancon Hill) and the island frogs are compared, 33 of 66 alkaloids (50%) are found in both sets, but often not in all frogs of each set. When trace alkaloids are excluded, 17 of 23 alkaloids (74%) are found in both sets.

The present results strongly suggest a contribution from alkaloids of leaf-litter prey to the profile of alkaloids in dendrobatid frogs. It should be noted that all of the decahydroquinolines, with one exception, and all of the pumiliotoxins and allopumiliotoxins that were present in wild-caught frogs from both the mainland leaf-litter site and the parental island site were completely absent in the captive-raised frogs. Isomers of the pyrrolizidine **251K**, one isomer of which occurs in certain ants (Jones et al., 1991), were present as major or minor alkaloids in all of the wild-caught frogs, but were absent in the captive-raised frogs. The absence of certain alkaloids suggests either that such alkaloids do

TABLE 1. ALKALOIDS IN SKIN EXTRACTS OF *Dendrobates auratus*

Alkaloids[a]	Captive-raised on leaf-litter insects	Wild-caught					Isla Taboga pooled skins
		Ancon Hill		Isla Taboga			
	1B	2A	2B	2C	2D	2E	1968–1975
Histrionicotoxins							
235A		+		+	+	+	++
259A		++		+	+	++	++
261A		+				+	
283A	++	++	++			+	
285A	++	+++	++	+	+	+	
285C	+++	+++	+++			+	
287A	++	+++	+++			+	
287B	+		+			+	
Pumiliotoxins							
237A				+			
251D				+	+	+	++
277B		+	+	+		+	
297B							+
307A		+	++	+	+	++	++
307B		+	+	+	+	+	++
323A		+++	+++	++	++	++	+++
Allopumiliotoxins							
253A				++	+		+
267A			+	++	++	+	+++
305A					+		
323B		++	++	++	+	++	+
325A		+		+		+	
339A		++	++	+	+		+
339B							+
341A							+
357							+

TABLE 1. CONTINUED

Alkaloids[a]	Captive-raised on leaf-litter insects	Wild-caught					
		Ancon Hill		Isla Taboga			Isla Taboga pooled skins
	1B	2A	2B	2C	2D	2E	1968–1975
Decahydroquinolines							
195A		++	++	+++	++	++	+++
211A			+	+			
219A				+++, +	+++, +	+++, +	+++
243A				++, +	++, +	+++, +	+++
269AB	++		+				
3,5-Pyrrolizidines							
223B							+
223H			+				
249I*			+	+			
251K(K′, K″)[b]		+++, ++	++, +	+++, ++	++, ++	++, ++	
3,5-Indolizidines							
195B			+				
223AB		+					
275C		++					
277C*			+		+		
5,8-Indolizidines							
167A							+
181B							+
203A				+	+	+	+
223A[c]				+, +	+, +	+, +	+
223D						+	
235B			+	++	+	+	
1,4-Quinolizidines							
181A							+
195G*				+	+	+	
223C							+
233G*	+	+	+				

235E	+, +		+, +	+			
249H*			+	++	++	++	
275A							+
Gephyrotoxins							
287C	+	+	+				
Pyrrolizidine oximes							
222	+	+	+	+	+	+	
236	+++	+	++	++	+	+	
252B	+		+	+	+	+	
Nitropyrrolizidines							
238[d]	+						
252C*	+						
254*	+						
Tricyclics[e]							
193C[f]	++	+	++	+	+	+	
205B				+			
205D*	++		+				
207F			+	+	+		
207J*			+	+	+		
207L*			+				
219H*	++		+				
219I*	++	+	+				
231H*	+	++	++				
263E*						+	
Piperidines							
223K*						++	
239L*						+	
Unknowns							
185							+
217C*				+	+		
243E*				+	+	+	
263D*		+	+	+	+	+	
265I*		+	+			+	

TABLE 1. CONTINUED

Alkaloids[a]	Captive-raised on leaf-litter insects	Wild-caught					Isla Taboga pooled skins
		Ancon Hill		Isla Taboga			
	1B	2A	2B	2C	2D	2E	1968–1975
Unknowns							
265J*			+	+	+		
267B							+
271C*		+	+				
295A							+
309B							+

[a] For structures see Daly et al. (1993), for distribution in dendrobatid frogs see Daly et al. (1987). The amounts (+++ = major, ++ = minor, + = trace) follow the notation used in prior publications. Where two or more entries are given separated by commas, this indicates two or more isomers in their order of elution from the gas chromatograph. Additional trace alkaloids were detected, but adequate data for characterization were not obtained. Previously unreported alkaloids are designated by asterisks and will be reported in detail elsewhere.

[b] Two isomers occur: the first to elute (K′) is the *exo,exo* (5*Z*,8*E*) isomer identical with an ant pyrrolizidine (Jones et al., 1991); the second is either the *exo,endo* or *endo,exo* isomer.

[c] The alkaloid(s) that have been designated **223A** may represent more than one structural class. Initially, **223A** was postulated to be a quinolizidine (Daly et al., 1992), but further analyses indicate that **223A** in many extracts is an 8-ethyl-5-propylindolizidine with in addition a 6-ethyl substituent (unpublished results). Alkaloid **223A** is now listed as 5,8-indolizidine in Table 1. Two isomers occur in these extracts.

[d] Alkaloid **238** is identical to nitropolyzonamine, an alkaloid known from a millipede (Meinwald et al., 1975).

[e] "Tricyclics" refer to compounds whose mass spectra appear related to those of the coccinellines, which occur in beetles (Ayer et al., 1976). Some of these alkaloids may prove to have uniquely different structures from the coccinellines.

[f] Alkaloid **193C** is identical to precoccinelline, an alkaloid known from beetles (Ayer et al., 1976).

not have a dietary origin in dendrobatid frogs or that they are present in insects or other prey that were not collected by the present technique using Berless funnels.

A combined extract from 21 collections of leaf-litter arthropods made over the seven-month period afforded one major alkaloid and two minor alkaloids (Figure 4). The major alkaloid was pyrrolizidine oxime **236**, while the minor alkaloids were the beetle alkaloid precoccinelline (**193C**) and the millipede alkaloid nitropolyzonamine (**238**). Because of the identity of the carbon skeleton to that of nitropolyzonamine, it seems likely that the pyrrolizidine oxime **236** and other such oximes (**222**, **252B**) are also of millipede origin. The demonstration in the present experiments that such pyrrolizidine oximes are nearly certainly of dietary origin, presumably from small neotropical millipedes, explains previously puzzling observations on the occurrence of such oximes in one population of *Dendrobates pumilio* from Isla Bastimentos, Bocas, Panama. Such oximes

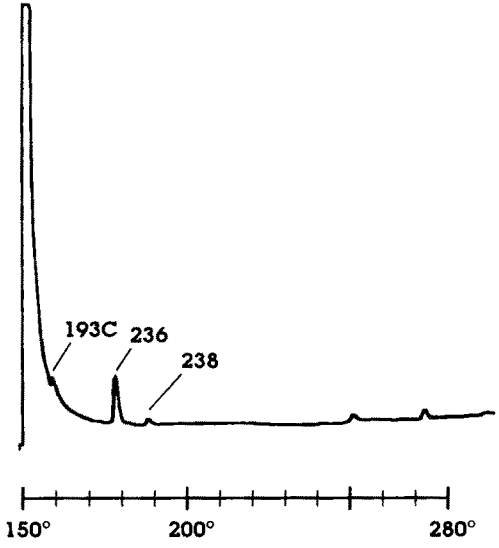

FIG. 4. Gas chromatographic profiles for alkaloids from combined extracts of leaf-litter arthropods. Combined extracts from 21 collections of leaf-litter arthropods made with Berless funnels over the seven-month period were extracted with ethanol and the pooled ethanol extract evaporated to a volume of 100 ml in vacuo at 30°C using a rotary evaporator. Alkaloids were separated by solvent partitioning as described (see Daly et al., 1987). The alkaloids were then dissolved in 0.5 ml methanol and analyzed (see Methods and Materials). The gas chromatogram was obtained with an injection of 0.2 μl. The code numbers for precoccinelline and nitropolyzonamine are **193C** and **238**, respectively.

were undetectable in skin extracts obtained in 1971 and 1972 (see Tokuyama et al., 1992), but three pyrrolizidine oximes (**222**, **236**, and **252A**) were significant, albeit minor, alkaloids in extracts obtained in 1981 and later years. The most likely explanation is that subtle changes in habitat have favored an increase in the abundance of millipedes that contain such pyrrolizidine oxime alkaloids.

The present preliminary study poses many questions, particularly with regard to the origin or source of several major classes of "dendrobatid alkaloids." Of the present alkaloids apparently accumulated from the diet in captive-raised frogs, precoccinelline most certainly originates from small beetles and the pyrrolizidine oximes, most likely from small millipedes. The histrionicotoxins, the pumiliotoxins, the gephyrotoxins, the decahydroquinolines, and 1,4-disubstituted quinolizidines are not known in nature except from frog skin. Although present in significant amounts in skin extracts of captive-raised frogs, neither the histrionicotoxins nor the quinolizidines were detected in extracts of the leaf-litter arthropods. Furthermore, levels of histrionicotoxins in the wild-caught frogs from the leaf-litter site were orders of magnitude higher than in frogs raised on leaf-litter arthropods. Thus, if histrionicotoxins do come from dietary sources, the present method for collection of arthropod prey is woefully inadequate. Clearly, an extensive study on the complete set of arthropods, including flying insects and other small creatures that could serve as food for dendrobatid frogs, and on the alkaloids present in such food sources needs to be conducted.

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A GROWTH INHIBITORY FACTOR FROM LAMBSQUARTERS (*Chenopodium album*)

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Abstract—Aqueous extract of air-dried lambsquarters (*Chenopodium album*) at 25 mg/ml significantly inhibited germination and growth of radish and wheat seeds. Soybean seed germination was not inhibited; however, hypocotyl growth was significantly reduced. Germination of radish seeds in sand amended with pulverized lambsquarters shoots at 2 and 4 mg/g was reduced 40 and 95%, respectively. Shoot dry weight and plant height were also reduced 30 and 9%, respectively, at 4 mg/g, but not at 2 mg/g concentration. Residues after extraction with water incorporated in sand were not inhibitory, indicating water solubility of the inhibitor(s). Aqueous extract of shoots decomposed for five days lost nearly 40% of its inhibitory effect; 20% of it still persisted in the extract of shoots decomposed for 30 days. The filtrate from ultrafiltration of aqueous extract through a pad of molecular-weight cutoff 1000 inhibited radish seeds germination and growth, indicating that the molecular weight of the inhibitor(s) was less than 1000. Partitioning of the aqueous extract by a series of solvents resulted in isolation of an inhibitor(s) in the butanol fraction. Seven phenolics were identified in this fraction using high-performance liquid chromatography (HPLC). Paper chromatographic analysis of the butanol fraction revealed six bands, of which one band with $R_f = 0.83$ inhibited germination and growth of radish seeds. Chlorogenic acid identified by HPLC appeared to be the principal component of the phytotoxin.

Key Words—*Chenopodium album*, lambsquarters, decomposition, inhibition, HPLC, paper chromatography, phenolics, radish, solvent extraction, ultrafiltration.

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INTRODUCTION

Although allelopathic interactions among plants are widely known (Rice, 1984), limited efforts have been made to exploit this phenomenon as a weed control mechanism. Concern for environmental damage caused by continued use of pesticides has prompted a renewed search for alternative methods of weed control. Naturally occurring compounds implicated in allelopathy may serve as a model or as a source from which an herbicide may be fashioned.

Singh et al. (1989) reported that aqueous extract of lantana (*Lantana camara*) leaves was strongly inhibitory to germination and growth of rye grass (*Lolium multiforum*). High-performance liquid chromatographic separation of an aqueous extract of this plant yielded 13 phenolic compounds, most of which strongly inhibited rye grass germination and growth. An aqueous extract of quackgrass [*Agropyron repens* (L.) Beau.] was reported to contain a phytotoxin effective against germination and radicle growth of several species of weed and crop plants (Weston et al., 1987). The phytotoxin contained two closely related flavonoids; both caused reduction of radicle growth in cress (*Lepidium sativum*) at less than 125 μg/ml concentration.

Lambsquarters (*Chenopodium album* L.) is a common weed in cultivated fields. McWhorter and Patterson (1979) reported this plant to be allelopathic to soybean. In our earlier experiments, we noted that residues of lambsquarters incorporated in sandy loam soil in pot culture severely reduced germination and nodulation of soybean. Aqueous extract of the residues equally affected soybean plants grown hydroponically (Mallik and Tesfai, 1988). The objective of this study is to isolate and identify the inhibitor(s) present in lambsquarters.

METHODS AND MATERIALS

Collection of Plant Materials. Lambsquarters was collected before and after anthesis from the horticultural farm of Langston University, Langston, Oklahoma, in early spring. The plant materials were brought to the laboratory, separated into different parts, air dried and oven dried briefly at 50°C before pulverizing in a Wiley mill, and stored in plastic bottles in a refrigerator (8°C).

Inhibitory Potential of Aqueous and Methanol Extracts. The pulverized residues were extracted with distilled water or 50% MeOH overnight on a shaker in a cold room, filtered through tissue paper, and centrifuged at 12,100*g* for 30 min. The MeOH extract after centrifugation was evaporated to dryness under reduced pressure at 45°C in a rotary evaporator; the residue in the flask was resuspended in water and restored to the original volume. The concentration of the extracts was 25 mg/ml.

Soybean (*Glycine max* cv. Essex), wheat (*Triticum aestivum* cv. Wrangler), and radish (*Raphanus sativus* cv. Snowbelle) seeds were used for bioassay.

Soybean seeds were surface sterilized with 25% Clorox for 5 min, and Clorox was washed off thoroughly with sterilized water. Surface sterilization was not necessary for wheat and radish seeds.

One hundred fifty grams of acid-washed (with 75% HCl acid followed by deionized water to wash off traces of acid) clean river-bed sand, after moistening with 25 ml extract, were placed in a Petri dish. Control plates received an equal volume of distilled water instead of the extract. The seeds for bioassay were planted in the moist sand. Replicate plates with 10 soybean or 20 wheat seeds in each were prepared. A plate for radish seeds contained 50 g sand moistened with 8 ml extract and 20 seeds were planted therein. Five replicate plates of each were prepared. The plates were incubated in a dark humid chamber (80% relative humidity) at 30°C. Percent germination and lengths of radicle and hypocotyl/coleoptile were determined after 65 hr of incubation.

Incorporation of Residues Before and After Aqueous Extraction in Sand. Pulverized plant residues before and after aqueous extraction were incorporated at 2 and 4 mg/g into river-bed sand. Water-extracted residue was air dried before incorporation into sand. Each pot received 400 g of this amended sand. Pots prepared similarly with an equivalent weight of peat moss as a substitute for the residue were included for comparison. Control pots received no amendment. Twenty-five radish seeds were planted per pot. Pots were watered at the base with distilled water as needed and fertilized with half-strength Hoagland and Arnon (1950) solution once a week. Pots were arranged in a randomized complete block design with five replicates in a phytotron (14 hr day length, photosynthetic photon flux density 490 μmol/m^2/sec at the top of plants, day–night temperature 30–25°C, 80% relative humidity). Seed germination count was taken four days after planting (DAP). The plants were harvested 21 DAP, at which time shoot height and dry weight were recorded.

Partial Decomposition of Residues. The pulverized residue was weighed and placed in designated beakers and moistened with a suspension of a fertile soil as a source of microorganisms for effective and uniform decomposition. The beakers were incubated at ambient temperature for 5, 10, 20, or 30 days. The whole content of the beaker was extracted with water after decomposition for the designated periods. Extracts were bioassayed with radish seeds in sand culture as described previously.

Ultrafiltration of Aqueous Extract. The crude aqueous extract after centrifugation (12,100g for 30 minutes) was ultrafiltered through a pad of molecular-weight cutoff (MWCO) 1000. The retentate on the pad was thoroughly washed and brought up to the original volume (40 ml) of the extract being filtered with distilled water. Both the filtrate and the retentate were bioassayed as described using radish seeds.

Partitioning of Aqueous Extract. Thirty grams of pulverized shoot were extracted with distilled water on a shaker overnight in a cold room, strained

through tissue paper, and centrifuged. To 500 ml of this extract were added 700 ml cold acetone, and the mixture was placed on a stir plate overnight in a cold room. The acetone extraction produced heavy precipitation consisting mostly of proteinaceous materials, which was removed by centrifugation. Acetone was removed by rotary evaporation at 35°C. The clear aqueous extract, reduced to 150 ml (by rotary evaporation at <50°C), was sequentially extracted with a series of organic solvents ranging from least polar to most polar (hexane, diethyl ether, dichloromethane, ethyl acetate, and butanol) (Figure 1). The extract was partitioned five times with 150 ml each (5 × 150 ml) of five solvents. Solvents from each fraction were evaporated to dryness and lyophilized. Each fraction was dissolved in distilled water except the ether fraction, which was dissolved in MeOH. An aliquot of this solution was further diluted with water to obtain a concentration of 3 mg/ml. Two and a half milliliters of this diluted solution were placed in a Petri dish lined up with a sterilized filter paper on which were placed 25 radish seeds for bioassay. A control plate prepared similarly received sterilized distilled water. Three replicate plates were prepared from each frac-

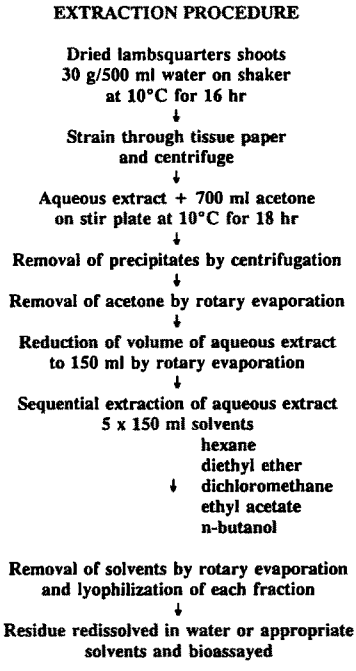

FIG. 1. Flow diagram of the procedure for extraction and partitioning of lambsquarters shoots.

tion. Percent germination and growth of seedlings were recorded after 65 hr of incubation at 30°C in a humid chamber.

Separation of Bioactive Fraction. Bioassay of the solvent fractions indicated that the BuOH fraction contained the phytotoxin. Freeze dried BuOH fraction was dissolved in water. A 25 μl portion containing approximately 1.2 mg of dry matter was streaked on pretreated Whatman 3M chromatographic paper (15 × 56 cm) and developed by 6% acetic acid. The chromatograms were examined under UV at 320 nm (long). Each band was marked, cut out, and divided in two equal halves. Two such pieces of each band were placed in a Petri dish and moistened with water. The strips cut from a chromatogram and developed similarly were used for control. Ten radish seeds were placed on top of the paper, covered by a third piece of the same band and incubated for 65 hr in a humid chamber at 30°C. Parameters for bioassay were the same as mentioned before.

Further separation of the butanol fraction was accomplished using a high-performance liquid chromatograph (HPLC) (Hewlett Packard model 1090M) fitted with a diode-array detector and Beckman Ultrasphere 5 μm C-18 column, 150 × 4.6 mm ID. Composition of the working buffer was 74.5:15.0:10.5 (2% acetic acid in water–methanol–tetrahydrofuran); buffer flow was set for 1 ml/min. Spectra of the samples and standards were collected to determine optimal detector setting and 300 nm was chosen for the separation. An aliquot of butanol fraction was dissolved in 3 ml of working buffer and 6 μl was injected in the column.

Eluate from the band with $R_f = 0.83$ indicated phytotoxicity (Table 6). Aqueous eluate from bands with $R_f = 0.83$ was pooled and filtered through a millipore membrane. This was condensed to near dryness by rotary evaporation at < 50°C. An aliquot of this eluate was dissolved in working buffer for analysis by HPLC. Presumptive identification of the compounds contained in the BuOH fraction and in the eluate was done by comparison of retention times of the unknown peaks with those of the phenolic standards (purchased from Sigma Chemical Co., St. Louis, Missouri).

RESULTS AND DISCUSSION

Inhibitory Potential. Crude aqueous extracts of lambsquarters shoots collected either before or after anthesis significantly reduced germination and growth of radish seeds (Table 1). Inhibition of radicle growth was accompanied by an almost complete absence of root hairs. Extract of inflorescence was similarly inhibitory. These results indicate that production of phytotoxin in lambsquarters shoots is independent of anthesis. Therefore, in subsequent experiments, shoot tissues collected after anthesis were used. Germination and growth of wheat

TABLE 1. GERMINATION, SHOOT AND ROOT GROWTH OF RADISH, WHEAT, AND SOYBEAN SEEDS TREATED WITH WATER EXTRACT OF LAMBSQUARTERS SHOOTS AND INFLORESCENCE

	Radish			Wheat			Soybean	
Extracts	Germ. (%)	Hypoc. (mm)	Rad. (mm)	Germ. (%)	Coleop. (mm)	Rad. (mm)	Germ. (%)	Hypoc. (mm)
Control (water)	84a[a]	9.2a	24.2a	90a	13.9a	31.8a	90a	42.3a
Before anthesis	29b	7.3b	12.0b	ND[b]	ND	ND	ND	ND
After anthesis	20b	6.5b	13.1b	74b	9.8b	17.2b	88a	31.6b
Control (water)	68a	12.7a	19.5a	ND	ND	ND	ND	ND
Inflorescence	17b	6.6b	5.0b	ND	ND	ND	ND	ND

[a]Data followed by a common letter in the same column are not significantly different at 1% level by Duncan's multiple-range test.
[b]Not determined.

seedlings were also significantly reduced; inhibition of radicle growth was pronounced. Soybean seed germination was not affected.

Methanol extracts of shoots and inflorescence were not inhibitory, nor were extracts of roots—methanol or water (not shown here). These results led us to infer that phytotoxin(s) contained in the aboveground parts of lambsquarters is water soluble. Water-soluble phytotoxin has been previously reported (Weston et al., 1987; Nicollier et al., 1985; Peters and Luu, 1985; Archhireddy et al., 1985).

To substantiate the above results, germination and growth of radish seeds were tested by planting seeds in sand amended with shoot tissues unextracted or extracted with water. Germination of radish seeds was reduced 40 and 95%, respectively, at 2 and 4 mg/g sand amended with unextracted shoots (Table 2, Figure 2). Shoot dry weight and plant height were also significantly reduced at 4 mg/g but not at 2 mg/g concentration. Germination and growth of seedlings were not affected when either water-extracted shoot tissues or peat moss were incorporated. Water extraction eliminated the phytotoxin(s) from shoot tissues. The phytotoxin(s) was more effective against germination than growth of radish seedlings. The results of the second experiment confirmed those of the first experiment. These results corroborate our earlier observation that the phytotoxin(s) in lambsquarters is water soluble. Lambsquarters shoot residues incorporated with silica sand at 0.75% inhibited soybean growth 80% compared with control; significant yield reduction occurred in the field when lambsquarters residues at 4725 kg/ha were incorporated into the soil (Bhowmik and Doll, 1982).

Partial Decomposition. The inhibitory effect of partially decomposed shoot

TABLE 2. GERMINATION, PLANT HEIGHT, AND SHOOT DRY WEIGHT OF RADISH SEEDS GROWN IN SAND AMENDED WITH PEAT MOSS AND LAMBSQUARTERS RESIDUE BEFORE AND AFTER EXTRACTION WITH WATER

Residues	Germination (%)		Height (mm/plant)		Dry weight (mg/plant)	
	Exp. I	Exp. II	Exp. I	Exp. II	Exp. I	Exp. II
Control	81a[a]	85ab	48ab	52d	11.0c	19.0c
Peat moss						
2 mg	84a	75ab	55ab	54cd	13.4ab	21.2ab
4 mg	85a	89a	55ab	57bc	11.7c	20.0bc
Lambsquarters shoot						
Before Ext.						
2 mg	49b	60c	56a	64a	13.2ab	22a
4 mg	4c	0d	34c	0e	10.0d	0d
After Ext.						
2 mg	88a	82ab	58a	59b	14.2a	20.8ab
4 mg	72a	62c	52ab	56bcd	12.2bc	19.7bc

[a]Data followed by a common letter in the same column are not significantly different at 1% level by Duncan's multiple-range test.

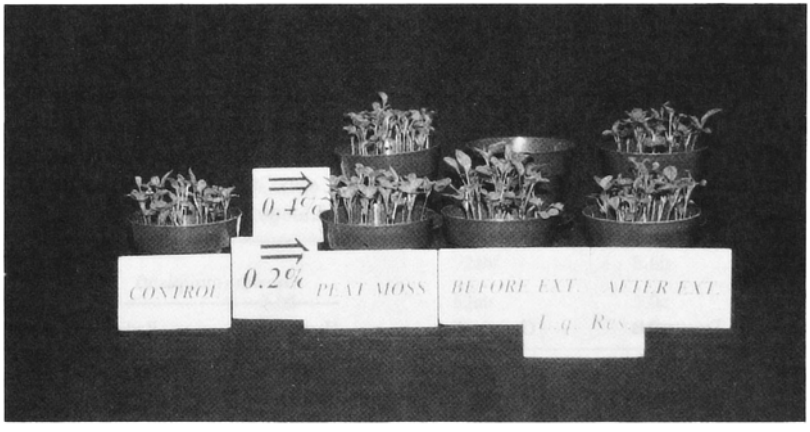

FIG. 2. Radish grown in sand (control) and sand amended with peat moss and pulverized lambsquarters shoots before and after extraction with water (left to right) at 2 (bottom) and 4 (top) mg/g sand.

tissues on germination and growth of radish seeds gradually decreased as the period of decomposition increased up to 30 days (Table 3). Percent germination increased from three at 0 day to 76 at 30 days. Hypocotyl and radicle growths similarly improved; however, radicle growth was still significantly inhibited even at 30 days of decomposition. It is noteworthy that phytotoxin(s) from lambsquarters shoots persisted even after 30 days of decomposition, albeit to a lesser degree. In contrast to these results, Kimber (1973) reported that the inhibitory effect of aqueous extracts of several legumes and grasses decomposed up to 21 days was negligible. Shorter persistence of toxicity of palmer amaranth (*Armaranthus palmeri*) incorporated into soil was also reported by Menges (1988). Toxicity of parthenium (*Parthenium hysterophorus*) to wheat seedling was diminished with increasing decomposition period (Mersie and Singh, 1987).

Ultrafiltration. The filtrate from ultrafiltration (MWCO 1000) of the aqueous extract significantly inhibited germination and growth of radish seeds (Table 4).

TABLE 3. RESPONSE OF RADISH SEED GERMINATION AND GROWTH TO AQUEOUS EXTRACTS OF PARTIALLY DECOMPOSED LAMBSQUARTERS SHOOTS

Decomposition period (days)	Germination (%)	Growth (mm/plant)	
		Hypocotyl	Radicle
0	2.2	25.0	4.7
5	56.5	79.4	20.4
10	32.6	66.2	19.8
20	60.9	69.1	17.4
30	80.4	91.2	19.5
Control	100.0	100.0	100.0

TABLE 4. RESPONSE OF RADISH SEED GERMINATION AND GROWTH TO ULTRAFILTRATE (MWCO 1000) OF AQUEOUS EXTRACTS OF LAMBSQUARTERS SHOOTS

Treatments	Germination	Growth (mm/plant)	
		Hypocotyl	Radicle
Control	57a[a]	9.3a	18.8a
Ultrafiltrate	8b	4.3b	5.2c
Retentate	59a	6.1b	11.1b

[a]Data followed by a common letter in the same column are not significantly different at 1% level by Duncan's multiple-range test.

We infer from these results that the molecular weight of the phytotoxin(s) present in lambsquarters shoots is < 1000.

Partitioning of Aqueous Extract. Water extraction of shoot materials was performed in a cold room to prevent any microbial breakdown of the phytotoxin. Among all the solvents, butanol (*n*) extraction yielded the greatest amount of precipitate (Table 5). The butanol fraction appeared pale yellow and was very hygroscopic. This fraction inhibited germination 78% of the control; likewise, growths of hypocotyl and radicle were inhibited 80 and 87%, respectively. Thus, the phytotoxin(s) appears to be polar and highly water soluble. It is noteworthy that residual aqueous extract, even after partitioning with several solvents, inhibited seed germination 25% and hypocotyl and radicle growths 35 and 42%, respectively. This might be additional evidence of water solubility of the phytotoxin(s). Ether and hexane fractions were also inhibitory, while ethyl acetate and dichloromethane fractions slightly stimulated hypocotyl growth.

Chromatographic Analysis of Bioactive Fraction. Chromatographic separation of butanol fraction revealed six bands under UV light (Table 6). Three of the bands were fluorescent and varied from very light blue to blue. The eluate of the band with $R_f = 0.83$ inhibited seed germination 64% and radicle growth 41% of control, indicating that this band contains a major portion of the phytotoxin(s). This band was fluorescent and light blue.

HPLC separation of the butanol fraction yielded 17 peaks, with retention times varying from 1.4 to 18.8 min. Of these 17 peaks, we could identify only seven phenolic acids because of availability of a very limited number of phenolic standards (Table 7). Gallic acid was the most dominant compound (51%), fol-

TABLE 5. RESPONSE OF RADISH SEED GERMINATION AND GROWTH TO DIFFERENT SOLVENT FRACTIONS OF PHYTOTOXIN(S) PRESENT IN AQUEOUS EXTRACT OF LAMBSQUARTERS SHOOTS

Solvent	Quantity (g/100g residue)	Germination (%)	Growth (mm/plant)	
			Hypocotyl	Radicle
Control		72ab[a]	8.6b	20.4a
Hexane	2.05	62ab	5.0c	10.3b
Diethyl ether	0.11	62ab	6.0c	11.3b
Dichloromethane	1.39	76a	11.1a	18.8a
Ethyl acetate	0.31	68ab	11.0a	21.3a
Butanol	2.96	16c	1.7d	2.7c
Resid. aq. extr.	15.00	54b	5.6c	11.9b

[a] Data followed by a common letter in the same column are not significantly different at 1% level by Duncan's multiple-range test.

TABLE 6. PAPER CHROMATOGRAPHIC SEPARATION OF BUTANOL FRACTION CONTAINING THE PHYTOTOXIN(S), AND EFFECT OF EACH COMPOUND ON GERMINATION AND GROWTH OF RADISH SEEDS

Compound	Rf.	Long-UV[a]	Germ. (%)	Growth (mm/plant)	
				Hypocotyl	Radicle
Blank = Control			83	10.4	22.5
#1	.17	ab., dk.violet	76	11.9	15.6
#2	.36	ab., v. lt-purple	ND[b]	ND	ND
#3	.43	ab., lt.-violet	63	11.1	18.1
#4	.57	fl., lt.-blue	76	10.5	13.4
#5	.68	br.fl., blue	70	10.7	14.3
#6	.83	fl., pale blue	30	10.3	13.2

[a]ab., absorbed; br., bright; dk., dark; fl., fluorescent; lt., light; v., very.
[b]Not determined.

TABLE 7. HPLC ANALYSES OF ELUATE FROM BAND WITH Rf 0.83 OF PAPER CHROMATOGRAM AND OF BuOH FRACTION OF SEQUENTIALLY EXTRACTED WATER EXTRACT OF LAMBSQUARTERS SHOOT

No.	Retention (time) (min)	Phenolic acids	% Relative concentration	
			BuOH fract.	Eluate Rf. 0.83%
1	2.14	Gallic	51.02	
2	2.74	Syringic	19.33	6.15
3	3.09	Chlorogenic	1.43	71.54
4	3.35	Vanillic	18.59	22.31
5	3.98	Caffeic	6.03	
6	4.66	Ferulic	2.90	
7	5.77	Coumaric	0.68	

lowed by syringic and vanillic acids. Caffeic, ferulic, chlorogenic, and coumaric acids appeared in traces. Singh et al. (1989) identified 13 phenolic compounds from an aqueous extract of *Lantana camara* by using a HPLC.

Three phenolic acids were identified in the eluate from $R_f = 0.83$ of the paper chromatogram (Table 7). Chlorogenic acid constituted 70%, followed by vanillic and syringic acids. The phytotoxicity of lambsquarters could be solely due to chlorogenic acid; however, a synergistic effect of all three acids could

not be ruled out. The phytotoxicity of chlorogenic and isochlorogenic acids isolated from sunflower plant was reported earlier (Wilson and Rice, 1968).

In conclusion, the water extract of lambsquarters shoots contains a highly polar phytotoxin(s), which is apparently more potent pregermination than postgermination. The phytotoxin is very slowly biodegradable. Partial purification of the phytotoxin was achieved by liquid–liquid extraction with organic solvents and paper chromatography. Presumptive identification of the phytotoxin by HPLC indicates chlorogenic acid as the principal component.

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EFFECTS OF TWO SESQUITERPENE LACTONES ISOLATED FROM *Artemisia annua* ON PHYSIOLOGY OF *Lemna minor*[1]

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Abstract—The effects of artemisinin and arteannuic acid extracted from *Artemisia annua* on the physiology of *Lemna minor* were evaluated. Changes in frond production, growth, dry weight, and chlorophyll content of *L. minor* were determined. Photosynthesis and respiration were evaluated with a differential respirometer. Artemisinin (5 μM) inhibited *L. minor* frond production and dry weight 82 and 83%, relative to methanol controls. Chlorophyll content was reduced 44% by artemisinin (2.5 μM). Arteannuic acid (10 μM) was less active, inhibiting frond production 61% and reducing chlorophyll content 66% at 5 μM. Artemisinin (1 μM) reduced *L. minor* photosynthesis 30% and 2.5 μM reduced respiration 39%. Arteannuic acid had no significant effect on photosynthesis or respiration at the levels tested.

Key Words—Artemisinin, arteannuic acid, photosynthesis, respiration, chlorophyll, *Lemna minor*.

INTRODUCTION

Artemisia annua L. (annual wormwood) was first used as a treatment for hemorrhoids in the Mawangdui Han dynasty in 168 BC (Klayman, 1985). In 1527, a

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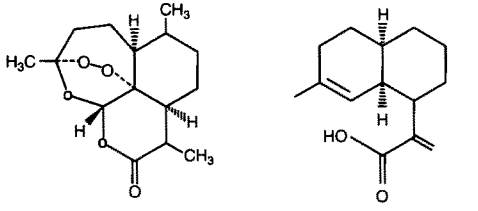
Chinese herb doctor, Li Shi-zen recommended the use of annual wormwood for the treatment of malaria (Klayman, 1985, 1989). Extracts from *A. annua* are also being used for the treatment of cerebral malaria and systemic lupus erythematosus (Klayman, 1985). These extracts are currently being studied extensively for their use as insecticides, herbicides, and other uses (Chen and Leather, 1990; Duke et al., 1987; Sherif et al., 1987).

The leaves of *A. annua* contain potent sesquiterpene lactones, which have a variety of biological properties including insect antifeedant and growth regulator activities (Chen and Leather, 1990; Duke et al., 1987; Fischer, 1986; Stevens, 1984; Thompson, 1985). Three sesquiterpene lactones isolated from the leaves of *A. annua* are artemisinin, arteannuic acid, and arteannuin B (Chen and Leather, 1990). The latter two are precursors to artemisinin. These three compounds have already been examined for some of their allelopathic properties (Chen and Leather, 1990).

Artemisinin is recognized by different names such as quinine, quinghaosu, or QHS (Klayman, 1985). The structure–activity relationships of artemisinin ($C_{15}H_{22}O_5$) have been investigated (Chen and Leather, 1990; Klayman, 1985, 1989; Luo and Shen, 1987). The pleasant odor of the compound is due to the terpene structure (Klayman, 1989). The linked pair of oxygen atoms (peroxide group) joining two parts of the carbon framework is unusual for a natural compound (Figure 1). Breaking the bond results in weak oxidizing properties similar to hydrogen peroxide (H_2O_2) (Klayman, 1989). A disadvantage of artemisinin as a biocontrol agent is its solubility of 1% or less in water and oil. The compound breaks down only in protic solvents by opening a lactone ring (Klayman, 1985). Artemisinin has a high thermal stability. The highest concentration of the compound in the leaves of *A. annua* occurs just prior to the onset of flowering (Chen and Leather, 1990; Klayman, 1985). Artemisinin has not been found in other species of *Artemisia* (Klayman, 1985, 1989).

Artemisinin's effect on the plasma membrane of *Plasmodium falciparum* appears to be linked to its effectiveness as an antimalarial drug. Klayman (1989) reported that the endoperoxide moiety of this compound is the crucial portion of the molecule for activity against the malaria parasite cell membrane. At a



ARTEMISININ ARTEANNUIC ACID

FIG. 1. Chemical structures of artemisinin and arteannuic acid.

concentration of 100.0 μM, the protein in the growth medium of *L. minor* was increased 12-fold and 20-fold over the controls after eight days for artemisinin and arteannuic acid, respectively (Chen and Leather, 1990). The response of plants and *Plasmodium falciparum* to artemisinin suggests that its mode of action may be similar in plants and microorganisms (Duke et al., 1987).

Chen and Leather (1990) reported that artemisinin inhibited root formation in bush bean (*Phasoleus vulgaris*) seedlings at levels as low as 10.0 μM. Duke et al. (1987) also reported that artemisinin inhibited roots and shoots of lettuce (*Lactuca sativa*), redroot pigweed (*Amaranthus retroflexus*), pitted morning glory (*Ipomoea lacunosa*), and common purslane (*Portulaca oleracea*) at 33.0 μM. Arteannuic acid stimulated short root formation on cuttings of bush and mung beans (*Phasoleus aureus*) (Chen and Leather, 1990). Artemisinin inhibited seed germination and seedling growth of several monocot and dicot vegetables and weeds, while arteannuic acid had no effect on seed germination, but increased seedling dry weight (Chen and Leather, 1990; Duke et al., 1987).

Previous work in our laboratory showed that artemisinin (10 μM) was more inhibitory to *Lemna* than 10 μM treatments of the herbicide 2,4-D (Chen et al., 1991). At this level, artemisinin inhibited chlorophyll production of *Lemna minor* and anthocyanin production of *L. obscura*. At 5.0 μM, artemisinin inhibited frond production 50% and created losses in both fresh and dry weight of *L. minor*, whereas arteannuic acid was less phytotoxic. However, the effect of artemisinin and arteannuic acid on physiological functions of intact plants has not been adequately investigated. The objective of the present research was to determine effects of low concentrations of artemisinin and arteannuic acid on photosynthesis and respiration of *L. minor* as possible causes of the effects of these compounds on plant growth.

METHODS AND MATERIALS

Culture Conditions. Axenic cultures of duckweed, *L. minor* strain 5, were grown in autoclaved, cotton-stoppered 125-ml Erlenmeyer flasks with 50 ml of E medium (Cleland, 1979). Stock cultures were maintained for 14 days in an environmental growth chamber at 28°C under constant (236 $\mu E/sec/m^2$) fluorescent and incandescent light before subculturing for bioassay. *Lemna* cultures were monitored for bacterial contamination by supplementing the E medium with a pancreatic digest of casein USP (Bacto-tryptone) and yeast extract and observing the medium for cloudiness.

Bioassay. The bioassay was performed according to the methods previously described by Einhellig et al. (1985) and Leather and Einhellig (1985). All procedures were performed in a laminar flow hood using aseptic techniques. Fresh E medium without sucrose and tartaric acid was prepared for each bioassay. Einhellig et al. (1985) demonstrated that using only a mineral medium during

the bioassay increased the sensitivity of *Lemna* to allelochemicals and eliminated growth of microorganisms. Using a sterile pipet, 1.5 ml of E medium was dispensed into each well of a sterile 24-well tissue culture plate. With a gas-tight microsyringe, 5.0 μl of test chemical (artemisinin or arteannuic acid) at 300 times the final concentration in 100% methanol was placed into each well according to a randomized block design with four replications. Concentrations of 0.0, 0.5, 1.0, 5.0, and 10.0 μM of artemisinin and arteannuic acid were tested. The control wells received 5.0 μl of methanol (carrier). *Lemna* colonies at the three-frond development stage were transferred by a flamed culture loop into each well. The culture plate was covered and placed in an environmental growth chamber (28°C and constant light, 236 μE/sec/M^2) for seven days until harvest.

Test for Bacteria. After the *Lemna* cultures had incubated seven days, 0.5 ml of the medium was removed from 10 randomly selected wells of each culture plate. The selected wells included two wells from each treatment. The medium was diluted 1:10, 1:100, and 1:1000 in sterile distilled water, and 0.1 ml of each was pipetted onto nutrient broth–yeast extract (NBY) agar medium and King's medium B agar (KB) (Schaad, 1988). The agar plates were incubated at 27°C for seven days to detect the presence or absence of bacteria.

Harvest. L. minor was gently removed by curved forceps from each well according to the randomized block design and replication and was placed in water for the frond count. Fronds were then placed in a small test tube, excess water was removed, and 1.5 ml of 90% ethanol was dispensed into each test tube. After 6 hr in the dark, the ethanol was removed by Pasteur pipet and fronds were reextracted in 1.5 ml of 90% ethanol for 16 hr. The ethanol extracts were pooled and the volume adjusted to 3.0 ml. After the ethanol extraction, fronds were air dried two days and dry weight determined on a balance accurate to 0.1 mg. Chlorophyll concentration was determined by measuring absorbance at 665 and 649 nm (Einhellig and Rasmussen, 1979). Values for chlorophyll *a*, chlorophyll *b*, total chlorophyll, and the ratio of chlorophyll *a* to chlorophyll *b* (*a*/*b*) were calculated according to the method of Wintermans and DeMots (1965). Data were subjected to analysis of variance and significant differences determined by nonoverlapping confidence intervals at $P = 0.1$.

Photosynthesis. This experiment was a randomized block design with eight replications of 1.5 ml of the modified E medium, four to five *L. minor* fronds, and 5.0 μl of the test chemical in each well of a 24-well tissue culture plate. Levels of 1.0 and 2.5 μM of artemisinin and arteannuic acid were used. The control received 5.0 μl of methanol (carrier). The culture plate was placed in an environmental chamber at 28°C under constant light for 48 hr. All of the fronds from a specific treatment were then placed in the transfer dish containing sterile distilled water. Fifteen fronds (three to four colonies) were transferred into a respirometer reaction flask containing 2.5 ml of the modified E medium. Each treatment consisted of four replications with one blank flask. All proce-

dures were performed in a laminar flow hood as described previously. Sodium carbonate–bicarbonate buffer (0.1 M) was added to the reaction flasks to maintain a 0.9% CO_2 phase (0.5 ml in the side well and 0.2 ml in the center well). A small fluted wick of filter paper was placed in each center well to increase absorbing surface (Umbreit et al., 1964). The reaction flasks were sealed and lowered simultaneously into the differential respirometer water bath and the micrometers were set on 250. The respirometer was placed in a photographic darkroom and black bags were taped over the bath to ensure darkness. The reaction flasks equilibrated in the dark for 30 min before any readings were recorded. Data were collected during 10-min intervals as follows: 0–10 and 10–20 in the dark phase, 30–40 and 40–50 in the light phase, and 60–70 and 70–80 min in the dark phase. All experiments were repeated at least once. Photosynthesis was calculated by using the formula (Umbreit et al., 1964):

$$h_{10} - \text{minute photosynthesis} = \left[\frac{3(h_{R_1} + h_{R_2}) - h_{P+R}}{2}\right]$$

where h is the manometer reading in millimeters, R_1 is the respiration at 10–20 min, R_2 is the respiration at 70–80 min, and $P + R$ is the photosynthesis plus respiration at 40–50 min.

RESULTS AND DISCUSSION

Test for Bacteria. No bacteria were detected in the medium of sampled culture plate wells. In separate experiments, wells containing artemisinin, arteannuic acid, or control medium were artificially contaminated at the beginning of a seven-day incubation. There was no effect on *L. minor* growth at levels of 3×10^6 microorganisms per well (data not shown).

Bioassay. We have successfully used *Lemna* for bioassay of many allelochemicals and plant growth regulators (Leather and Einhellig, 1988; Toro et al., 1988; Nyberg, 1986; Einhellig et al., 1985). This plant is extremely useful because of its small size, rapid reproduction, and sensitivity to small microvolumes of the test chemical. Leather and Einhellig (1985) reported that the *Lemna* bioassay was a more sensitive test for low levels of allelochemicals than bioassays measuring seedling growth, seed germination, or radicle elongation. The results of this research showed that *L. minor* is sensitive to artemisinin and arteannuic acid at levels of 0.5–10.0 μM.

Artemisinin and arteannuic acid inhibited frond production at levels of 5.0 and 10.0 μM (Table 1). Artemisinin at 5.0 and 10.0 μM exhibited a stronger inhibition than arteannuic acid as it reduced frond growth by 82 and 98% of the control, respectively, during the seven-day growth test (Table 1). *Lemna* colonies with these levels of artemisinin had fronds that were reduced in size, bleached at their outer edge, and showed little to no root formation. Artemisi-

TABLE 1. EFFECTS OF ARTEMISININ AND ARTEANNUIC ACID ON GROWTH AND CHLOROPHYLL CONTENT OF *Lemna minor* IN A 7-DAY BIOASSAY[a]

Treatment (μM)	Frond production (fronds/well)	Total dry weight (mg)	Total chlorophyll (μg)	Chlorophyll (μg) per mg tissue	Ratio chlorophyll *a*/ chlorophyll *b*
Artemisinin					
0.0	42.1c	3.5b	54.0d	15.9a	2.3a
0.5	48.1c	3.6b	48.0d	13.6a	2.7b
1.0	46.6c	3.2b	47.0d	15.0a	2.9b
2.5	34.6c	2.2b	30.2c	14.8a	3.8b
5.0	7.5b	0.6a	6.5b	10.7a	2.9ab
10.0	1.0a	0.3a	2.5a	9.9a	3.1ab
Arteannuic acid					
0.0	40.6b	3.3b	51.6b	15.9b	2.9a
0.5	38.8b	3.3b	47.6b	15.1b	3.0a
1.0	36.2b	3.0b	43.4b	14.7b	3.2a
2.5	34.0b	3.2b	38.5b	12.2b	3.1a
5.0	23.8ab	2.5ab	17.7a	7.2a	3.2a
10.0	15.9a	1.5a	8.7a	6.1a	3.5a

[a]Values in a column for each chemical having the same letter are not significantly different at $P = 0.1$ by nonoverlapping confidence intervals.

nin's effects on dry weight closely followed its effect on frond production. Levels of 2.5, 5.0, and 10.0 μM reduced the dry weight 37, 83, and 94% of control, respectively. When dry weight was determined on a per frond basis, no significant inhibition was observed at the levels of artemisinin tested.

Arteannuic acid inhibited frond production 41 and 61% of the control at 5.0 and 10.0 μM, respectively (Table 1). Fronds were small, clumped in their growth, and exhibited a visible loss of chlorophyll and shortened roots. A dry weight reduction of 56% from the control plants was recorded at 10.0 μM arteannuic acid. The activity threshold was much higher than artemisinin's for this parameter (arteannuic acid, 5.0 μM; artemissinin, 2.5 μM) (Table 1). Chen and Leather (1990) reported similar results using lettuce seedling growth. Artemisinin inhibited both fresh and dry weight of lettuce seedlings whereas arteannuic acid appeared to increase lettuce seedling dry weight.

Chlorophyll content was the most sensitive indicator of phytotoxicity by artemisinin. Total chlorophyll content was reduced 12.5% at 0.5 and 1.0 μM, and levels of 2.5, 5.0, and 10.0 μM of artemisinin reduced total chlorophyll content 44, 88, 96%, respectively (Table 1). Reduction of chlorophyll in these experiments was less than previous reports indicated (Chen and Leather, 1990; Duke et al., 1987). The threshold for reduction of chlorophyll was less than the threshold for frond production (0.5 μM compared to 2.5 μM) (Table 1). Although

a trend to less chlorophyll (micrograms per milligram of tissue) was observed with artemisinin, the differences were only significant at 5 and 10 μM levels of compound. Not understood at present, but an indicator of perturbation by allelochemicals, is the alteration of the ratio of chlorophyll *a* to chlorophyll *b*. The *a*/*b* ratio increased in all artemisinin treatments and was highest at 2.5 μM concentration (Table 1).

Arteannuic acid reduced chlorophyll content to a greater extent than frond production. At 2.5, 5.0, and 10.0 μM, total chlorophyll content was reduced to 75, 66, and 17% of the control plants, respectively (Table 1). The threshold concentration (2.5 μM) for chlorophyll reduction appeared to be lower than the threshold for frond number inhibition (5 μM). The chlorophyll (micrograms per milligram of tissue) was reduced 23, 55, and 62% for 2.5, 5.0, and 10.0 μM arteannuic acid, but no differences were observed for the chlorophyll *a*/*b* ratio (Table 1).

Photosynthesis. The effects of allelochemicals in photosynthesis and respiration have been well studied. Nyberg (1986) identified many allelochemicals that successfully inhibited net photosynthesis in *Lemna*. The minimum concentration needed for inhibition varied from 0.1 mM to 2.0 mM. Einhellig et al. (1970) and Putnam (1983) also reported photosynthesis inhibition of several plant species treated with scopoletin. This compound caused the plant's stomata to close. This factor creates uncertainty as to whether the stomata closure occurred because of photosynthesis inhibition or as a result of other physiological effects. Kaempferol inhibited coupled electron transport and photophosphorylation in isolated pea chloroplasts (Arntzen et al., 1974; Stenlid, 1970). The inhibition of respiration appeared to be related to the uncoupling of electron transport in mitochondria. Research with juglone (a naphthaquinone) showed it also uncoupled electron transport in the mitochondria of corn during oxidative phosphorylation (Putnam, 1983; Koeppe, 1972). This chemical inhibited respiration in corn plant roots (90% reduction in 1 hr) and depressed photosynthesis of *Lemna* at very low concentrations (Nyberg, 1986; Hejl et al., 1993).

Artemisinin and arteannuic acid at levels of 1.0 and 2.5 μM were observed for changes in net photosynthesis and respiration of *L. minor*. Artemisinin exhibited a greater effect on photosynthesis (oxygen evolution) inhibition than arteannuic acid. The data collected for artemisinin's effect on photosynthesis could not be combined for experiments 1 and 2 because analysis of variance indicated there was significant difference between them. At 1.0 μM artemisinin, photosynthesis was reduced by 30% (Figure 2). Its effect at the 2.5 μM level was even greater, reducing photosynthesis 70%. Arteannuic acid affected photosynthesis differently. At the 1.0 μM level, net photosynthesis was stimulated by 11% above the control plants. No inhibition was observed at the 2.5 μM level (Figure 2).

The percent reduction in photosynthesis was twofold that observed for total chlorophyll loss at 1.0 and 2.5 μM artemisinin. The plants used for photosyn-

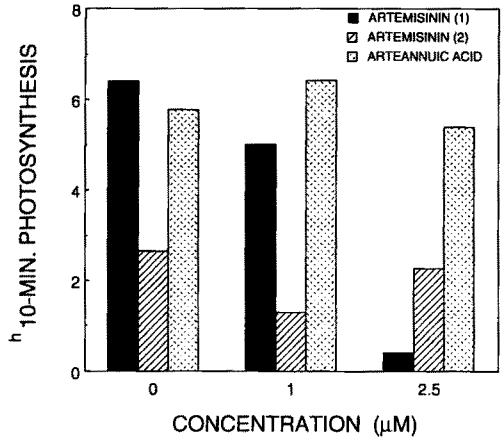


FIG. 2. Photosynthesis of *Lemna minor* measured as oxygen evolution per flask of 15 fronds after treatment with artemisinin or arteannuic acid during an 80-min test period. Treatment concentrations are recorded in micromoles.

thesis were only exposed to the chemical two days, while those that were extracted for chlorophyll were treated seven days. Thus, reduction in photosynthesis could not be totally attributed to chlorophyll loss. Additionally, at 1.0 and 2.5 μM, artemisinin, no reduction was observed in the amount of chlorophyll per tissue weight, which was the basis for comparison among samples in the respirometer. It is noteworthy, however, that the increases in the chlorophyll *a*/*b* ratio at 1.0 and 2.5 μM artemisinin (Table 1) corresponded closely with the reductions in photosynthesis (Figure 2).

Arteannuic acid did not affect photosynthesis at 1.0 or 2.5 μM concentrations (Figure 2). Although growth effects were similar for arteannuic acid and (μg/mg of tissue) artemisinin, total chlorophyll from arteannuic acid-treated plants was only reduced at 2.5 μM and higher concentrations. Chlorophyll (μg/mg of tissue), however, was reduced at 2.5 μM arteannuic acid, but only at the more toxic level of 5 μM artemisinin. These results suggest that the primary effects on *Lemna* are different for artemisinin and arteannuic acid.

Respiration of *L. minor* was significantly reduced only by artemisinin at 2.5 μM during the light phase of measurement in the respirometer (Figure 3). Arteannuic acid did not affect respiration at the levels tested (data not shown).

In summary, this study demonstrates that the allelochemicals isolated from *Artemisia annua* are biologically active and can contribute to interference in *Lemna* plants. In most of the parameters tested with artemisinin and arteannuic acid, there existed a concentration relationship in which toxicity increased with higher concentrations of the test chemical. The inhibitory effect with artemisinin was greater than arteannuic acid in all parameters tested.

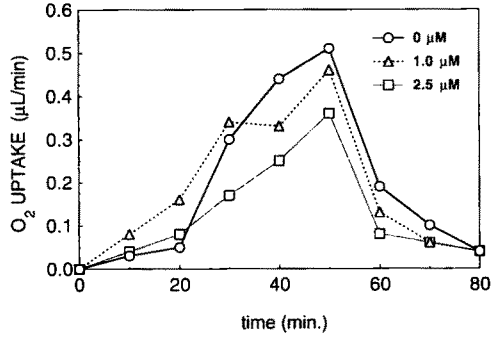


FIG. 3. Respiration of *Lemna minor* measured in microliters of oxygen uptake per minute after treatment with artemisinin during an 80-min test period. Dark intervals were 0–10, 10–20, 60–70, and 70–80 min. Light intervals were 30–40 and 40–50 min. Treatment concentrations are recorded in micromoles.

The exact mechanism of action of artemisinin remains unknown. However, changes in chlorophyll levels, photosynthesis, and respiratory activity occurred. These results agree with observations by Chen and Leather (1990) that there may be a disturbance to the cell membrane by these allelochemicals. In the future, *Artemisia annua* and its allelochemicals may have potential for weed control. With the increase in environmental awareness, society and technology are pursuing natural compounds for commercial use. *Artemisia annua* and its allelochemicals may have great potential as environmentally desirable herbicides (Rice, 1984; Einhellig and Leather, 1988).

Acknowledgments—This paper is dedicated to Dr. D. Klayman, who died on November 23, 1992. His support and encouragement for this research will be missed.

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BEHAVIORAL AND ELECTROPHYSIOLOGICAL RESPONSE OF CABBAGE SEED WEEVILS (*Ceutorhynchus assimilis*) TO CONSPECIFIC ODOR

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Abstract—The responses of *Ceutorhynchus assimilis* Payk. (Coleoptera, Curculionidae) to the odor of overwintered and new generation weevils were studied using an olfactometer, choice tests in a laboratory cage, field tests using sticky traps baited with live weevils, and by electroantennograms (EAG's). Unmated male weevils and, to a lesser extent, female weevils of the overwintered generation were attracted to the odor of live unmated overwintered female weevils. New generation weevils exhibited no behavioral response to conspecific odor. Male and female weevils of the overwintered generation exhibited positive EAGs to hexane extracts of overwintered female weevils, whereas EAGs of new-generation weevils of either sex were unresponsive to these extracts. This suggests that the unmated female weevils from the overwintered generation produce a volatile chemical or chemicals that attracts unmated male and female weevils. The new generation of female weevils does not produce this attractive chemical before overwintering, and male and female weevils of this generation can not detect the chemical(s) via their antennal chemoreceptors until they have undergone their overwintering period.

Key Words—*Ceutorhynchus assimilis*, Coleoptera, Curculionidae, olfactometer, sex pheromone, aggregation pheromone, electroantennogram.

INTRODUCTION

The cabbage seed weevil (*Ceutorhynchus assimilis* Payk.) is an important pest of oilseed brassicas in Europe, especially oilseed rape, *Brassica napus* ssp.

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oleifera DC. (Lamb, 1989). Other weevil species such as the boll weevil, *Anthonomus grandis* Boh. (Dickens, 1984), sweet potato weevil, *Cylas formicarius elegantulus* Summ. (Mason et al., 1990), American palm weevil, *Rhynchophorus palmarum* (L.) and other *Rhynchophorus* spp. (Rochat et al., 1991, 1993; Weissling et al., 1993), and pea and bean weevil, *Sitona lineatus* (L.) (Blight and Wadhams, 1987), have been shown to utilize pheromones for sexual attraction or aggregation. Although there is evidence for an oviposition-deterring pheromone in seed weevils (Kozlowski et al., 1983; Ferguson and Williams, 1991), there have been no reports of a sex or aggregation pheromone in this species.

Seed weevils overwinter as adults at the site of brassica crops from the previous season and usually emerge in March–April the following year (Dmoch, 1965). After emergence, the weevils disperse to a flowering rape crop. The beginning of mating and oviposition by female weevils depends on a period of supplementary feeding on rape, which is necessary for the maturation of the eggs (Dmoch, 1965; Williams and Free, 1978; Ni et al., 1990). After oviposition and larval development, the new generation appears in August, and these weevils overwinter until the following spring.

The response of seed weevils to conspecific odor was observed using a four-choice olfactometer based on the design of Vet et al. (1983). Laboratory choice experiments and field trapping in an oilseed rape crop with weevil-baited sticky traps were undertaken to establish whether seed weevils produce a volatile attractant. Electroantennograms (EAGs) of both overwintered and new-generation seed weevils were recorded to determine whether the weevils could detect any stimulatory compounds in the hexane extracts of female weevils.

METHODS AND MATERIALS

Insects. Overwintered seed weevils were sweep-netted from oilseed rape crops as soon as weevils were observed at their emergence sites (rape crops from the previous season) in traps baited with attractive oilseed rape extracts (Evans and Allen-Williams, 1989). These weevils were assumed to be unmated, as seed weevils feed on rape or other cruciferous plants for several days before mating and laying eggs (Dmoch, 1965; Williams and Free, 1978; Ni et al., 1990). The weevils were kept in single-sex cultures at 20°C in a 16-hr light–8-hr dark light regime and fed on rape pods and flowers. New generation weevils were collected later in the season and kept under similar conditions.

Description of Olfactometer. The basic design of the olfactometer was identical to that used by Vet et al. (1983). The exposure chamber was made from two sheets of transparent Perspex, which comprised the ceiling and floor of the chamber, with four Perspex crescents glued to the ceiling to form the

walls (1.5 cm high) of the chamber. Each point of the star-shaped chamber ran without obstruction into a tube (inside diameter 0.5 cm) with each tube connected to an insect collection flask. Four odor fields were created within the chamber by drawing air through the central hole in the ceiling by a vacuum pump (Edwards High Vacuum, Crawley, U.K.). The flow rate of air through each of the four arms of the olfactometer was equilibrated by a flowmeter so that four kite-shaped odor fields were generated within the chamber at a flow rate of approx. 300 ml/min through each arm. The shape of the odor fields was determined by generating NH_4Cl smoke from NaOH and HCl vapor, as suggested by Vet et al. (1983).

The odor to be tested was introduced through one of the olfactometer arms, the three other arms introducing filtered air. The olfactometer was housed in a room with no natural light, the lighting being supplied by strip lights in the ceiling of the room, which gave an even illumination of 230 lux over the whole of the olfactometer apparatus. The room temperature was within the range 20–25°C.

Odorless control tests were performed with the weevils to check for any directional bias in the olfactometer chamber and to test that the cleaning of the chamber after an odor treatment was effective. Equal numbers of weevils were used for each of the four replicates in each treatment (with fresh weevils for each replicate), but the numbers between treatments varied from 20 to 50 per replicate due to the availability of weevils.

The responses of seed weevils to conspecific odor was studied by using 20 individuals of each sex as the odor source. The position of the weevils in the olfactometer chamber was recorded at 5-min intervals for 30 min. The number of weevils that had passed into the tube leading to the insect collection flask or that were congregated around the entry to the tube were recorded as those exhibiting movement to the odor source, "attraction" as defined by Dethier et al. (1960). Any positional bias of the olfactometer chamber was overcome by rotating the chamber through 90° after each replicate, thus providing four replicates for each odor tested. After each replicate the weevils were removed from the chamber and replaced by an equal number of naive weevils. After each set of four replicates, the olfactometer chamber, glassware, and tubing were washed with 70% industrial methylated spirit and soaked in Decon overnight.

Laboratory Choice Tests. In choice tests within an evenly illuminated insect cage (50 × 50 × 50 cm), single-sex overwintered, unmated seed weevils were given a choice of four perforated white plastic circular containers (7 cm diam. × 6 cm height) in a square design, two containers containing no weevils, and two containing 10 unmated female weevils or 10 unmated male weevils. New-generation male seed weevils were given a choice of two empty containers and two containers containing new-generation female or male seed weevils. The number of weevils on or within 1 cm of each container after 10 min were

recorded. Thirty weevils were released at a time, with four replicates of each test, the position of the containers being moved between each replicate.

Baited Sticky Traps. Within a week after the arrival of overwintered seed weevils at a flowering oilseed rape crop, sticky traps baited with unmated female weevils (six traps), unmated male weevils (six traps), or no weevils (controls, six traps), were placed in the headlands of a rape crop. The sticky traps consisted of 14 × 10-cm transparent sheets covered with Tanglefoot (The Tanglefoot Company, Grand Rapids, Michigan) on both sides. Live weevils ($N = 5$) were placed in small cotton bags tied to the top of the traps containing a food source of rape flowers and pods. The unbaited (control) traps also contained the food source. The traps were collected a week later and the weevils caught on the traps counted and sexed. In most cases the weevils in the bags were still alive when the traps were collected.

Statistical Analysis of Data. In the olfactometer tests, the mean number of weevils in each odor field every 5 min over a 30-min period can be assumed to be an indication of how often the weevils were visiting a particular odor field. Analysis of odorless control replicates showed no significant difference in the responses of the weevils to the position of the olfactometer chamber. Thus, data from each of the four replicates was pooled to obtain the overall mean response of the weevils to the test odor.

The null hypothesis was that if there was no effect of test odor, the mean numbers of weevils attracted to the olfactometer arm containing the odor would be equal to the mean numbers attracted to each of the other olfactometer arms, i.e., $\mu_{\text{Arm-1}} = \mu_{\text{Arm-2}}$, $\mu_{\text{Arm-3}}$, and $\mu_{\text{Arm-4}}$, where Arm-1 is the olfactometer arm providing the odor stimulus, and arms 2, 3, and 4 provide no odor (filtered air).

A one-way analysis of variance of the means was carried out, and if evidence of significant differences between the means were present ($P < 0.001$), Tukey's honestly significant difference method (or T-method) was applied to compare the means (Sokal and Rohlf, 1981). This test calculates the 95% comparison intervals for each mean, so that if the mean and comparison intervals of $\mu_{\text{Arm-1}}$ do not overlap with any of those of $\mu_{\text{Arm-2}}$, $\mu_{\text{Arm-3}}$, and $\mu_{\text{Arm-4}}$, then, $\mu_{\text{Arm-1}} \neq \mu_{\text{Arm-2}}$, $\mu_{\text{Arm-3}}$, and $\mu_{\text{Arm-4}}$ ($P < 0.05$).

The results of the choice tests using the white plastic containers were analyzed by χ^2, assuming and equal distribution of weevils if there were no difference between treatments. The field trap data was analyzed by analysis of variance and Student's t tests (Sokal and Rohlf, 1981).

EAGs. EAGs were obtained using the method of Evans and Allen-Williams (1992). The EAG responses were recorded and analyzed using the PC-EAG recording and evaluating system (Syntech, Hilversum, The Netherlands). Several hexane (BDH Chemicals Ltd., Poole, England) extracts of weevils of both overwintered and new generations were obtained: OFW, overwintered female weevils ($N = 20$), which were kept in a glass tube (2 cm diam. × 10 cm) for

24 hr after which the tube walls washed with 1 ml of hexane; OMW, overwintered male weevils prepared in the same way as OFW; NFW, new-generation female weevils prepared in the same way as OFW; NMW, new-generation male weevils prepared in the same way as OFW; LOFW, live overwintered female weevils ($N = 290$) suspended in 1 ml hexane for 72 hr; LOMW, live overwintered male weevils ($N = 246$) prepared as LOFW; LNFW, live new-generation female weevils ($N = 190$) prepared as LOFW; LNMW, live new-generation male weevils ($N = 224$) prepared as for LOFW; HOFW, live overwintered female weevils ($N = 198$) homogenized in 1 ml hexane and filtered; and HOMW, live overwintered male weevils prepared as for HOFW.

The EAG responses to the weevil extracts (measured as maximum amplitude in millivolts) were expressed as a percentage of the EAG response to the standard compound 10^{-2} *cis*-hex-3-en-1-ol (C3H), which has been used previously as a standard in the EAGs of seed weevils (Kozlowski, 1984; Evans and Allen-Williams, 1992). Weevil antennae were treated every three stimulations with the C3H standard, and the amplitude of the response of the weevil extracts expressed as a percentage of the mean of the two adjacent standard response amplitudes (Guerin and Visser, 1980). Six replicates were obtained for each sex and generation of weevil tested.

RESULTS

Olfactometer Tests. Seed weevils were very active within the olfactometer, and very often an individual weevil would wander throughout the whole olfactometer chamber during the 30-min test. The responses of male and female seed weevils in control tests (where only filtered air was presented) indicated that the null hypothesis of equal choice of each odor field of the olfactometer was supported.

Preliminary observations of overwintered seed weevils indicated a high level of sexual activity (copulation) between weevils, and initially, when weevils of both sexes were used together in the olfactometer control (air only) studies, aggregation of copulating weevils was seen (Evans, unpublished observations). This aggregation was absent when males and females were tested separately. When the response of overwintered male weevils to overwintered female weevil odor was tested in the olfactometer, a significant level of attraction to the source of the female odor was obtained (Figure 1). Female weevils were unresponsive to male odor (Figure 1).

Later in the season when the new generation of weevils had emerged at the rape crop, the response of male weevils to female odor was again tested. In this instance no behavioral response was observed, and when males and females were observed together, no copulation was observed.

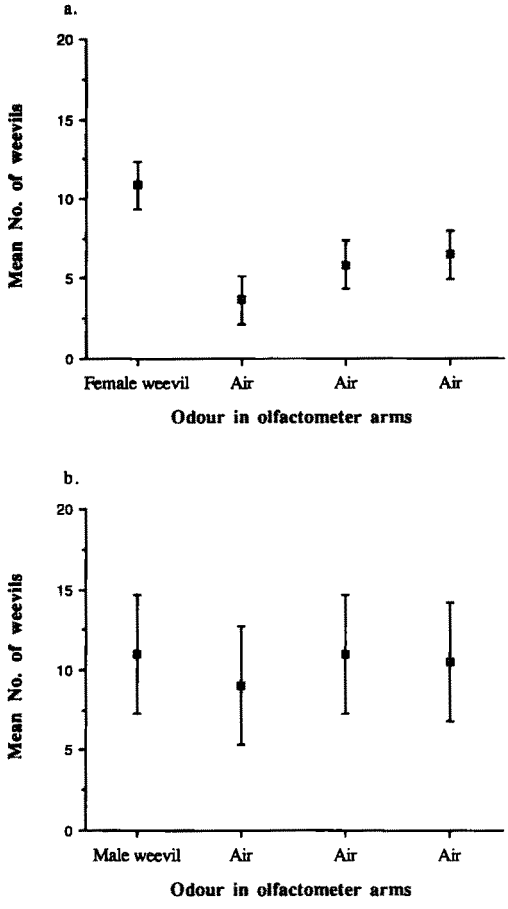

FIG. 1. Response of male seed weevils to the odor of unmated overwintered female weevils (a) and the response of female seed weevils to the odor of unmated overwintered male weevils (b) in a four-choice olfactometer (mean of four replicates). Error bars refer to the 95% comparison intervals obtained from Tukey's T-method (Sokal and Rohlf, 1981). Overlapping comparison intervals denote no significant difference in choices of olfactometer arms, whereas no overlap of comparison intervals indicates a significant difference between choices of olfactometer arms ($P < 0.05$).

Choice Tests. The response of overwintered and new-generation weevils to conspecific odor in choice tests is summarized in Table 1. There was a significant level of attraction of overwintered generation unmated male seed weevils to the containers containing unmated female weevils ($P < 0.001$, χ^2

analysis, Table 1); unmated female weevils were not attracted to the odor of male weevils. However, female weevils were attracted to the odor of unmated female weevils ($P < 0.001$, Table 1). New-generation male seed weevils exhibited no detectable response to the odor of new-generation female weevils, and new-generation females were not attracted to the odor of other new generation females (Table 2).

Weevil-Baited Traps. The results from the weevil-baited sticky traps are shown in Table 3. Significantly more male weevils ($P < 0.025$) were captured on traps baited with female weevils than the unbaited and male-weevil-baited traps. There were also significantly more female weevils ($P < 0.025$) caught on traps baited with female weevils than the male-weevil-baited traps (Table 3).

EAGs. Significant EAG responses were only obtained from overwintered seed weevils in response to extracts from overwintered female weevils (Figure 2). There was no significant EAG response from new-generation seed weevils to any of the extracts tested. EAGs obtained from overwintered male weevils in response to hexane extracts from overwintered females were significantly

TABLE 1. RESPONSES OF OVERWINTERED SEED WEEVILS TO CONSPECIFIC WEEVIL ODOR IN CHOICE EXPERIMENT

Sex of weevil	Test odor	No. reacting[a]	No. reacting to test odor
Male	Male weevil	61	33
	Female weevil	103	86[b]
Female	Male weevil	52	23
	Female weevil	90	61[b]

[a] Combined data from four replicates, $N = 120$.
[b] $P < 0.001$ determined by χ^2 analysis.

TABLE 2. RESPONSES OF NEW-GENERATION SEED WEEVILS TO CONSPECIFIC WEEVIL ODOR IN CHOICE EXPERIMENT

Sex of weevil	Test odor	No. reacting[a]	No. reacting to test odor
Male	Male weevil	60	27
	Female weevil	59	36
Female	Male weevil	63	35
	Female weevil	59	26

[a] Combined data from four replicates, $N = 120$.

TABLE 3. MEAN NUMBERS OF SEED WEEVILS CAUGHT ON STICKY TRAPS BAITED WITH LIVE WEEVILS

Bait in trap	Weevils caught (mean ± SE)[a]	
	Males	Females
Unbaited	0.80 ± 0.37b	1.60 ± 0.40bc
Male weevils	2.00 ± 0.55b	0.80 ± 0.58b
Female weevils	7.00 ± 1.30c	3.40 ± 0.75c

[a] Means in each column followed by the same letter are not significantly different, $P < 0.025$, Student's t test.

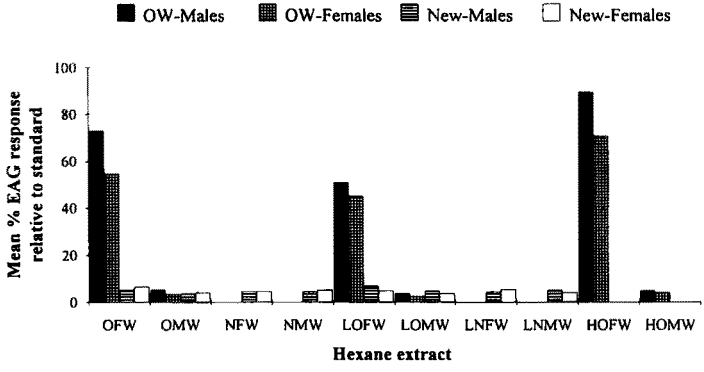

FIG. 2. Mean percent EAG response (relative to the standard of 10^{-2} *cis*-hex-3-en-1-ol) of seed weevils to several hexane extracts of overwintered (OW) and new-generation weevils. $N = 6$ for each sex and generation of weevil tested. See Methods and Materials for description of the hexane extracts.

stronger than EAGs from overwintered females ($P < 0.05$, analysis of variance) except for the LOFW extract. The homogenized hexane extract of overwintered female weevils (HOFW) was the most stimulatory extract (Figure 2).

DISCUSSION

Overwintered male seed weevils are attracted to the odor of sexually active overwintered female weevils and exhibit significant EAG responses to hexane extracts of overwintered females. New-generation male weevils were not attracted to the odor of new-generation females or exhibit EAG responses to hexane extracts of these female weevils. New-generation weevils do not mate until they

have overwintered (Williams and Free, 1978). The results obtained suggest that there is an attractive volatile compound or compounds associated with female weevils that are used in sexual communication.

Overwintered female weevils were not attracted to the odor of overwintered or new-generation male weevils, but in the choice tests using baited containers (Table 1) and in the field traps (Table 3), they were attracted to the odor of overwintered unmated female weevils. As overwintered females also exhibited significant EAG responses to hexane extracts of overwintered females (Figure 2), the chemical(s) emitted by overwintered female seed weevils may be involved in aggregating female weevils as well as attracting male weevils for mating.

Seed weevils possess olfactory receptors on their antennae that are sensitive to odors associated with their host plants (Blight et al., 1989; Evans and Allen Williams, 1992), but no specific receptors have as yet been shown to respond to conspecific odor. Seed weevils have been shown to use an oviposition-deterring pheromone to prevent larval competition for food (Kozlowski et al., 1983; Ferguson and Williams, 1991), but this pheromone is thought to be detected by contact chemoreceptors on the antennae and to be of relatively low volatility (Ferguson and Williams, 1991).

Sex pheromones identified in other weevil species tend to be produced by the female weevil (Trung et al., 1988; Mason et al., 1990), whereas aggregation pheromones are usually produced by males only (Dickens, 1984; Blight and Wadhams, 1987; Trung et al., 1988; Roseland et al., 1990; Rochat et al., 1991, 1993; Weissling et al., 1993).

Seed weevils feed for several days after emergence from overwintering (Dmoch, 1965; Williams and Free, 1978; Ni et al., 1990). During this time the weevils are likely to disperse from their emergence site in search of cruciferous food plants. The possession of a sex pheromone released when female weevils are sexually mature would increase the likelihood of mate location. As the chemical emitted by female weevils also attracts female weevils to some extent as well as males, then the aggregation of both sexes would lead to greater mating success.

The fact that the sexually inactive new-generation weevils are not attracted to each other suggests that the aim of the pheromone emitted by overwintered female weevils is to increase the chance of successful mating rather than attracting weevils to a patch of food. Male and female weevils are attracted to host-plant odor from a distance of at least 20 m over open ground (Evans and Allen-Williams, 1993), so the use of the pheromone for attraction to a food source is unlikely.

Scanning electron microscopy of weevil antennae (Evans, 1991) indicated several types of sensilla, some of which are similar in morphology to sensilla in other weevil species that have been shown to be pheromone receptors (Mustaparta, 1975). There appeared to be no difference in antennal morphology

between male and female weevils (Evans, 1991); however, further research needs to be carried out to determine whether there are separate sensilla for pheromone and host-plant odor perception on seed weevil antennae.

Identification and synthesis of the sex or aggregation pheromone in seed weevils will enable pheromone-baited traps to be used to monitor the arrival of seed weevils at the rape crop in the same way that the aggregation pheromone-baited traps are used to monitor spring dispersal of *S. lineatus* (Nielsen and Jensen, 1993). Use of yellow water traps baited with rape extracts, while trapping large numbers of seed weevils as they arrive at the crop (Evans and Allen-Williams, 1989), are not species-specific and much time is spent sorting through the trap catches, whereas a pheromone trap would be species-specific.

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LEAF SURFACE EXTRACTS OF *Solanum berthaultii* HAWKES DETER COLORADO POTATO BEETLE FEEDING

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Abstract—Leaf rinses of *S. berthaultii* PI 473334 with methylene chloride were deterrent to feeding by the Colorado potato beetle when applied to *S. tuberosum* tuber and leaf disks. When the leaf rinse was separated into its nonvolatile and volatile fractions and applied to tuber disks, the nonvolatile fraction was highly deterrent, while the volatile fraction reduced consumption, but not significantly compared to the controls. A hexane leaf rinse was not deterrent to feeding, while an acetone rinse was approximately twofold more deterrent than the methylene chloride rinse when applied to leaf disks. Three cycles of bioassay-guided, reversed-phase open-column fractionation of an acetone leaf rinse yielded a relatively polar fraction with low deterrent activity, and two nonpolar fractions exhibiting higher specific activity. Reversed-phase preparative HPLC of these fractions yielded seven active fractions among the 10 assayed. Subsequent analytical HPLC indicated that two fractions each contained a single UV-absorbing compound, while another represented a mixture of at least four compounds. The remaining fractions were composed of complex mixtures of possibly ionic or polymeric compounds that were poorly resolved by HPLC.

Key Words—*Leptinotarsa decemlineata*, Colorado potato beetle, Coleoptera, Chrysomelidae, *Solanum berthaultii*, glandular trichomes, feeding deterrents, host preference, plant resistance to insects.

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INTRODUCTION

The wild Bolivian potato, *Solanum berthaultii* Hawkes, is resistant to a number of arthropod pests including mites, leafminer flies, the potato tuber moth complex, aphids, leafhoppers, fleabeetles, and the Colorado potato beetle (Gibson, 1971; Tingey and Gibson, 1978; Casagrande, 1982; Lapointe and Tingey, 1984; Tingey and Laubengayer, 1986; Neal et al., 1989, 1990; Tingey, 1991). Resistance of *S. berthaultii* is associated with the type A and B glandular trichomes present on the foliage of this plant. The morphological and physiochemical insect defensive attributes of the trichomes have been previously described (Gregory et al., 1986; King et al., 1987; Steffens and Walters, 1991; Tingey, 1991). Several studies have elucidated the physical and chemical factors associated with the type A and B glandular trichomes that result in the feeding deterrence, entrapment, and immobilization of small-bodied insects such as aphids and leafhoppers (Tingey et al., 1982; Lapointe and Tingey, 1984; Avé et al., 1987; Neal et al., 1990; Kowalski et al., 1992). However, for the Colorado potato beetle, which is much larger and capable of maneuvering through the mechanical/adhesive barrier presented by the trichomes, the factors that impart resistance are less understood (Dimock and Tingey, 1988).

Resistance of *S. berthaultii* to the Colorado potato beetle is associated with the type A and B glandular trichomes (Dimock and Tingey, 1988; Neal et al., 1989; Pelletier and Smilowitz, 1990). Neal et al. (1989) postulated that the type A trichomes were required for the expression of resistance, while the sucrose esters produced by the type B trichomes enhanced resistance in the presence of the type A trichomes. Pelletier and Smilowitz (1990) demonstrated that methylene chloride leaf rinses from *S. berthaultii* deterred Colorado potato beetle feeding when they were applied to *S. tuberosum* leaflets. However, differences in the deterrent activity of leaf rinses obtained from sibling clones of *S. berthaultii*, which both had type A trichomes but were segregated for the presence or absence of secretory type B trichomes, suggested that *S. berthaultii* possessed more than one mechanism of resistance to the Colorado potato beetle (Pelletier and Smilowitz, 1990).

In order to better understand the nature of glandular trichome-mediated resistance to the Colorado potato beetle, we have focused our studies on *S. berthaultii* accession PI 473334. This accession bears only type A trichomes and is among the most resistant accessions of *S. berthaultii* identified to date (Flanders and Radcliffe, 1992; W.M.T., personal observations). Previous studies have demonstrated that removing the type A trichomes from PI 473334 results in increased adult feeding initiation, preference, and consumption of foliage in choice and no-choice situations. Furthermore, when foliage of PI 473334 was appressed to *S. tuberosum* leaflets, which transferred the contents of the type A trichomes to the *S. tuberosum* leaflets, fewer adults fed on the

appressed leaflets (Yencho and Tingey, 1994). These studies indicated that *S. berthaultii* PI 473334 possessed compounds deterrent to Colorado potato beetle feeding and that the deterrents were associated with the outer epidermis of the plant.

This report describes the extraction, fractionation, and partial characterization of a complex of feeding deterrents from the foliage of *S. berthaultii* PI 473334. Information about the nature of the feeding deterrents present in *S. berthaultii* will facilitate their identification and site of production. This information can be used to increase our understanding of the relative importance of the type A and B trichomes in mediating resistance to the Colorado potato beetle. In addition, knowledge of the active components of resistance will facilitate the development of biochemical assays to identify resistant progeny in segregating populations and will provide useful information for future studies of the molecular genetic basis of glandular trichome-mediated insect resistance.

METHODS AND MATERIALS

Insects

A colony of Colorado potato beetles was established by collecting overwintered adults (> 100) from the Cornell University, H.C. Thompson Vegetable Research Farm, Freeville, New York. The laboratory colony was reared on *S. tuberosum* (cv. Atlantic) in a greenhouse programmed to provide a 16-hr photoperiod and a daily temperature and relative humidity ranging from 20°C to 27°C, and 50% and 80%, respectively. The natural lighting of the greenhouse was supplemented by 1000-W metal halide multivapor growth lamps. The colony was reared in 0.7-m^3 cages. Prepupae were collected from the cages, placed in 31-cm × 16-cm × 8-cm plastic boxes containing moistened Pro-Mix BX soil mix (Premier Brands Inc., Stamford, Connecticut) and transferred to a model E-30B Percival Growth Chamber (Percival Manufacturing Co., Boone, Iowa) illuminated with fluorescent lights set to the same photoperiod and a constant temperature and relative humidity of 27°C and ca. 65%, respectively. Adults were collected within 24 hr of emergence, transferred to plastic boxes containing moistened cellulose sponges, and held in the growth chamber for up to 48 hr prior to bioassay.

Plants

Plants of *S. tuberosum* (cv. Atlantic) were grown from certified seed tubers. *S. berthaultii* accession PI 473334 was grown first from botanical seed obtained from the USDA Potato Introduction Station, Sturgeon Bay, Wisconsin. These

plants were then vegetatively propagated to ensure that the leaf rinses were obtained from a uniform population. All plants were grown in Pro-Mix BX soil mix and fertilized with 20–10–20 Peters Pete-Lite Professional (W.R. Grace and Co., Fogelsville, Pennsylvania) liquid fertilizer weekly. The natural lighting of the greenhouse was supplemented by metal halide growth lamps similar to those of the insect colony.

Bioassays

Two bioassay procedures were developed for these experiments. The tuber disk bioassay was used to establish an initial leaf rinse activity curve and to determine if the deterrent factor was volatile. The leaf disk bioassay was used to further define the activity of the leaf rinse, to determine if the deterrent components were associated with the external or internal regions of the leaves, and to examine the deterrent activity of the nonvolatile fractions.

All bioassays were conducted with unfed adults obtained within 24–48 hr of emergence. The tuber and leaf disk bioassays were conducted for 24 hr and 4 hr, respectively, in a Scientific Systems, TWINcubator plant growth chamber (Scientific Systems Corporation, Baton Rouge, Louisiana) illuminated with a 400-W metal halide lamp set to a 16-hr photoperiod and a constant temperature and relative humidity of 27°C and ca. 65%, respectively.

Tuber Disk Bioassay

The tuber disk assay employed the tissue of lyophilized potato tubers, which provided a porous medium that was readily accepted by adults and was compatible with a variety of polar and nonpolar solvents. To prepare the tuber disks, *S. tuberosum* (cv. Superior) tubers were removed from 5°C cold storage, washed, and bored with a No. 13 (2 cm ID) cork borer. The cores were cut into a 3-cm-diameter × 4-mm disks using a Cuisinart DLC-8 Plus food processor fitted with a DLC-844 blade (Cuisinarts Inc., Greenwich, Connecticut). The disks were transferred to 15-cm × 3-cm polystyrene Petri dishes and frozen overnight at −80°C. They were then dried under vacuum in a Labconco model 75015 lyophilizer (Labconco Corp., Kansas City, Missouri) for ca. 48 hr and stored in a vacuum desiccator until use.

The tuber disks were removed from the desiccator ca. 30 min prior to use and allowed to equilibrate to ambient humidity. They were then weighed to eliminate unusually light or heavy disks, transferred to aluminum weighing trays and treated with leaf rinse extracts dissolved in 200–400 μl methylene chloride. Controls were treated with solvent. The solvent was evaporated from the tuber disks under ambient conditions for ca. 30 min and the tuber disks were reweighed and placed into 6-cm × 1.5-cm Petri dishes containing a moistened cotton dental wick. Two adults (one female, one male) were placed in each dish and the

dishes were transferred to the incubator. The adults and cotton wicks were removed after 24 hr, and the dishes were dried in the lyophilizer for about 1 hr. The tuber disks were then examined visually for evidence of feeding, and the percent adults initiating feeding was calculated for each treatment by dividing the number of disks for which feeding was evident by the number of disks prepared. Feeding by the adults resulted in the formation of three distinct fractions: the partly consumed tuber disk; pellets of frass produced by the adults; and dust produced by adult biting and feeding on the semibrittle tuber disks. The frass was separated from the dust by sieving the fractions through No. 25 (0.7 mm) and No. 36 (0.6 mm) U.S.A. Standard Testing Sieves (W.S. Tyler, Inc., Mentor, Ohio), which retained the frass. Each fraction was weighed to the nearest 0.1 mg and the amount consumed was calculated as: mean consumption $= w_i - (w_f + w_d)$, where w_i was the postapplication disk weight, w_f the postfeeding disk weight, and w_d the weight of the dust fraction.

Leaf Disk Bioassay

Leaves of *S. tuberosum* (cv. Atlantic) were excised from greenhouse-grown plants and their petioles immediately immersed in tap water. Leaf disks were cut from the foliage with a No. 13 (2 cm ID) cork borer. The leaf rinse extracts were dissolved in either 100 μl acetone or 200 μl 3:1 methanol–water, while the control leaf disks were treated with only solvent. The treatments were applied to the disks with a 200-μl micropipet. The solvent was evaporated from the leaf disks under ambient conditions for ca. 20 min, and the disks were transferred to 6-cm × 1.5-cm Petri dishes containing moistened Whatman No. 1 filter paper. A single female or male was placed in each dish and the dishes transferred to the incubator. Ten females and 10 males were generally used for each bioassay. The amount of leaf disk remaining after 4 hr of feeding was determined with a Li-Cor model LI-3000 Leaf Area Meter equipped with a model LI-3005A Belt Conveyor (Li-Cor, Inc., Lincoln, Nebraska). Consumption was calculated by subtracting the amount of leaf disk remaining from the mean of a duplicate set of solvent-treated disks prepared to account for shrinkage during the assay.

Leaf Extraction and Fractionation Procedures

Volatile and Nonvolatile Deterrents. Leaves of *S. berthaultii* PI 473334 (1000 g fresh weight) were obtained from the upper half of prebloom greenhouse grown plants. The leaves were rinsed in groups of three to five for 10, 10, and 5 sec in three 250-ml volumes of ice-chilled methylene chloride. The rinses were pooled, filtered through glass fiber filter paper, and concentrated to ca. 5 ml under reflux in a round-bottom flask immersed in a 55–60°C water bath. The concentrated leaf rinse was transferred to a Hickman still and the remaining solvent distilled into a collection flask under low heat (ca. 40°C). The volatile

and nonvolatile components of the mixture were separated by removing the collection flask and applying a vacuum (760 mm Hg) to the still for 1.5 hr, which condensed the volatiles on a Dry Ice-chilled cold finger (i.e., short-path vacuum distillation). The volatile condensate was collected by removing the vacuum, reattaching the collection flask to the still and refluxing with methylene chloride (ca. 40°C) to wash the volatiles condensed on the cold finger into the flask. Because complete evaporation of the raw and volatile fractions risked the loss of volatiles, these fractions were evaporated to ca. 3 ml under reflux. The raw leaf rinse was then prepared to provide concentrations equivalent to 0.3, 1, 3, 5, and 8 gram leaf equivalents (gle)/tuber disk (e.g., 1 gle/tuber disk was equivalent to 100 g fresh weight of rinsed foliage in 10 ml solvent, with 100 μl of this being applied to a tuber disk), while the volatile and nonvolatile fractions were adjusted to 8 gle, and each was subjected to tuber disk bioassay.

Effect of Solvent on Deterrent Extraction. Six hundred grams of *S. berthaultii* PI 473334 leaves were collected from the upper half of prebloom greenhouse-grown plants and separated into three samples of 200 g each. The leaves from each sample were rinsed as described above in acetone, methylene chloride, or hexane. After rinsing, the leaves were flattened and placed on paper towels to determine the total leaf area rinsed. The rinses were chilled to precipitate waxes, vacuum filtered through Whatman No. 1 qualitative filter paper, evaporated at 50°C, and weighed to determine the amount of material removed per unit area. Each sample was dissolved in acetone and subjected to leaf disk bioassay at 83 $\mu g/cm^2$, which represented an amount equivalent to ca. 3.5 gle applied per leaf disk.

Internal and External Deterrents. Six hundred grams of *S. berthaultii* PI 473334 leaves were collected as described above and separated into two samples of 300 g each. The leaves from one sample were rinsed as above in acetone, flattened, and placed on paper towels for area determinations. The second sample was processed similarly; however, after rinsing, the leaves were immediately macerated in a Waring blender containing ca. 500 ml acetone. The leaf rinse (external extract) was vacuum filtered through a Whatman No. 1 qualitative filter, and evaporated at 50°C. The macerated extract (internal extract) was vacuum filtered through glass wool and Whatman No. 1 qualitative filter paper, evaporated to remove the acetone, and the aqueous portion (ca. 50 ml) was extracted 3× with 100 ml of chloroform and the organic phase retained and evaporated. The internal and external fractions were subjected to leaf disk bioassay at 83 $\mu g/cm^2$ per disk.

Fractionation of Feeding Deterrents. A *S. berthaultii* PI 473334 acetone leaf rinse was separated into fractions using the protocol illustrated in Figure 1. The leaf rinse was chilled to precipitate waxes, vacuum filtered through Whatman No. 1 qualitative filter paper, evaporated to dryness at 50°C, dissolved in methanol, and separated into fractions by nitrogen-pressurized flash chromatog-

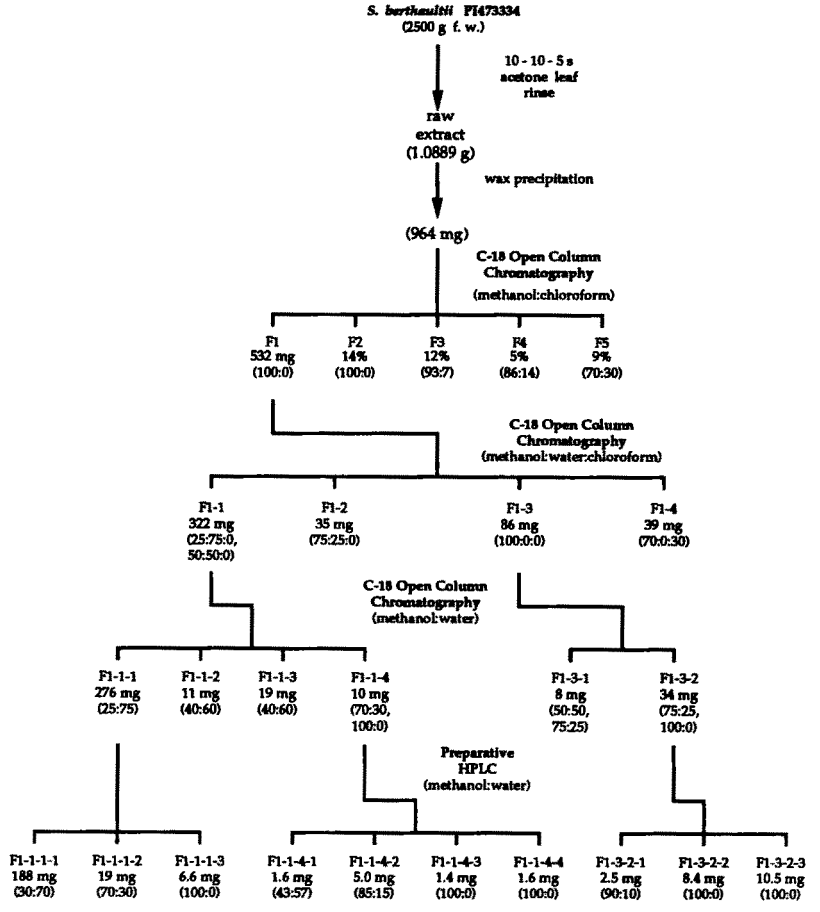

FIG. 1. Fractionation procedure used to separate Colorado potato beetle feeding deterrents from *S. berthaultii* PI 473334 foliage. Yields obtained at each step and approximate elution concentrations are given. All fractions were tested at a concentration of 10 gle, with the exception of C_{18} chromatography step 2, which was tested at 3 gle. Ithaca, New York, 1992.

raphy on a 40-μm bead size, 2.5-cm × 25-cm Bakerbond C_{18} open column (J.T. Baker Chemical Co., Phillipsburg, New Jersey), and eluted with methanol, water, and chloroform in varying ratios (Figure 1). Subfractions (15–20 ml) were collected as the material eluted from the column. These were evaporated, and an aliquot of each was applied to 20-cm × 20-cm, precoated, fluorescent indicator silica gel thin-layer chromatography (TLC) plates (Kieselgel 60 F-254,

220-μm layer thickness; EM Laboratories, Inc., Elmsford, New York). The TLC plates were developed in methanol–chloroform (3:97) and fractions grouped according to similar solvent mobilities of the UV-quenching constituents present in each subfraction. The resulting fractions were dissolved in acetone and methanol–acetone (13:87) for the first and second leaf disk bioassays, respectively; all others were in methanol–water (75:25). The leaf disks were treated with 10 gle of leaf extract in all bioassays excepting the second C_{18} open-column chromatography step, which was treated with 3 gle (Figure 1).

The deterrent fractions were then subjected to reversed-phase high-pressure liquid chromatography (HPLC) on a 50-cm × 8-mm Micropak MCH-10 C_{18} preparative column (Varian, Palo Alto, California) with a Varian model 5000 HPLC, and a Waters model 490 Programmable Multiwavelength Detector set to a wavelength of 220 nm. The mobile phase was methanol–water and the flow rate was 3 ml/min. Solvent conditions for the fractions were as follows: fraction F1-1-1, 0–5 min 100% H_2O, a linear gradient reaching 100% CH_3OH at 30 min, and 30–60 min 100% CH_3OH; fraction F1-1-4, 0 min 25% CH_3OH in water, a linear gradient reaching 100% CH_3OH at 30 min, and 30–40 min 100% CH_3OH; and fraction F1-3-2, 0–10 min 80% CH_3OH in water, a linear gradient reaching 100% CH_3OH at 20 min, and 20–30 min 100% CH_3OH.

The most active fractions identified by preparative HPLC were subjected to analytical HPLC using a 4.6-mm × 25-cm, 5-μm Beckman Ultrasphere ODS analytical column (Beckman, San Ramon, CA) attached to a Varian Vista 5500 liquid chromatograph and a Hewlett-Packard Diode Array Detector with monitoring at 220 nm and 254 nm. The mobile phase was acetonitrile–water and the flow rate was 1 ml/min. The solvent conditions were: 0–2 min 100% H_2O, a linear gradient reaching 100% acetonitrile at 20 min, and 20–30 min 100% acetonitrile.

RESULTS

Volatile and Nonvolatile Deterrents. Increasing concentrations of *S. berthaultii* PI 473334 methylene chloride leaf rinses applied to tuber disks resulted in decreased adult consumption (Figure 2). When the leaf rinse was separated into its volatile and nonvolatile components by fractional distillation and tested at a concentration of 8 gle, the nonvolatile fraction deterred feeding, but it was not as deterrent as the raw extract, while the volatile fraction tended to decrease consumption (ca. 15%) but not significantly ($P < 0.1987$) when compared to the control (Table 1). Adults feeding on tuber disks treated with the nonvolatile and raw leaf rinse extracts produced less frass compared to those feeding on the volatile and control tuber disks.

Tuber and Leaf Disk Activity Curves. As observed with the tuber disks,

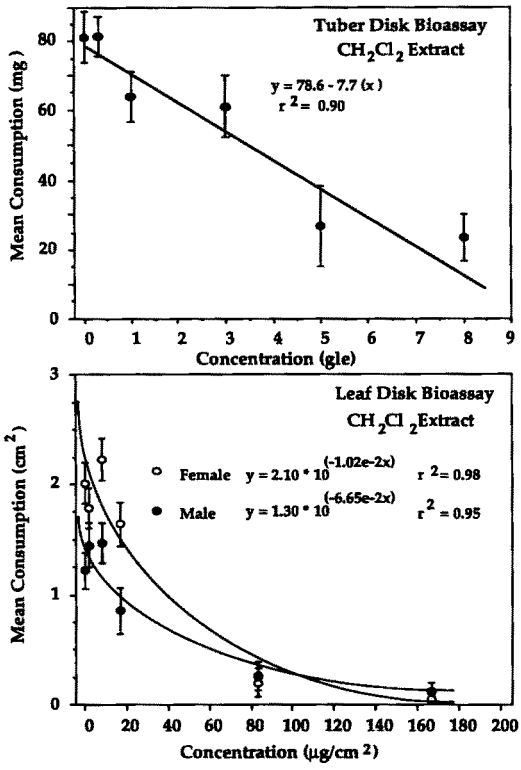

FIG. 2. Mean consumption of adult Colorado potato beetles exposed to tuber and leaf disks treated with *S. berthaultii* PI 473334 methylene chloride (CH_2Cl_2) leaf rinses. The gle concentration of extract applied to the tuber disks approximates the concentration of extract applied per leaf disk. Vertical bars are standard error of the mean. Ithaca, New York, 1992.

adult feeding decreased when methylene chloride leaf rinses were applied in increasing concentrations to leaf disks. However, the response curves differed considerably (Figure 2). For the leaf disks, consumption decreased exponentially, compared to a linear decrease in consumption observed for the tuber disks. Furthermore, compared to the tuber disk assay, the variability in leaf disk feeding was greatly reduced, as is indicated by the width of the SEM bars and the higher coefficients of determination for females and males, respectively.

Solvent Extract Comparisons. The hexane, methylene chloride, and acetone leaf rinses removed 8, 9, and 6 $\mu g/cm^2$ of surface components, respectively. When applied at a concentration of 83 $\mu g/cm^2$, the hexane leaf rinse was not

deterrent, and the acetone leaf rinse was ca. twofold more deterrent than the methylene chloride rinse (Figure 3). When leaf disks were treated with external and internal acetone leaf extracts obtained from identical *S. berthaultii* PI 473334 leaves and applied at 83 $\mu g/cm^2$, adults fed less on the disks treated with the external extract, while the internal extract stimulated feeding (Figure 4).

Deterrent Fractionation. Preliminary fractionation of the *S. berthaultii* PI 473334 acetone leaf rinse by reversed-phase open-column chromatography with methanol–chloroform (Figure 1 and Table 2) indicated that the most polar fraction (F1) possessed significant ($P < 0.05$) deterrent activity when assayed at 10 gle. Refractionation with methanol–water–chloroform yielded two equally active fractions: F1-1 and F1-3. The most polar fraction, F1-1, represented 67% of the total material recovered. However, fraction F1-3, which comprised 18% of the total weight of the fraction and eluted from the column with 100%

TABLE 1. EFFECT OF VOLATILE AND NONVOLATILE *S. berthaultii* PI 473334 LEAF RINSE FRACTIONS APPLIED TO LYOPHILIZED POTATO TUBER DISKS ON CONSUMPTION BY ADULT COLORADO POTATO BEETLES[a]

Fraction	*N*	Initiating feeding (%)	Amount consumed (mg)	Frass (mg)
Control	30	100	69 ± 3 a	39 ± 2 a
Nonvolatile	30	93	42 ± 5 b	25 ± 3 b
Volatile	20	100	59 ± 4 a	33 ± 3 b
Raw Extract	30	67	26 ± 5 c	15 ± 4 c

[a]Means ± standard error followed by the same letter in a column are not significantly different ($P \leq 0.05$, Fisher's protected LSD).

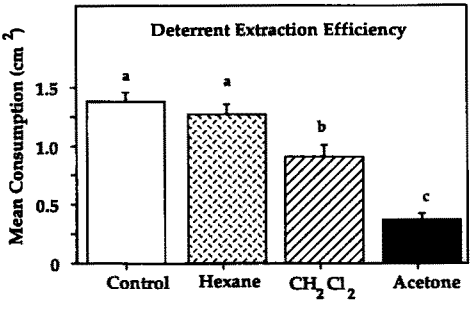

FIG. 3. Effect of hexane, methylene chloride (CH_2Cl_2), and acetone leaf rinses on adult Colorado potato beetle feeding deterrent extraction. Extracts were tested at 83 $\mu g/cm^2$. Vertical bars are standard error of the mean. Ithaca, New York, 1992.

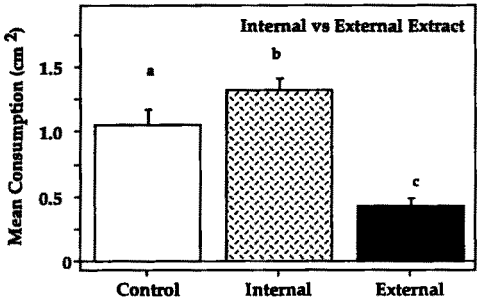

FIG. 4. Effect of internal and external *S. berthaultii* PI 473334 acetone leaf extracts on adult Colorado potato beetle consumption. Extracts were tested at 83 $\mu g/cm^2$. Vertical bars are standard error of the mean. Ithaca, New York, 1992.

methanol, had approximately 27-fold higher specific activity compared with the former when the amount of material applied per unit area was calculated. Fraction F1-3 was separated into two fractions. The second of these, F1-3-2, eluted from the column in 100% methanol, contained 80% of the material recovered and was deterrent at ca. 37 $\mu g/cm^2$. Fraction F1-1 was separated into four fractions, and two deterrent fractions were identified. Fraction F1-1-1 eluted from the column at 25:75 methanol–water, and F1-1-4 eluted from the column with addition of 100% methanol. These fractions were equally deterrent at 10 gle. However, the specific activity of fraction F1-1-4 (Figure 1) was much greater because F1-1-4 was tested at a concentration of ca. 11 $\mu g/cm^2$, while F1-1-1 was tested at ca. 300 $\mu g/cm^2$. The three most deterrent fractions, F1-1-1, F1-1-4, and F1-3-2, were further separated using preparative reversed-phase HPLC, yielding seven fractions that were deterrent to feeding (Figure 1). All the deterrent fractions eluted from the column at concentrations of aqueous methanol greater than 70%, and they were deterrent at rates between 4 $\mu g/cm^2$ and 25 $\mu g/cm^2$.

Examination of the UV spectra of the predominant compounds in F1-3-2-1 and F1-3-2-3 (Figure 1) (retention times of 20.14 and 26.33 min, respectively) revealed distinct similarities. Absorption maxima at 265 nm for both compounds and minima at 230 nm (F1-3-2-1) and 240 nm (F1-3-2-3), suggested the presence of an aromatic structure possibly differing in ring substitution. The UV spectra of the predominant compounds in F1-3-2-2, which eluted between F1-3-2-1 and F1-3-2-3, indicated that two of the five constituents (retention times of 23.17 and 26.07 min) were related and that these were also aromatic. The remaining fractions were composed of complex mixtures of possibly ionic or polymeric compounds that were poorly resolved by HPLC. However, the

TABLE 2. MEAN CONSUMPTION OF ADULT COLORADO POTATO BEETLES EXPOSED TO LEAF DISKS TREATED WITH *S. berthaultii* PI 473334 LEAF RINSE FRACTIONS PRESENTED IN FIGURE 1[a]

	Mean consumption (4 hr) (cm^2 ± SE)						
N	Control	F1	F2	F3	F4	F5	Raw extract
20	1.3 ± 0.2 a	0.1 ± 0.1 b	1.0 ± 0.1 a	1.2 ± 0.2 a	1.2 ± 0.1 a	1.0 ± 0.2 a	0.4 ± 0.1 a
		F1-1	F1-2	F1-3	F1-4		
20	1.7 ± 0.1 a	1.2 ± 0.2 b	1.9 ± 0.1 a	1.1 ± 0.1 b	1.9 ± 0.2 a		
		F1-1-1	F1-1-2	F1-1-3	F1-1-4		
20	1.4 ± 0.1 a	0.4 ± 0.1 b	1.3 ± 0.1 a	1.2 ± 0.1 a	0.4 ± 0.2 b		
		F1-1-1-1	F1-1-1-2	F1-1-1-3			
15	1.2 ± 0.1 a	1.3 ± 0.1 a	0.6 ± 0.1 b	0.7 ± 0.1 b			
		F1-1-4-1	F1-1-4-2	F1-1-4-3	F1-1-4-4		
20	1.1 ± 0.1 a	1.1 ± 0.1 a	0.7 ± 0.1 b	0.6 ± 0.1 c	1.0 ± 0.1 ab		
		F1-3-1	F1-3-2				
20	1.4 ± 0.1 a	0.8 ± 0.2 b	0.6 ± 0.1 b				
		F1-3-2-1	F1-3-2-2	F1-3-2-3			
20	1.2 ± 0.1 a	0.7 ± 0.2 bc	0.6 ± 0.1 b	0.9 ± 0.1 c			

[a]All fractions were tested at a concentration of 10 gle, except the second cycle of C_{18} open-column chromatography (F1-1, etc.), which was tested at 3 gle. Control leaf disks were treated with solvent alone. Means ± standard error followed by the same letter in a row are not significantly different (Fisher's protected LSD, $p \leq 0.05$,).

use of 0.05% acetic acid in water considerably improved peak resolution of the most polar fractions suggesting the presence of ionic compounds.

DISCUSSION

These studies clearly demonstrate that *S. berthaultii* PI 473334 contains chemicals that are deterrent to Colorado potato beetle feeding. Increasing concentrations of methylene chloride leaf rinse applied to both tuber and leaf disks decreased Colorado potato beetle consumption proportionately. When the leaf rinse was separated into its nonvolatile and volatile constituents and applied to tuber disks, the nonvolatile fraction was deterrent, while the volatile fraction tended to reduce consumption, but not significantly. Neither fraction was as deterrent as the raw extract, suggesting that the volatile components may enhance the activity of the nonvolatile chemicals. Recombining the volatile and nonvolatile fractions and testing them for an additive or synergistic response would have been an appropriate test of this hypothesis, but sufficient material was not available for this experiment.

Rinsing the leaves of *S. berthaultii* PI 473334 in methylene chloride typically removed ca. 5–10 $\mu g/cm^2$ of surface chemicals. The leaf rinse treatment, in addition to removing surface chemicals from the leaves, probably also extracted some of the lower molecular weight allelochemicals produced by the type A glandular trichomes, although the trichomes themselves remained intact. When applied to *S. tuberosum* leaf disks, the raw leaf rinse was deterrent at a concentration of 18 $\mu g/cm^2$. However, the magnitude and variability of adult consumption was greatly reduced when the leaf rinse was applied at 83 $\mu g/cm^2$. The fact that the extracts were only moderately deterrent at a concentration twofold greater than that removed from the foliage of *S. berthaultii* was not surprising because the leaf rinse was applied to comparatively glabrous, susceptible *S. tuberosum* leaf disks. Previous studies have shown that resistance is associated with both physical (Dimock and Tingey, 1988) and chemical factors (Neal et al., 1989; Pelletier and Smilowitz, 1990; Yencho and Tingey, 1994). Moreover, the extracts in these studies were applied to leaf disks that were offered to adults searching for their first postemergence meal, and the adults repeatedly probed the leaf disks with their mouthparts. This behavior undoubtedly released leaf sap containing feeding stimulants, which were perceived along with the deterrents. At the lower concentrations, the combination of high internal excitatory feeding inputs and relatively low negative external inhibitory inputs resulted in a slight reduction in feeding compared to the controls, whereas at the higher deterrent concentrations, the negative external inhibitory stimuli probably overcame the internal excitatory stimuli and feeding was greatly reduced. This behavior corresponds to Dethier's (1982) and Miller and Strickler's (1984)

internal versus external stimuli or "rolling fulcrum" model of arthropod investment behavior.

When the deterrent activities of the hexane, methylene chloride, and acetone leaf rinses were compared at equal concentrations, the hexane leaf rinse was not deterrent to Colorado potato beetle feeding, and the acetone leaf rinse was ca. twofold more active than the methylene chloride leaf rinse. When the acetone extract was subjected to the first C_{18} column chromatography step, the deterrent fractions eluted in the first fraction. These results suggest that the active components of resistance are of intermediate polarity. The deterrents are not associated with leaf waxes because these were precipitated before the extracts were applied to the leaf disks. Because the most polar fractions appeared to consist of complex mixtures of ionic or polymeric compounds that were poorly resolved by HPLC, our current efforts are focused on determining the structural characterization of the three most nonpolar fractions (F1-3-2-1, F1-3-2-2, and F1-3-2-3).

The plant surface is one of the most critical junctures influencing arthropod host acceptance. Therefore, it is not surprising that a remarkable array of chemical and physical factors deterrent to feeding are concentrated at this surface (Juniper and Southwood, 1986; Chapman and Bernays, 1989). We hypothesize that the feeding deterrents extracted in this study are produced by the type A trichomes. However, validation of this awaits their identification and extraction from individual type A trichomes. The type A trichomes in particular are chemically complex structures. Polyphenol oxidase comprises up to 60% of the total type A trichome protein (Kowalski et al., 1992). This enzyme, in addition to being a proteinase inhibitor, catalyzes the formation of quinoid reaction products that are highly reactive molecules that covalently modify and cross-link with a variety of plant cellular constituents (Duffey and Felton, 1991; Steffens and Walters, 1991). It is possible that some of these products are deterrent to feeding. The type A trichomes also produce low-molecular-weight volatile substances, including a number of sesquiterpenes that act to deter aphid settling (Avé et al., 1987). It is possible that volatile components of *S. berthaultii* may enhance the activity of the nonvolatile Colorado potato beetle feeding deterrents. For *S. berthaultii* accessions that contain functional type B trichomes, it is postulated that the volatile sesquiterpenes are absorbed and slowly released by the viscous sugar ester droplets that the type B trichomes secrete (Avé et al., 1987), and the sugar esters are themselves apparently deterrent to feeding by adult Colorado potato beetle (Pelletier and Smilowitz, 1990).

The wild Bolivian potato, *S. berthaultii*, is a valuable source of insect resistance for *S. tuberosum* cultivars (Plaisted et al., 1992). Determining the mechanisms of resistance to the Colorado potato beetle in this species will further facilitate the incorporation of this trait into adapted cultivars. The bioas-

says used in these studies provided complementary information on the nature of resistance to this insect. The tuber disk bioassay, which was more labor intensive than the leaf disk bioassay, provided a uniform, porous medium that absorbed a variety of solvents. In addition, the tuber disks probably released the volatile extracts for a longer period of time compared to leaf disks and thus provided an ideal substrate for studies of the effects of the volatile deterrents on Colorado potato beetle feeding. Because complete evaporation of the volatile fraction risked the loss of volatiles and the porous nature of this substrate prevented accurate estimates of the quantities remaining at the surface, the concentration of the leaf rinse applied to the tuber disks was expressed in gram leaf equivalents, rather than a microgram per square centimeter basis. Another advantage of the tuber disk assay was that adults feeding on the tuber disks produced distinct pellets of frass that could be separated from the remains of tuber disk and weighed. This, combined with data on initial and final starting weights could be used to calculate efficiency of conversion of ingested food (ECD) (Slansky and Rodriguez, 1987). However, in comparison to the tuber disks, the leaf disk bioassay probably provided more accurate and precise estimates of the effects of the nonvolatile deterrents on adult feeding because leaves are the preferred feeding substrate of the Colorado potato beetle and the concentration of the extract at the leaf surface could be estimated more accurately compared to the tuber disks. Furthermore, the present studies and others (Neal et al., 1989; Yencho and Tingey, 1994) indicate that the feeding deterrents of *S. berthaultii* are probably produced in the outer epidermal regions of the leaves. Therefore, the leaf disk bioassay approximated the effect of the deterrents on adult feeding more realistically because the extracts applied to the leaf disks were distributed on the leaf surface, while the extracts applied to the tuber disks were absorbed into the starch matrix of the tuber disks.

Clearly, the leaf surface of *S. berthaultii* is a very complex substrate. Precise identification of the compounds responsible for the expression of resistance to the Colorado potato beetle will enable us to determine how the type A and B trichomes interact and to ascertain the relative importance of each in determining resistance to this insect. In addition, knowledge of the specific chemical constituents that impart resistance to Colorado potato beetle will facilitate the development of biochemical assays for screening for resistant progeny in segregating populations and will provide useful information for ongoing studies aimed at determining the molecular genetic basis of resistance to this insect in *S. berthaultii*.

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ALLELOPATHIC POTENTIAL IN BILBERRY–SPRUCE FORESTS: INFLUENCE OF PHENOLIC COMPOUNDS ON SPRUCE SEEDLINGS

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Abstract—Regeneration failure of *Picea abies* in a subalpine bilberry–spruce forest was studied in relation to phenolic compounds, their occurrence and toxicity. Germination bioassays with natural leachates of bilberry (*Vaccinium myrtillus*) and spruce showed negative effects on root elongation of spruce seedlings. Growth bioassays on litter and humus demonstrated inhibitory effects of these organic layers. *p*-Hydroxyacetophenone, a spruce-specific metabolite, was isolated in spruce throughfall (10^{-6} M), in water extracts of litter (between 1 and 8 μg/g dry wt) and organic layer (less than 1 μg/g dry wt) in addition to tannins and several common phenolic acids. Potential relationships between vegetation cover and phenolic pattern of the soil are discussed, since organic layers under bilberry heath exhibited higher amounts of phenolic acids and tannins than those under spruce. *p*-Hydroxyacetophenone and caffeic acid reduced, even at 5×10^{-5} M, spruce seedling growth, especially root development, with additive effects for these two monomers. Autotoxicity involving spruce trees and allelopathy of understory species, mediated by *p*-hydroxyacetophenone and other phenolic compounds, including tannins, deserves further attention in regeneration studies.

Key Words—Allelopathy, *p*-hydroxyacetophenone, phenolic patterns, *Picea abies*, regeneration failure, tannins, *Vaccinium myrtillus*.

INTRODUCTION

Despite numerous reports of spruce (*Picea abies* L. Karst) regeneration failure in mountain forest stands (Lachaussee, 1947; Weissen, 1979), it remains unclear

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whether climatic, biological, or chemical factors are responsible for such failure. Nevertheless, good seedling establishment on soil from which the organic layer (moder or mor humus) had been removed suggests that humus may be a factor. In particular, toxicity of soil substances could be suspected, and there is now evidence that some forest regeneration problems could be associated with biochemical interference between species (Fisher, 1980). For example, seedling growth of coniferous species such as *Pinus elliottii* and *Pinus taeda* (Hollis et al., 1982), *Picea mariana*, (Jobidon et al., 1989), and *Pinus sylvestris* (Nilsson and Zackrisson, 1992) could be affected by undergrowth species, but only a few investigations have dealt with allelopathic interferences between spruce trees or understory species [*Vaccinium myrtillus* L., *Athyrium filix-femina* (L.) Roth, etc.] and spruce seedlings. Phytoxicity of spruce litter, root extracts (Leibundgut, 1976; Andre et al., 1987), and soil solutions (Weissen and Van Praag, 1991) on spruce growth have, however, been demonstrated, although identification of associated allelochemicals remains rare (Van Praag et al., 1991).

The objective of this research was to assess the allelopathic potential of throughfall, litter, and organic layer samples of mountain spruce–bilberry forests, and of the prevailing water-soluble phenolic compounds found in these substrates, on seed germination and growth of spruce seedlings.

METHODS AND MATERIALS

Sampling

Sampling was made at two altitudes of a bilberry–spruce forest in Tarentaise (Savoy-France): site 1, 1630 m, on colluvial ranker and spruce regeneration by spots; and site 2, 1860 m, on podzol with stagnogley and poor regeneration on decaying stumps. For each stand, the vegetation and associated humus have been described by Bernier et al. (1993) and two major vegetation units were specially studied: around an adult tree (> 100 years) with pure litter of spruce needles and fragments (2–3 cm thick at the lower site, > 5 cm thick at the upper site), on a dysmull humus (pH > 4.5); and bilberry heath with a mixed litter made of spruce needles, bilberry leaves and moss fragments (3 cm thick), on a mor humus (pH 4.2).

Litter and soil samples (organic horizon A0) were collected in triplicate (random sampling in three locations) on four dates during the growing period (August and September 1990, May and June 1991), and frozen at −18°C after removal of visible fragments. Each sample was treated separately. Throughfalls were collected at site 2 with PVC gutters under three different adult trees (trees I, II, III) and with micro PE gutters connected to PE bottles under bilberry heath. Since preservatives could not be used without chemical alteration of the

solution, leachates were utilized as soon as possible after each rain event (less than one day of storage).

Extraction and Analysis of Water-Soluble Phenolic Compounds from Litter and Soils

Intact samples of litter and soil were shaken in distilled water (10 g/500 ml for litter, 30 g/250 ml for soil) for 20 hr under N_2, at room temperature. After centrifugation (5000 rpm), the supernatant was filtered with Whatman No. 1 filter paper. The resulting solutions, as well as natural leachates, were analyzed for their concentration of total phenolics following the procedure of Marigo (1973) (Folin-Ciocalteu reagent) with gallic acid as the standard. Tanning capacity was determined by the colorimetric method of Bate-Smith (1973), modified by Schultz et al. (1981), with tannic acid used as the standard.

Monomeric phenols were extracted from sample solutions after acidification (pH 2.5, HCl) of a given volume of the solution, and three extractions with ethyl ether. After evaporation to dryness, the residue was redissolved with ethanol. The ethanolic solutions were subjected to HPLC. A Waters 5000 A and M 660 liquid chromatograph, equipped with a multirange UV detector (Waters M 490) and a 20-μl valve loop injector were used. Peak areas and retention times of separated solutions were obtained with a Waters 730 computing integrator. A short column of Novapak 18 C was placed immediately before a 300-mm × 3.9-mm-ID column, filled with μBondapak 18 C. Linear gradient elution was carried out at a flow rate of 1.5 ml/min. Solvent A was 0.5% acetic acid in distilled water. Acetonitrile with 0.5% acetic acid was used as solvent B. A gradient from 0% to 20% B over 45 min, followed by 15 min reequilibration with A, was used. Identification of phenolic acids (duplicate analysis) was performed by comparison of retention times, wavelength detection, and cochromatography. The amounts were determined by comparison with reference curves of mixtures of standard compounds.

Bioassays on Germination and Early Growth: Throughfalls and Standard Solutions

All bioassays were performed with high-altitude (1100-m) spruce seeds (6–8 mg) (Office National des Forêts, Sécherie de la Joux, Lot H1-81-0682).

Aqueous solutions (10 ml/dish) were placed into 12-mm-diam. Petri dishes lined with Whatman No. 1 filter paper. In five replicate dishes, 12 seeds were exposed to native throughfalls (without any treatment), standard solutions, or distilled water. Caffeic acid, *p*-hydroxyacetophenone, and picein (*p*-hydroxyacetophenone-β-D-glucopyranoside) were used as standards, in three concentrations: 10^{-3}, 10^{-4}, and 10^{-5} M. Water potentials of the standard solutions were measured on a Fiske QF Osmometer (model 330 D). The Petri dishes were

sealed and maintained in a growth chamber (18°C) under 14 hr illumination (white light, 15 $\mu E/m^2/sec$).

Germinated seeds, with a >1-mm radicles, were recorded daily, and after 18 days, the lengths of the radicles and aerial parts were measured.

Growth Bioassays: Litter, Humus, and Standard Solutions

Method 1. Pregerminated seeds (30 seeds per pot, two pots per treatment) were placed into plastic pots (1000 ml) filled with a mixture of vermiculite and materials (1:2 v/v for litter and ground litter, 1:4 for humus v/v). Two controls were made: vermiculite and vermiculite + sand (1:4 v/v). All pots were watered to field capacity with a nutrient solution (Norkrans, 1949) and maintained in a growth chamber under 16 hr illumination (white light, 15 $\mu E/m^2/sec$) and at a temperature of 20°C during the day and 12°C during the night.

After three months and regular addition of dilute nutrient solution (1/10), the seedlings were harvested, the number of secondary roots was measured, and the dry weight (after drying at 70°C) of shoots and roots was determined.

Method 2. Pregerminated seeds (30 seeds per pot, two pots per treatment) were placed into pots (1000 ml) filled with vermiculite. The pots were watered to field capacity with nutrient solution (Norkrans, 1949). Regular watering with nutrient solution 1/10 were made, and the pots were maintained in a growth chamber under 16 hr illumination (white light, 15 $\mu E/m^2/sec$) and a temperature of 20°C (day) and 12°C (night). After two months, the pots were exposed to three successive treatments with phenolic solutions (250 ml/pot). Solutions of caffeic acid and *p*-hydroxyacetophenone were used at 10^{-5}, 10^{-4}, and 2×10^{-4} M. Possible synergistic effects between the two molecules were tested with equimolar solutions of the two acids: $5 \times 10^{-5} + 5 \times 10^{-5}$ M, and $10^{-4} + 10^{-4}$ M.

Four months after germination, the seedlings were harvested, the number of secondary roots, and the weights of shoots and roots after drying at 70°C were recorded.

Statistical Analysis

The data were tested for homogeneity with Bartlett's test and analyzed by one-way analysis of variance (bioassays with natural substrates) or two-way analysis of variance (bioassays with different standards at different concentrations). The least significant difference (LSD) was used to determine significant differences between treatments and the control treatment (Sokal and Rohlf, 1981).

RESULTS

Litter and Soil Phenolics under Picea *and* Vaccinium. Analysis of throughfalls (Table 1) showed that the nature of the vegetation greatly influences leachate composition. Bilberry leachates were less acidic than incident rain and

TABLE 1. PHENOLIC COMPOSITION OF THROUGHFALL UNDER BILBERRY AND SPRUCE, SAMPLED SEPTEMBER 13 (A) AND 25 (B), 1991, IN SUBALPINE SPRUCE–BILBERRY FOREST (1860 m, Site 2)

	pH	Total phenols (mg/liter^{-1})[a]	Tanning capacity (mg/liter^{-1})[b]	Phenolic acids (mg/liter^{-1})[c]
Incident rain	5.6	0.3	0	0
Bilberry A	6.4	4.6	0	0
Bilberry B	6.0	2.6	tr.	caffeic acid = tr.
Spruce I B	4.4	21.1	670	pha = 80 (+phb, pro = tr)
Spruce II B	4.1	30.2	720	pha = 216 (+phb, pro = tr)
Spruce III B	4.1	31.2	750	pha = 257 (+phb, pro = tr)

[a] Expressed as gallic acid equivalent.
[b] Expressed as tannic acid equivalent.
[c] pha = *p*-hydroxyacetophenone, phb = *p*-hydroxybenzoic acid, pro = protocatechuic acid, tr. = trace.

contained about 10 times more total phenols. Caffeic acid, the most abundant phenolic monomer in bilberry foliage (Gallet, 1992) was present. In contrast, throughfall acidification was observed under spruce canopy, as well as a marked brown coloration. Phenolic content was 100 times greater than that of incident rain. Phenolic monomers, especially *p*-hydroxyacetophenone, and polymers with tanning activity were abundant enough to be quantified.

Analysis of litter and soil (Table 2) also showed differences between spruce and bilberry, with more tannins and phenolic monomers under the latter. The difference in concentrations of tannins between litter and humus was greater (about four times) than for total phenols (about two times), showing faster transformations for the tannins during humification processes. Phenolic acid concentration was four times lower in spruce humus than in spruce litter, and 2.5 times lower in bilberry humus than in bilberry litter. *p*-Hydroxyacetophenone, a specific spruce metabolite, is prevalent in litter when the degradation products vanillic and protocatechuic acids become prevalent in humus. Analysis of water-soluble fractions of litter showed that water-soluble compounds represented only a small part of total phenols (less than 10%). In contrast, more than 20% of tannins were water soluble. Mixed litter of needles, leaves, and moss found under bilberry, especially at higher elevation, were more leachable than pure spruce hydrophobic needles. Except in one case (sum of phenolic acids in spruce litter), litter and soil of the highest site (1860 m) contained more phenols, tannins, and monomers than the lower site (1630 m).

Effects of Leachate on Seed Germination. Root elongation of spruce seed-

TABLE 2. PHENOLIC COMPOSITION OF SPRUCE AND MIXED (Spruce + Bilberry + Moss) LITTER (L), ORGANIC HORIZON (H) OF TWO SITES (Site 1: 1630 m, Site 2: 1860 m) IN SUBALPINE BILBERRY–SPRUCE FOREST

	Water-soluble phenols (mg/g dry wt)[a]	Water-soluble tanning capacity (mg/g dry wt)[b]	Water-soluble phenolic acids	
			Sum (μg/g dry wt)	Monomers[c]
Spruce L site 1	0.8 ± 0.3[d](3%)[e]	29 ± 8(20%)	10.7 ± 3.6	pha > phb > van
Spruce L site 2	0.9 ± 0.2(5%)	34 ± 11(23%)	7.3 ± 4.4	> pro = p-coum
Mixed L site 1	1.0 ± 0.5(7%)	53 ± 13(30%)	12.7 ± 5.4	pha > phb = van = pro
Mixed L site 2	1.1 ± 0.3(7%)	57 ± 30(24%)	17.5 ± 3.0	> p-coum > caf
Spruce H site 1	0.3 ± 0.1	7 ± 4	2.2 ± 1.1	van > phb > p-coum
Spruce H site 2	0.5 ± 0.3	8 ± 5	2.2 ± 0.9	= pha > pro
Mixed H site 1	0.4 ± 0.2	12 ± 6	5.6 ± 4.2	van > phb > p-coum
Mixed H site 2	0.5 ± 0.2	13 ± 5	6.6 ± 3.7	= pro > caf > pha

[a] Expressed as gallic acid equivalent.
[b] Expressed as tannic acid equivalent.
[c] pha = *p*-hydroxyacetophenone, phb = *p*-hydroxybenzoic acid, pro = protocatechuic acid, van = vanillic acid, p-coum = *p*-coumaric acid, caf = caffeic acid.
[d] Mean and standard deviation of four sampling dates, three replicates per date.
[e] Expressed in percent of total amounts (500 mg ground litter twice extracted with ethanol/water mixture, and redissolved in water after filtration and vaccum evaporation).

lings exposed to leachates was significantly inhibited (Figure 1): the length of the root was only 34% of the control for bilberry leachate, and about 74% for spruce leachate, except in one case: bilberry throughfall B (September 25, 1991). In contrast, bilberry leachate A (September 13, 1991), with the greatest phenolic content, produced the greatest inhibition, as well as seed mortality: compared to the control (92%), total germination was significantly reduced (83%). Spruce throughfalls did not affect germination, but inhibited root elongation, with a degree of reduction correlated ($r = 0.97$) to total phenolics (in gallic acid equivalents) (Table 1). Shoot elongation was significantly affected for only one spruce and one bilberry throughfall. Distilled water control or incident rain sampled from a clearing gave similar results.

Seedling Growth on Litter and Humus. Total seedling biomass of the 3-month-old seedling grown on vermiculite + sand (18.3 mg) was not different when compared to the vermiculite control (19.0 mg), but root biomass (8.9 mg) was significantly higher (+59%) than the control (5.6 mg) (Figure 2). Thus the sand produced an increase in root growth, but a decrease in secondary root number ($N = 13.5$) compared to the vermiculite control ($N = 20.6$).

All other treatments significantly reduced both total and root biomass.

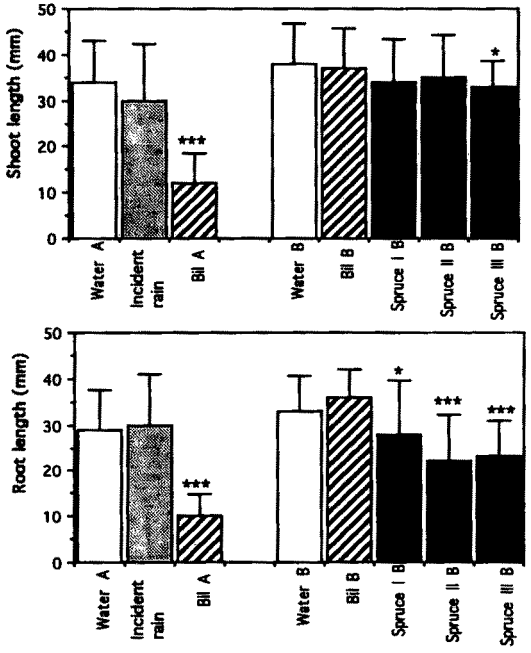
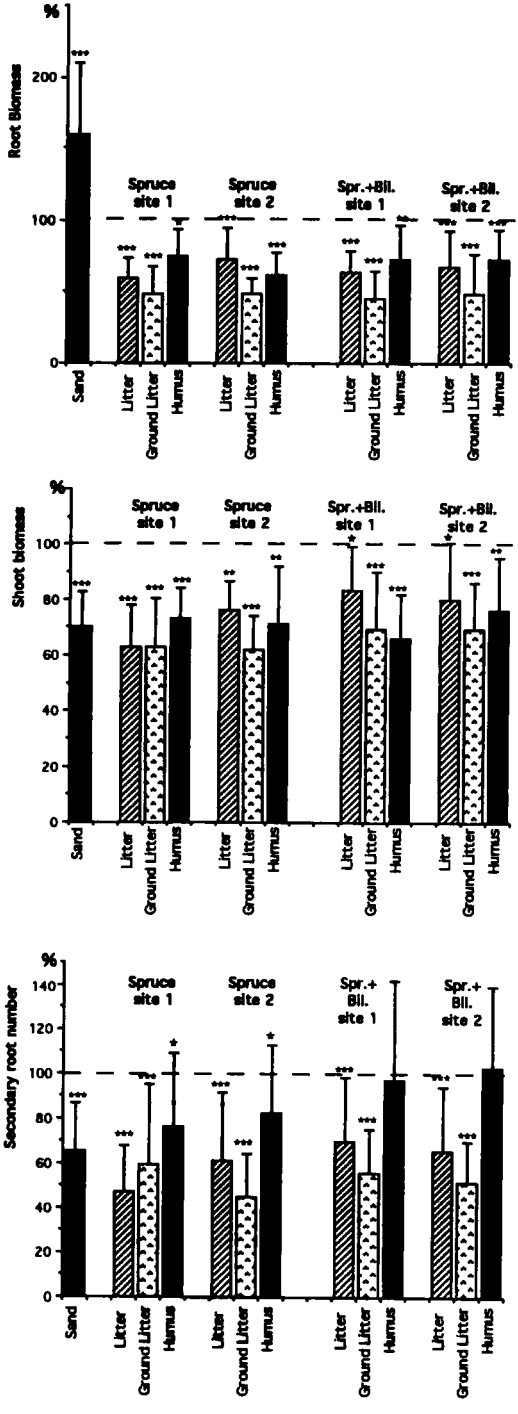


FIG. 1. Shoot and root length (mean and SD) of spruce seedlings (60/treatment) germinated in contact with different throughfalls and control solutions. Spruce throughfalls were collected under three different trees: I, II, III. Bil. = under bilberry heath. Date of sampling: A = September 13, 1991 (control shoot length: 34.1 ± 2.0 mm, root length: 29.0 ± 1.9 mm), B = September 25, 1991 (control shoot length: 38.1 ± 2.1 mm, root length: 32.4 ± 1.8 mm). *, **, *** = significantly different from control at 0.05, 0.01, and 0.001, respectively.

Ground litter was the most inhibiting material for root growth (reduction of 52% for spruce, 53% for spruce + bilberry) and shoot growth (reduction of 37% for spruce, 31% for spruce + bilberry). Humus was the least inhibiting material for root biomass (reduction of 32% for spruce and 27% for spruce + bilberry) and number of secondary roots (reduction of 20% for spruce and no significant reduction for spruce + bilberry). Mixed medium (spruce + bilberry) had less inhibitory effects on shoot biomass and secondary roots than pure spruce medium, but seedlings grown on mixed litter exhibited chlorosis and thin shoots, as well as brown-colored roots.

Effects of Three Phenolic Monomers on Seed Germination and Early Growth. Primary root elongation was the variable most affected by the three standards used against spruce seed germination (Figure 3). Neither of the phe-


%
Root Biomass
200
100
0
Spruce site 1
Spruce site 2
Spr.+Bll. site 1
Spr.+Bll. site 2
Sand
Litter
Ground Litter
Humus
%
Shoot biomass
100
80
60
40
20
0
%
Secondary root number
140
120
100
80
60
40
20
0
Spr.+ Bll. site 1
Spr.+ Bll. site 2

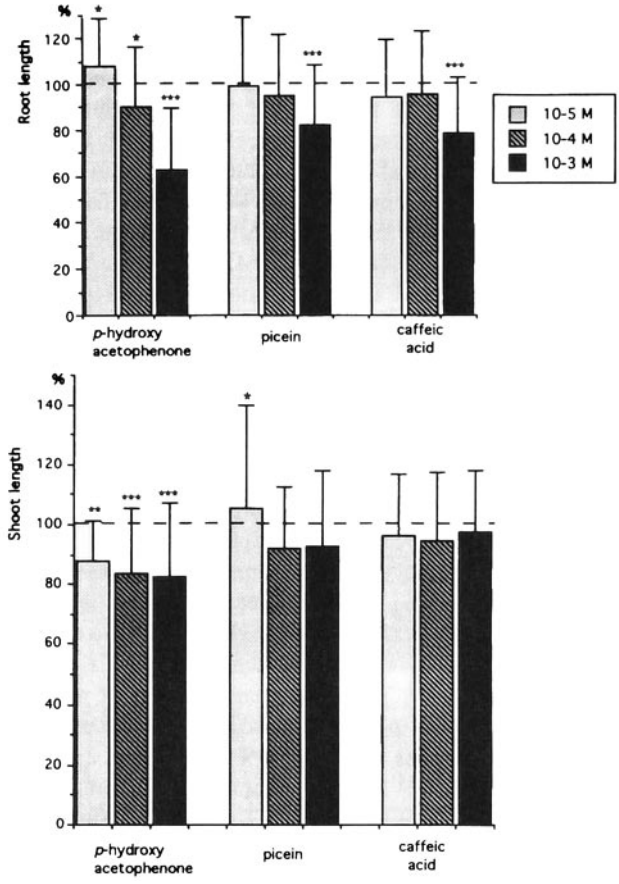


FIG. 3. Shoot and root length (mean and SD) of spruce seedlings (60/treatment) germinated on contact with three phenolic compounds: *p*-hydroxyacetophenone, picein (*p*-hydroxyacetophenone-β-D-glucopyranoside), and caffeic acid, as percent of control (distilled water). *, **, *** = significantly different from control (control root length: 37.3 ± 1.9 mm, shoot length: 43.7 ± 2.3 mm) at 0.05, 0.01, and 0.001, respectively.

FIG. 2. Shoot and root biomass and secondary root number (mean and SD) of 3-month-old spruce seedlings grown on litter and humus, as percent of control (vermiculite). Litter and humus were sampled at site 1 (1630 m) and site 2 (1860 m) under spruce and under spruce + bilberry (Spr. + Bil.). All treatments were regularly watered with nutrient solutions. *, **, *** = significantly different from control (control root biomass: 5.6 ± 0.6 mg, shoot biomass: 13.4 ± 1.3 mg, *N*: 20.6 ± 1.9) at 0.05, 0.01, and 0.001, respectively.

nolic monomers influenced total seed germination, even at the highest concentration. A reduction of root length of, respectively, 37%, 18%, and 21% was observed for *p*-hydroxyacetophenone, picein, and caffeic acid at the highest concentration (10^{-3} M), and of 10% at 10^{-4} M for *p*-hydroxyacetophenone. This standard also showed a stimulatory effect (+8%) on root length at the lowest concentration. When combined with glucose in picein form, it appeared to have a lower activity. Shoot elongation was less affected than root elongation, but *p*-hydroxyacetophenone significantly reduced shoot length 18%, 15%, 12%, respectively, at the three concentrations 10^{-3} M, 10^{-4} M, and 10^{-5} M. At 10^{-5} M, a slight stimulatory influence of picein on shoot elongation (+5%) was observed. Osmotic potentials of all these standard solutions were below 15 mosmol.

Effects of p-*Hydroxyacetophenone and Caffeic Acid on Seedling Growth.* Dry weights of 4-month-old seedlings grown on nutrient cultures with addition of phenolic acids were reduced even for the lowest concentration of 5×10^{-5} M (reduction of 33% and 39% of root biomass by *p*-hydroxyacetophenone and caffeic acid) (Figure 4). Root biomass was more influenced than shoot biomass, with a reduction of about 50% at the highest concentration of *p*-hydroxyacetophenone (2×10^{-4} M). No significant differences could be seen between the two monomers, except for the number of secondary roots, which was significantly reduced only by caffeic acid ($N = 29.2$) compared to the control ($N = 37.2$).

The single treatments *p*-hydroxyacetophenone or caffeic acid and the equimolar mixture treatment with *p*-hydroxyacetophenone + caffeic acid caused similar root biomass reductions of 46%, 39%, and 40%, respectively (for a total concentration of 2×10^{-4} M). The joint action of the mixture of the two phenolic monomers is superior to the single action only for secondary root number and total concentration of 10^{-4} M; the single treatments had no effect, while the mixture caused a decrease of 30%. The joint action seems to be additive, rather than synergistic.

DISCUSSION

Root and shoot development appear to be more sensitive than germination *sensu stricto* to phytotoxins released by spruce and bilberry. Germination inhibition was only found with one bilberry throughfall but not with any of the other samples. This could be due to variation in solution chemistry associated with rate and timing of precipitation events (Parker, 1983). For instance, the September 13 rain, following a long drought period, may have leached a greater amount of exudation products accumulated at the leave surfaces, thus containing a larger amount of toxic substances. Influence of physical factors, such as pH

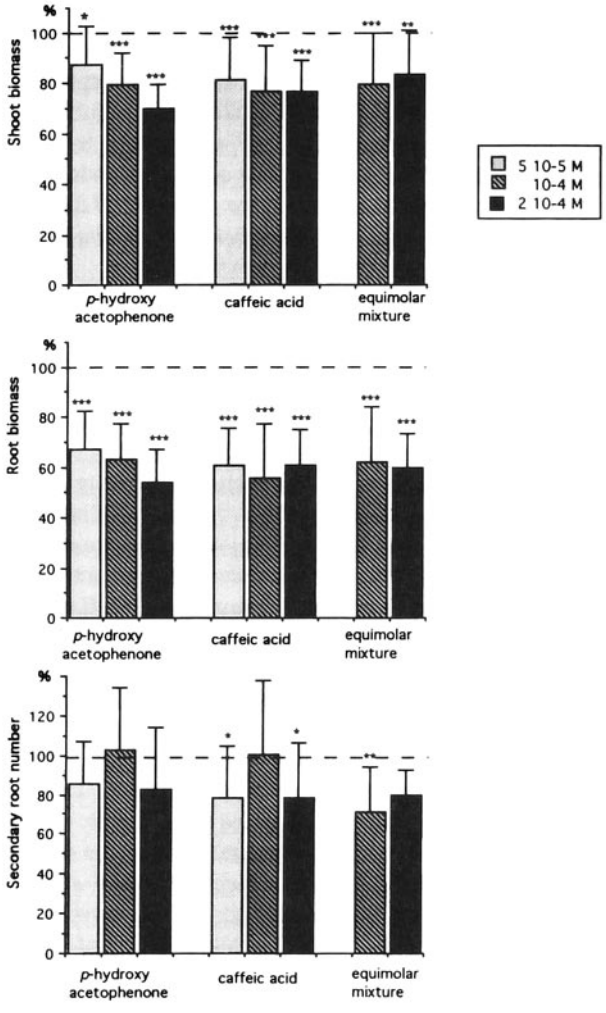


FIG. 4. Shoot and root biomass, secondary root number (mean and SD) of 4-month-old spruce seedlings grown on vermiculite in the presence of *p*-hydroxyacetophenone, caffeic acid, and a mixture of the two, as percent of control (distilled water). All treatments were regularly watered with nutrient solution. *, **, *** = significantly different from control (control shoot biomass: 18.0 ± 2.0 mg, root biomass: 13.9 ± 1.9 mg, *N*: 37.2 ± 4.2) at 0.05, 0.01, and 0.001, respectively.

and ionic strength, as well as toxicity of inorganic components could not be ruled out by these experiments. The chemical nature of the germination inhibitor(s) is in this instance not known, but it must be water soluble. Since caffeic acid did not affect germination in bioassays in vitro, it was probably not responsible for the observed inhibition. A number of Ericaceae species are known to contain germination inhibitors (Chou and Muller, 1972; Carbareilla, 1980; Tinnin and Kirkpatrick, 1985; Wepplo, 1987). Phenolic acids have frequently been cited as the inhibitors. In addition to phenolic acids, less widespread compounds such as 2-hydroxyphenyl acetic acid in *Erica scoparia* (Ballester et al., 1977) and substituted dihydrostilbene in *Empetrum hermaphroditum* (Odén et al., 1992) have also been isolated.

During heterotrophic growth, root elongation, and, to a lesser extent, shoot elongation were affected by application of the phenolic monomers *p*-hydroxyacetophenone and caffeic acid. Ionic strength of these solutions (< 15 mosmol) was below the toxic level of 75 mosmol found by Bell (1974) for *Bromus rigidus*. Root growth inhibition could then be ascribed to these substances; even cellular mechanisms responsible for the toxicity are not known. These two substances have been isolated in natural solutions inducing deleterious effects on seedling early growth (spruce and bilberry leachates). Since their concentrations were lower (about 10^{-6} M for *p*-hydroxyacetophenone in throughfall) than those in standard solutions, other compounds might be active. Synergistic or additive actions may also have occurred (Rasmussen and Einhellig, 1977; Lyu et al., 1990) between monomers and between monomers and tannins.

The results of growth bioassays on litter and humus suggested that the first centimeters of the soils studied are not favorable to spruce seedling establishment. Occurrence in the materials of toxic monomers (*p*-hydroxyacetophenone, phenolic acids) and of polymers (tannins) does not limit this inhibition to biochemical interactions. For instance, in spite of greater amounts of phenolic compounds, bilberry–spruce substrates seemed to be slightly less inhibiting than pure spruce substrates. The physical characteristics of the medium should be taken in account. For example, the occurrence in bilberry–spruce substrates of moss fragments creates favorable moisture conditions, compared to the hydrophobic structures of spruce litter and organic layers. One of the main problems in studying interference between plants is to separate allelopathy from competition and reduced resources (Fuerst and Putnam, 1983). Biomass reduction of seedlings observed on litter and humus could be related to nutrient deficiency, especially on poor substrates such as mor humus. Regular nutritional additions, as applied in the bioassays, should prevent this problem. On the other hand, low fertility conditions could enhance some phytotoxic manifestations (Stowe and Osborn, 1980). The increase of inhibition shown with litter grinding could be explained by biochemical factors; even the physical differences (hardness, porosity) between intact litter and ground litter could modify root development.

However, such operation equally enhances the release of metabolites stocked in senescent tissues (especially phenols) and can increase tenfold the leaching of water-soluble organic compounds (Nykvist, 1963).

This is the first identification of *p*-hydroxyacetophenone in spruce organic layers and throughfall. It is the prevailing monomer in both litters and in spruce throughfall. This compound, and to a lesser extent its glucoside picein, exhibits strong biological activity and is also assumed to be a chemical indicator for stress in *Picea abies* (Jensen and Lokke, 1990). It was inhibitory to rice and wheat seedlings and caused accelerated needle senescence and loss of apical dominance in 2-year-old spruce seedlings (Hoque, 1985). Fungitoxic activity towards forest pathogens (Osswald et al., 1987) and mycorrhizal species (Pellissier, 1993) had also been ascribed to this compound. This leads us to the possibility of autotoxicity for spruce seedlings in spruce stands.

In addition, widespread phenolic acids were also isolated in litter and soils: *p*-hydroxybenzoic, vanillic, protocatechuic, *p*-coumaric acids. Concentrations (micrograms per gram) were similar to other studies on free water-soluble phenolic acids (Whitehead et al., 1981; Jalal and Read, 1983; Kuiters and Denneman, 1987). Higher amounts of phenolic compounds under bilberry heath than under spruce could be associated with the more active phenolic metabolism characteristic of Ericaceae (Hegnauer, 1966). Caffeic acid, the major phenolic acid in bilberry leaves, disappeared rapidly from the brown leaves as concentrations of degradation metabolites (*p*-coumaric and protocatechuic acids) increased. In humus, vanillic acid, often considered an important lignin degradation metabolite, becomes preponderant. There are now numerous reports of inhibiting effects of these phenolic acids towards seedlings growth; caffeic acid seemed to be one of the less active against herbaceous (Kuiters, 1987) or cultivated species (Reynolds, 1978). Spruce seedlings were, however, greatly affected by this compound (even at 5×10^{-5} M), and an additive effect with *p*-hydroxyacetophenone was possible. Qualitative differences observed in shoots (chlorosis) and roots (marked brown coloration) of seedlings grown on bilberry–spruce mediums could be associated with specific bilberry compounds, emphasizing the need for further experiments on the physiological activity (impact on photosynthesis, nutrient uptake) of such compounds.

The ecological importance of allelopathic interactions in the field by low-molecular-weight compounds is often questioned because of the low concentrations of the free forms of these compounds in soil solutions (Proksch et al., 1985). The resulting data obtained suggest that greater attention should be given to local heterogeneity related to vegetation and specific metabolite patterns. *p*-Hydroxyacetophenone and picein synthesis present great variability associated with tree, needle age, and environment (Esterbauer et al., 1975). In high-altitude stands, increased light intensity enhanced the synthesis of such phenolic compounds (MacClure, 1979), when extracellular microbial degradation is slowed

down by environmental factors. In addition, heath development led to mor humus formation (Handley, 1952) and phenolic compound accumulation, particularly tannins. Some reports on tannin impact on soils and ecosystems are now available (Tiarks et al., 1989), but further work on the nature and the extracellular evolution of tannins appears appropriate, under heath cover as well as around spruce trunks.

The "synergistic," or "additive" impact on the two dominant species on spruce establishment, bilberry and spruce, need to be studied, since germination-inhibiting substances appear to be released by living bilberry leaves and growth-inhibiting compounds by both spruce litter and throughfall.

Other experiments, preferentially carried out under field conditions, are necessary to attribute regeneration failure in mountain spruce forest to biochemical interactions. Nevertheless, identification in the spruce forest ecosystem of significant amounts of a particular biologically active structure, *p*-hydroxyacetophenone in free water-soluble form could be of interest for studies of mechanisms in which living organisms are involved: rate of litter decomposition, nitrification, ammonification and mycorrhizal relationships.

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RELATIVE ACTIVITIES OF GLUCOSINOLATES AS OVIPOSITION STIMULANTS FOR *Pieris rapae* AND *P. napi oleracea*

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Abstract—The relative activities of 10 glucosinolates in stimulating oviposition by *P. rapae* and *P. napi oleracea* were compared under the same conditions. When tested at the same concentration, the structurally different glucosinolates stimulated both butterfly species to widely varying degrees. In most cases, *P. rapae* was more sensitive to aromatic and indole glucosinolates than to aliphatic representatives. This species responded even less to alkyl thio and sulfinyl glucosinolates. However, *P. napi oleracea* responded strongly to these aliphatic and sulfur-containing members of the group, and the relative activities of aromatic and aliphatic glucosinolates did not show a clear pattern for this species. *P. napi oleracea* was much more sensitive to low concentrations of sinigrin than was *P. rapae*. The threshold concentration for response of *P. napi oleracea* to sinigrin was 10^{-8} M, which was 100 times lower than for *P. rapae*, but *P. rapae* was more sensitive than *P. napi oleracea* to changes in glucosinolate concentrations. For both *Pieris* species, an optimal concentration was reached, above which the response remained constant or tended to decrease.

Key Words—*Pieris rapae*, *Pieris napi oleracea*, Lepidoptera, Pieridae, oviposition, stimulants, glucosinolates.

INTRODUCTION

The glucosinolates are a well-known group of thioglucosides found in plants and are constituents of many crop species, especially members of the Cruciferae, that are used as vegetables or sources of condiments, relishes, and oil seeds

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(Jackson, 1991). These compounds have long been of toxicological and pharmacological interest (Daxenbichler et al., 1991) and are known to play a role in the chemical mediation of interactions between plants and other organisms. Glucosinolates are important determinants of herbivory (Feeny, 1977) and play a role in the host selection behavior of adapted insects (Nielsen, 1978). About 100 glucosinolates are now known (Louda and Mole, 1991), and these differ only in the structure of the side chain attached to the basic thioglucose-sulfonated oxime part of the molecule. However, little is known about the structural requirements for biological activity.

The behavioral "decision" of most herbivorous insects to accept or reject a potential host plant as oviposition substrate or food source is based largely on chemical input from the plant (Dethier, 1982; Miller and Strickler, 1984). Verschaffelt (1910) found that the host ranges of *Pieris* butterflies are restricted to members of Cruciferae and a few related plant families that contain glucosinolates. Recent studies have emphasized such correlations and confirmed that glucosinolates play an important role in host selection by a number of insect species. For example, oviposition and larval feeding by *Pieris napi macdunnoughii* correlate with glucosinolate profiles of plant species in a natural community (Rodman and Chew, 1980). An indole glucosinolate (glucobrassicin) on the surface of cabbage (*Brassica oleracea*) leaves is a potent stimulant inducing oviposition by the cabbage butterflies, *Pieris brassicae* (van Loon et al., 1992) and *P. rapae* (Renwick et al., 1992). Various glucosinolates have been shown to be responsible for recognition of *Tropaeolum majus*, *Carica papaya*, *Brassica juncea*, and *Isatis tinctoria* by *P. rapae* (Sachdev-Gupta et al., 1992). More recently, it has been clearly demonstrated that glucosinolates are involved in differential acceptance of *Erysimum cheiranthoides* (Huang et al., 1993a), *Iberis amara* (Huang et al., 1993b), and *Barbarea vulgaris* (Huang et al., 1994) by *P. rapae* and *P. napi oleracea*. Host selection by these *Pieris* species is controlled by a balance of information provided by chemoreceptors sensitive to deterrents and stimulants in the plants (Dethier, 1982). Different glucosinolates may play different roles in host selection by a particular insect species. Traynier and Truscott (1991) found that learning behavior of *P. rapae* could be triggered by sinigrin, but glucobrassicin produced a much stronger effect. Sachdev-Gupta et al. (1992) suggested that aromatic glucosinolates are very stimulatory to *P. rapae* and that glucosinolates containing an alkenyl side chain may also stimulate oviposition, but those with a sulfur atom in the side chain are inactive. More studies are apparently needed to reveal the structure–activity relationship of glucosinolates as oviposition stimulants for herbivorous insects.

This study compares the relative activities of 10 glucosinolates, representing different structural groups, in stimulating oviposition by *P. rapae* and *P. napi oleracea*. Differences in sensitivity and threshold level for response of the

two *Pieris* species to these compounds were examined to explain differential acceptance of glucosinolate-containing plants.

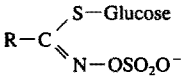
METHODS AND MATERIALS

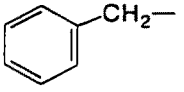
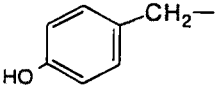
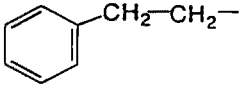
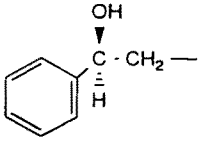
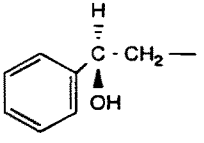
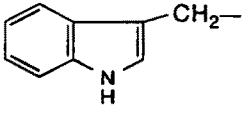
Chemicals. Glucoerucin, glucoiberin, and glucocapparin were purchased as the potassium salts from Roth Chemical Co., Karlsruhe. Sinigrin was purchased also as the potassium salt from Sigma Chemical Co. Glucotropaeolin, glucosinalbin, and glucobrassicin were supplied by G.R. Fenwick as tetramethyl ammonium salts. These were converted to the potassium salts by passing through an ion-exchange column packed with Amberlite IR-120-P (K^+ form).

Gluconasturtiin, (2*S*)-glucobarbarin, and (2*R*)-glucobarbarin were isolated from foliage of watercress (*Nasturtium officinale*) (Stokes Seeds Inc., Buffalo, New York), *Barbarea vulgaris*, and from seeds of a *B. vulgaris* subspecies from Denmark (a gift from J.K. Nielsen), respectively. Samples of fresh foliage or dry seeds were extracted in boiling ethanol for 5 min, cooled, homogenized, and filtered. The ethanolic extract was evaporated to dryness under reduced pressure and lipids were removed with *n*-hexane. The defatted residue was dissolved in water and the aqueous extract partitioned three times with 1-butanol. The postbutanol water extracts were concentrated under reduced pressure at ca. 50°C and subjected to preliminary separation by open column flash chromatography using reversed-phase columns (45 × 2 cm) packed with 30 g of 55- to 105-μm preparative C_{18} silica (Millipore Corporation, Milford, Massachusetts). Twenty-five fractions (15 ml each) were collected by sequentially eluting the column with 0.5% potassium sulfate (150 ml), water (150 ml), 25% (15 ml), 33% (15 ml), 50% (15 ml) and 100% (30 ml) methanol in water. These fractions were tested for the presence of glucosinolates by HPLC of desulfoglucosinolates (Huang et al., 1993a). Those fractions containing the glucosinolate of interest were combined, evaporated under reduced pressure, and separated by HPLC on a semipreparative reversed-phase C_{18} column (50 cm × 8 mm) using a linear water–acetonitrile gradient from 0% CH_3CN at 0 min to 100% CH_3CN at 30 min. The glucosinolates were collected and their purities were confirmed by HPLC of desulfoglucosinolates and by thin-layer chromatography on 5 × 10-cm, 0.25-mm-thick, Whatman K6 silica gel plates, with ethyl acetate–methanol–acetic acid–water (4 : 1 : 1 : 0.5) as the solvent system. The plates were dried with a hairdryer immediately after development. Spots were visualized by spraying with 1% ceric sulfate solution followed by heating at 110°C for ca. 15 min. The structures of these glucosinolates are shown in Table 1.

Insects and Plants. P. rapae and *P. napi oleracea* butterflies for behavioral assays were obtained from colonies started from field-collected insects each summer and maintained in the laboratory at ca. 22°C under fluorescent lights

TABLE 1. STRUCTURES OF GLUCOSINOLATES USED IN THIS STUDY



Trivial name	Semisystematic name	Formula R
Glucocapparin	methyl	CH_3
Sinigrin	propenyl	$CH_2{=}CHCH_2$
Glucoerucin	4-methylthiobutyl	$CH_3S(CH_2)_4$
Glucoiberin	(*R*)-3-methylsulfinyl	$CH_3SO(CH_2)_3$
Glucotropaeolin	benzyl	
Glucosinalbin	*p*-hydroxybenzyl	
Gluconasturtiin	2-phenylethyl	
(2*S*)-Glucobarbarin	(2*S*)-hydroxy-2-phenylethyl	
(2*R*)-Glucobarbarin	(2*R*)-hydroxy-2-phenylethyl	
Glucobrassicin	3-indolylmethyl	

providing a photoperiod of 16:8 hr light–dark. Oviposition occurred in the greenhouse, with supplementary lighting, at ca. 25°C. *P. rapae* were reared on cabbage (*Brassica oleracea* L. var. Golden Acre) and *P. napi oleracea* on *Conringia orientalis* plants. Pupae were separated by sex (Richards, 1940) and kept in screen cylinders until eclosion, and equal numbers of each sex were placed in each bioassay cage. Watercress plants (4 weeks old) for isolating gluconasturtiin, and cabbage plants (5 weeks old) for aqueous extracts (containing stimulants, used as control) were grown in an air-conditioned greenhouse at ca. 25°C. Supplemental light was provided by 400-W multivapor high-intensity discharge lamps. *Barbarea vulgaris* foliage for the isolation of (2*S*)-glucobarbarin was collected from the field in mid-July in Ithaca, NY.

Bioassays. Oviposition bioassays for stimulatory activity were conducted in screen cages (48 × 48 × 48 cm) in a greenhouse as described by Renwick and Radke (1988). Both *Pieris* species were tested at the same time to minimize the possible effects of differences between plant batches, intensity of sunlight and other factors on oviposition behavior. Eight pairs of newly emerged butterflies were transferred to each cage in the greenhouse. Each cage was supplied with a vial of 10% sucrose solution containing yellow food coloring and a cotton wick to facilitate feeding. During the pre-oviposition period, a cabbage plant was placed in each cage. When more than 50 eggs in one day were observed (ca. two days after emerging), the plant was removed and the butterflies were used for testing the next day. Bioassays were started at 9:30 AM and the eggs laid were counted at 3:30 PM. Treated and control plants were placed in opposite corners of the cage. Positions of plants were alternated in each cage to control for possible position effects. Neutral (stimulants were not present) bean (*Phaseolus vulgaris* var. Sieva) plants were used as the oviposition substrate as single plants at the two-leaf stage in plastic pots (6.25 × 6.25 cm). Treated plants were sprayed with glucosinolates dissolved in 70% methanol. Control plants were sprayed with cabbage postbutanol water extract (as a standard stimulant) also dissolved in 70% methanol, and one extract from the same batch of cabbage plants was used as the control throughout. The solutions (2 ml/plant) at different concentrations were applied in a fine mist with a chromatographic sprayer to both upper and lower leaf surfaces.

Design and Analysis. A replication consisted of one cage with eight pairs of butterflies and 4–12 replications were performed for each concentration of each compound. Each batch of butterflies was used for bioassays over a period of about five days, and in most cases different compounds were tested on the same butterflies on successive days. When different concentrations of a glucosinolate were compared with a constant amount of cabbage extract, the same batch of butterflies was used, and lower concentrations were tested before the higher ones. The proportion of eggs laid on the treated plant relative to the total on both treated and control plants was calculated, and the data were subjected

to arcsine square root transformation. Differences between treatments and controls were analyzed using a one-sample t test on the transformed proportions, under the null hypothesis that the total number of eggs was distributed evenly over treated and control plants. A paired t test on the transformed proportions was used to assess significance of differences between insect species. The relative stimulatory activities of glucosinolates were expressed by an oviposition stimulant index, where:

$$\text{OSI} = 100\ (\text{treated} - \text{control})/(\text{treated} + \text{control})$$

An OSI of zero indicates that the tested glucosinolate and the control (cabbage postbutanol water extract) are equally stimulatory to the insects. A positive OSI suggested that the treated plant was preferred and a negative OSI indicated that the control plant was preferred.

RESULTS

As shown in Figure 1, aromatic glucosinolates including glucotropaeolin, gluconasturtiin, (2*S*)-glucobarbarin, (2*R*)-glucobarbarin, and glucobrassicin were more stimulatory to *P. rapae* at a concentration of 10^{-4} M (2 ml, ca. 0.1 mg/plant) than were the cabbage extracts [0.2 gram leaf equivalents (gle)/plant]. Glucosinalbin, which is also aromatic, was apparently an exception. Significantly fewer eggs were laid on bean plants treated with glucosinalbin than on plants treated with cabbage extract (one-sample t test, $P = 0.05$), so that an OSI of -31.3 was obtained (Figure 1). *P. rapae* was more stimulated by cabbage extract than by any of the aliphatic glucosinolates including glucocapparin, sinigrin, glucoerucin, and glucoiberin (Figure 1). Among these compounds, glucoerucin and glucoiberin were least preferred by *P. rapae*, as indicated by negative OSIs of -62.9 and -61.2, respectively. The highest OSI for *P. napi oleracea* was obtained with sinigrin, and the lowest with gluconasturtiin (Figure 1). However, no clear-cut relationship between activity and aliphatic or aromatic structure was noted for *P. napi oleracea*. Significant differences were found between OSIs for the two *Pieris* species for glucocapparin, sinigrin, glucoerucin, glucoiberin, glucosinalbin, gluconasturtiin, and (2*S*)-glucobarbarin (Figure 1).

When glucobrassicin was tested at different concentrations while the concentration of cabbage extract (control) remained constant (0.2 gle/plant), significant differences were detected between OSIs for *P. rapae* and *P. napi oleracea* (Figure 2). When compared with *P. napi oleracea*, *P. rapae* had a higher response threshold, and the stimulatory activity of glucobrassicin to this species increased rapidly from 10^{-5} M (OSI = -27.8) to 10^{-4} M (OSI = 33.4). However, when the concentration of glucobrassicin was further increased (10^{-3} and 3×10^{-3} M), the response of *P. rapae* no longer increased. In contrast, *P. napi oleracea* had a relatively low threshold for response (Figure 2). This

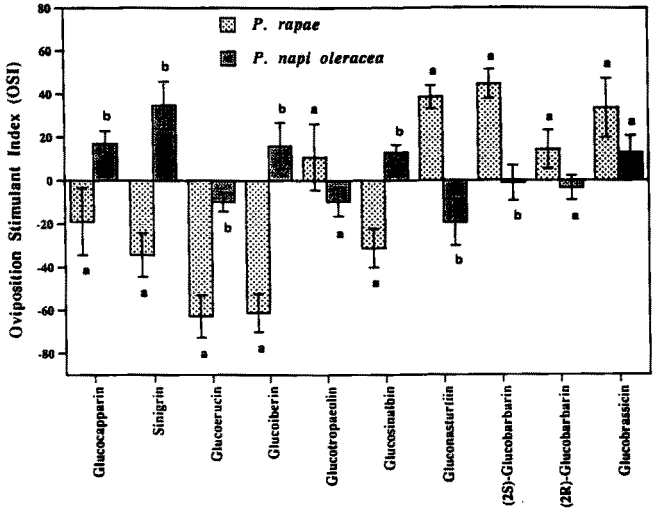


FIG. 1. Oviposition stimulant index (OSI) of glucosinolates at a concentration of 10^{-4} M for *P. rapae* and *P. napi oleracea* on bean plants. OSI = 100 (treated − control)/(treated + control). Two milliliters from a glucosinolate solution were used in each replication. Postbutanol water extract from cabbage at a dose of 0.2 gle/plant was used as control in all cases. Replicated eight times with the exception of glucosinalbin which was replicated twelve times. A replication consisted of one bioassay cage with eight pairs of butterflies. Means (±SE) with the same letters in each series are not significantly different according to a paired *t* test ($P < 0.05$).

insect was stimulated to a similar extent by glucobrassicin at the lowest concentration (10^{-6} M) and by cabbage extract, but its response changed little as the concentration of this glucosinolate was increased to 3×10^{-3} M. Significant differences in OSIs between *P. rapae* and *P. napi oleracea* were found at concentrations of 10^{-6}, 10^{-3} and 3×10^{-3} M.

P. rapae responded to increasing concentrations of gluconasturtiin in a similar manner as to glucobrassicin (Figure 3). The stimulatory effect was lower than that of the cabbage extract at a concentration of 10^{-6} M (OSI = −38.6). However, gluconasturtiin at 10^{-4} M was more stimulatory to *P. rapae* than was the cabbage extract, giving an OSI of 38.5. When the concentration was 10 or 30 times higher (10^{-3} and 3×10^{-3} M), no increase in stimulatory activity was obtained. Instead, the OSIs declined somewhat at these higher concentrations. In the case of *P. napi oleracea*, the mean numbers of eggs laid on plants treated with gluconasturtiin at all the concentrations were smaller than on plants treated with cabbage extract, so that negative OSIs were obtained in all cases (Figure 3). Unlike glucobrassicin (Figure 2), gluconasturtiin at the lowest con-

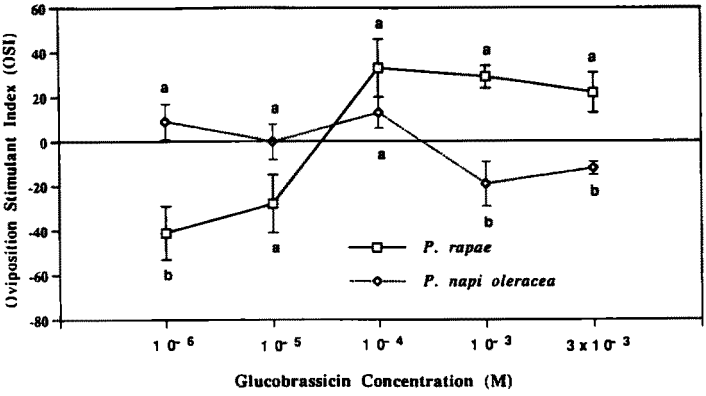


FIG. 2. Oviposition stimulant index (OSI) of glucobrassicin at different concentrations for *P. rapae* and *P. napi oleracea* on bean plants. OSI = 100 (treated − control)/(treated + control). Two milliliters from each concentration were used in each replication. Post-butanol water extract from cabbage at a dose of 0.2 gle/plant was used as control in all cases. Replicated eight times for the assay with 10^{-4} M glucobrassicin and four times for all the others. A replication consisted of one bioassay cage with eight pairs of butterflies. Means (±SE) with the same letters at each concentration are not significantly different according to a paired *t* test ($P < 0.05$).

centration (10^{-6} M) was much less stimulatory to *P. napi oleracea* than was the cabbage extract. Responses of *P. rapae* and *P. napi oleracea* to 10^{-4} or 10^{-3} M gluconasturtiin was significantly different (Figure 3).

The response threshold to sinigrin was lower for *P. napi oleracea* than for *P. rapae* (Figure 4). At the lowest concentration (10^{-6} M) of sinigrin, no significant activity was detected for *P. rapae* (OSI = −98.8; between treatment and control, $P = 0.0129$). *P. napi oleracea* was also stimulated more by the cabbage extract control than by 10^{-6} M sinigrin (OSI = −61.9, $P = 0.0021$). However, the two OSIs were significantly different between the two species (Figure 4). The response of *P. napi oleracea* was much stronger when the concentration of sinigrin was increased from 10^{-6} to 10^{-5} M, but leveled off at higher concentrations. There was also an increased response of *P. rapae* to concentrations of 10^{-5} M or higher, although the slope of the dose–response curve was much lower than for *P. napi oleracea*. A maximum OSI (41.1) for *P. rapae* occurred at 10^{-3} M, and as in the cases of glucobrassicin and gluconasturtiin, the OSI dropped slightly at higher concentrations.

To compare the absolute response thresholds of the two *Pieris* species to sinigrin, bioassays using different concentrations of sinigrin were performed using solvent alone (70% methanol, rather than cabbage extract) as a control. The number of eggs laid by *P. napi oleracea* on bean plants treated with 10^{-6}

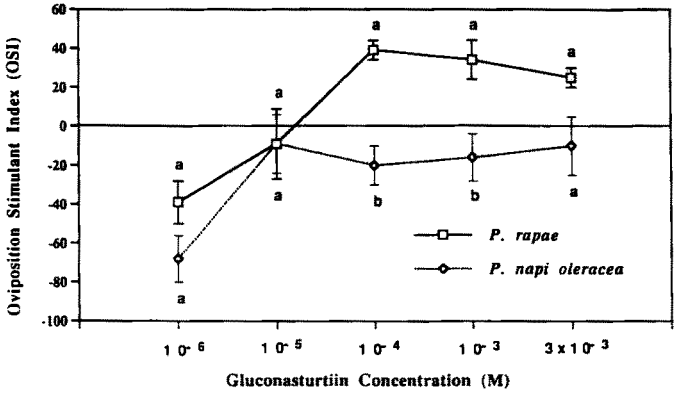


FIG. 3. Oviposition stimulant index (OSI) of gluconasturtiin at different concentrations for *P. rapae* and *P. napi oleracea* on bean plants. OSI = 100 (treated − control)/(treated + control). Two milliliters from each concentration were used in each replication. Post-butanol water extract from cabbage at a dose of 0.2 gle/plant was used as control in all cases. Replicated eight times for the assay with 10^{-4} M gluconasturtiin and four times for all the others. A replication consisted of one bioassay cage with eight pairs of butterflies. Means (±SE) with the same letters at each concentration are not significantly different according to a paired *t* test ($P < 0.05$).

M sinigrin was significantly higher than that on control plants (Figure 5, $P = 0.0009$). The stimulatory activity for *P. napi oleracea* was still significant when the concentration was decreased to 10^{-8} M. Only when the concentration was lowered to 10^{-9} M did *P. napi oleracea* cease to discriminate between the treatment and control. For *P. rapae*, although a significant difference was found between 10^{-6} M sinigrin and the control (Figure 5, $P = 0.0366$), the stimulatory effect of the treatment was very weak because the mean number of eggs laid by this species on treated plants was abnormally small. Sinigrin was not stimulatory to *P. rapae* at 10^{-7} M. Therefore, the response threshold to sinigrin was about 100 times higher for *P. rapae* than for *P. napi oleracea*.

DISCUSSION

When offered a choice between the glucosinolates (10^{-4} M) and cabbage extract containing stimulants (0.2 gle/plant), *P. rapae* was more sensitive to the aromatic glucosinolates including glucotropaeolin, gluconasturtiin, (2*S*)-glucobarbarin, (2*R*)-glucobarbarin, and the indole glucosinolate, glucobrassicin (Table 1, Figure 1). Both saturated (glucocapparin) and unsaturated (sinigrin) aliphatic representatives were less stimulatory to this species when compared with the

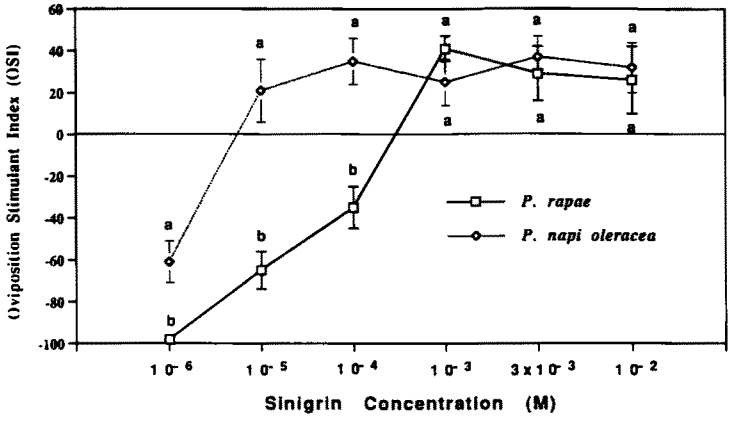


FIG. 4. Oviposition stimulant index (OSI) of sinigrin at different concentrations for *P. rapae* and *P. napi oleracea* on bean plants. OSI = 100 (treated − control)/(treated + control). Two milliliters from each concentration were used in each replication. Post-butanol water extract from cabbage at a dose of 0.2 gle/plant was used as control in all cases. Replicated eight times for the assay with 10^{-4} M sinigrin and four times for all the others. A replication consisted of one bioassay cage with eight pairs of butterflies. Means (±SE) with the same letters at each concentration are not significantly different according to a paired *t* test ($P < 0.05$).

aromatic glucosinolates. Two compounds with a sulfur atom in the side chain (glucoerucin and glucoiberin) were almost inactive when compared with cabbage extract. These results seem to fit quite well with the gradation suggested by Sachdev-Gupta et al. (1992). The only exception found in this study was the response of *P. rapae* to glucosinalbin, which, although aromatic, has a hydroxyl group on the benzyl ring. Because the two glucobarbarins also have a hydroxyl group in the side chain, but were much more stimulatory to the same species, the position of the OH may be important for activity. The pattern of activity of aliphatic and aromatic glucosinolates in the case of *P. napi oleracea* was not as evident as for *P. rapae*, but this insect did respond differently to the various compounds tested (Figure 1). Therefore, although all glucosinolates tested so far had some stimulatory activity for the two *Pieris* species, their importance as cues for oviposition by these insects may differ according to structural types.

Significant differences were found between the responses of *P. rapae* and *P. napi oleracea* to most of the glucosinolates tested (Figure 1). These two species, one naturalized and the other indigenous, co-occur in large populations in northern New England. Their distinctly different host ranges (Richards, 1940; Chew, 1977a,b; Huang and Renwick, 1993) would suggest that they have

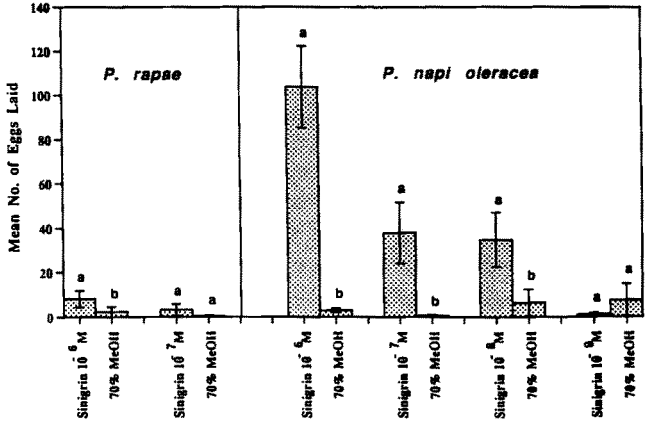


FIG. 5. Oviposition by *P. rapae* and *P. napi oleracea* on bean plants treated with sinigrin at different concentrations, with 70% methanol as control in all cases. Two milliliters from each concentration were used in each replication. Replicated eight times. A replication consisted of one bioassay cage with eight pairs of butterflies. Means (±SE) with the same letters in each series are not significantly different according to a one-sample *t* test ($P < 0.05$), under the null hypothesis that eggs were distributed evenly over control and treated plants.

evolved different sensitivities to allelochemicals, including glucosinolates that are characteristic of their host plants.

Indole glucosinolates such as glucobrassicin have been shown to be powerful oviposition stimulants for crucifer specialists including *P. rapae*, *P. brassicae*, and some other insect species (van Loon et al., 1992). In the present study, gluconasturtiin and (2*S*)-glucobarbarin were as stimulatory as glucobrassicin to *P. rapae*.

P. napi oleracea was stimulated by a much lower concentration of sinigrin than was *P. rapae* (Figure 4). This species responded to a concentration of sinigrin of 10^{-8} M (2 ml/plant), a threshold which was ca. 100 times lower than for *P. rapae* (Figure 5). *P. napi oleracea* was also much more sensitive than *P. rapae* to the low natural concentrations of glucoiberin in *Erysimum cheiranthoides* (Huang et al., 1993a) and *Iberis amara* (Huang et al., 1993b). However, butterflies of this species were less sensitive to the changing concentrations of glucobrassicin, gluconasturtiin, and sinigrin. In contrast, although *P. rapae* had a higher "discriminative threshold" for response to glucobrassicin and sinigrin when compared with cabbage extract, it was more sensitive to the changing concentrations. The response threshold concentration (in the absence of cabbage extract) of sinigrin for *P. rapae* was ca. 10^{-6} M (Figure 5; 2 ml/plant), similar to that for glucobrassicin for the same species in Australia (Tray-

nier and Truscott, 1991). For both species, there was an optimal concentration of each glucosinolate for the strongest stimulation. When the concentration increased beyond this optimum, the response remained constant or even tended to decline. This effect is understandable, because the higher concentrations used in this study were beyond the range found at the surface of host plants of the two *Pieris* species.

Sinigrin has been widely tested as a feeding stimulant for a number of crucifer specialists (Nielsen, 1989; Nielsen et al., 1989; Hagerup et al. 1990). As an oviposition stimulant, sinigrin has been found to be less active than glucobrassicin for *P. rapae* (Renwick et al., 1992). Traynier and Truscott (1991) also reported that solutions of 10^{-5} M glucobrassicin and 10^{-2} M sinigrin were equally stimulatory to the same insect species. However, in our study, the response of *P. rapae* to sinigrin increased almost linearly within a range of concentrations from 10^{-6} to 10^{-3} (Figure 4). At 10^{-3} M, ca. 0.8 mg of sinigrin was applied to a bean plant (2 ml/plant), and significantly more eggs were laid on the treated plant than on the control plant sprayed with 0.2 gle of cabbage extract. The amount of glucobrassicin applied to the control plant, based on an average of 50 mg/100 g (van Loon et al., 1992), was only eight times less than the amount of sinigrin on treated plants. Therefore sinigrin may act as an important stimulant in host recognition by *P. rapae* when the concentration is high enough, as in the case of *I. amara* (Huang et al., 1993b).

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CARDENOLIDES AS OVIPOSITION DETERRENTS TO TWO *Pieris* SPECIES: STRUCTURE–ACTIVITY RELATIONSHIPS

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Abstract—Oviposition responses of *Pieris rapae* and *P. napi oleracea* to 18 cardenolides were compared under the same conditions. Effects of different concentrations of selected cardenolides were also tested. Most of the compounds were deterrent to oviposition by both insects, but to significantly different degrees. *P. rapae* were strongly deterred by K-strophanthoside, K-strophanthin-β, cymarin, convallatoxin, oleandrin, erysimoside, erychroside, and gitoxigenin. The most deterrent compounds for *P. napi oleracea* were erychroside, cymarin, erysimoside, convallatoxin, and K-strophanthoside. Strophanthidin-based glycosides were more deterrent than digitoxigenin-based ones, and the number and type of sugar substitutions can have profound effects on activity. Both similarities and contrasts were found in responses of *P. rapae* and *P. napi oleracea* to these cardenolides. Cymarin was equally deterrent to both *Pieris* species at all concentrations tested. However, when compared with *P. rapae*, *P. napi oleracea* was less sensitive to most of the cardenolides. *P. napi oleracea* was insensitive to K-strophanthin-β and oleandrin at 0.5×10^{-4} M, which were highly deterrent to *P. rapae*.

Key Words—*Pieris rapae*, *Pieris napi oleracea*, Lepidoptera, Pieridae, oviposition, deterrents, cardenolides.

INTRODUCTION

Host selection by ovipositing adults of many phytophagous insects is critical for the survival and development of their offspring, especially when these are relatively immobile. Plant chemistry is undoubtedly an important source of infor-

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mation affecting the final decision by the adults to accept or reject the potential plant (Schultz, 1988). Specialists will oviposit if stimulants characteristic of suitable plants are present, whereas acceptance by generalists seems to be governed to a large extent by the absence of deterrents (Renwick, 1983). However, oviposition stimulants and deterrents may often co-occur in a particular plant species (Renwick and Radke, 1987), and chemoreceptors sensitive to either stimulants or deterrents may play a major role in determining the final decision by an insect (Dethier, 1982).

Cardenolides, a group of C_{23} steroid derivatives having an $\alpha:\beta$-unsaturated γ-lactone (butenolide) ring, have long been thought to serve as a defense against herbivory. These secondary metabolites are present in several families of angiosperms and are particularly abundant in the Apocynaceae and Asclepiadaceae (Roeske et al., 1976). Their biological properties that might be involved in herbivore defense include bitterness, emeticity, cardiotonic activity, Na^+, K^+-ATPase inhibition, and cytotoxicity (reviewed by Nelson et al., 1981). Cardenolides may be toxic to insects (Duffey et al., 1978; Seiber et al., 1980) or act as insect feeding deterrents (Rothschild, 1972). For example, cardenolides in *Erysimum cheiranthoides* were recently found to be responsible for the rejection of this plant by *Pieris rapae* larvae (Sachdev-Gupta et al., 1993). However, several insect species that feed on cardenolide-containing plants can sequester and store cardenolides for their own defense against vertebrate predators (Brower, 1970; Malcolm, 1992).

Cardenolides in plants may also be important in mediation of discriminatory behavior of ovipositing butterflies. Recent studies have shown a relationship between cardenolide content of milkweeds and oviposition by the monarch butterfly, *Danaus plexippus* (Oyeyele and Zalucki, 1990; Zalucki et al., 1990). Females of this species accepted milkweeds that had significantly lower mean cardenolide levels compared with rejected or control plants. Oviposition by the cabbage butterfly, *P. rapae*, is deterred by cardenolides in the unacceptable crucifer, *E. cheiranthoides*, and the active compounds were recently identified as strophanthidin glycosides (Renwick et al., 1989; Sachdev-Gupta et al., 1990). When a butanol extract containing the deterrent cardenolides from *E. cheiranthoides* was applied onto cabbage plants in the field, significantly fewer eggs were laid by *P. rapae* on treated plants than on the controls (Dimock and Renwick, 1991). Renwick et al. (1989) and Sachdev-Gupta et al. (1990) also tested some commercially available cardenolides and found that cymarin was active, but digitoxin, ouabain, and helveticoside were not deterrent to *P. rapae*. The importance of structure–activity relationships for cardenolides in mediating oviposition behavior of the insect was therefore suggested.

Oviposition deterrents for one insect may affect the behavior of associated insects in different ways. Renwick and Radke (1990) found that a chemical fraction from *E. cheiranthoides* containing cardenolides that deter *P. rapae*

oviposition were actually stimulatory to *Plutella xylostella*. When compared with *P. rapae* under similar conditions, *P. napi oleracea* was less sensitive to a combination of the deterrent cardenolides isolated from *E. cheiranthoides* (Huang et al., 1993a). Furthermore, *P. napi oleracea* was not sensitive to 2-*O*-β-D-glucosyl cucurbitacin I and 2-*O*-β-D-glucosyl cucurbitacin E from *Iberis amara*, which were identified as oviposition deterrents to *P. rapae* (Huang et al., 1993b). More studies are needed to better understand the host selection mechanisms of phytophagous insects and in particular to evaluate the involvement of deterrents in insect–plant interactions. The structural requirements for activity of cardenolides as oviposition deterrents to *P. rapae* are not yet known, and the extent to which differential sensitivities to these compounds have evolved in *P. rapae* and *P. napi oleracea* remains to be determined.

This study was designed to test the relative oviposition deterrent activities of 18 cardenolides for *P. rapae* and *P. napi oleracea* under the same conditions and to compare the sensitivities of the two *Pieris* species to different concentrations of selected cardenolides.

METHODS AND MATERIALS

Chemicals. Erysimoside and erycordin were isolated from butanol extracts of *E. cheiranthoides* as described by Sachdev-Gupta et al. (1990) and Huang et al. (1993a). K-Strophanthin-β was purchased from Roth Chemical Co. All the other cardenolides were purchased from Sigma Chemical Co. The structures of these cardenolides are shown in Figure 1.

Insects and Plants. P. rapae and *P. napi oleracea* butterflies for behavioral assays were obtained from colonies as described by Huang and Renwick (1994). Cabbage plants (5 weeks old) used as oviposition substrate were grown individually in 10-cm cord pots in an air-conditioned greenhouse at ca. 25°C. Supplemental light was provided by 400-W multivapor high-intensity discharge lamps.

Bioassays. Cardenolides were tested for deterrent activity in oviposition bioassays under the same conditions as for stimulant assays (Huang and Renwick, 1994). Treated cabbage plants were sprayed with compounds dissolved in methanol. Control plants were sprayed with solvent alone. The solutions were applied in a fine mist with a chromatographic sprayer to both upper and lower leaf surfaces. Deterrent activity was measured by applying 4 ml test solution at 0.031×10^{-4}, 0.125×10^{-4}, 0.5×10^{-4}, 2.0×10^{-4}, or 8.0×10^{-4} M on cabbage plants.

Design and Analysis. Experimental design and data transformation were the same as those described in Huang and Renwick (1994). A one-sample *t* test,

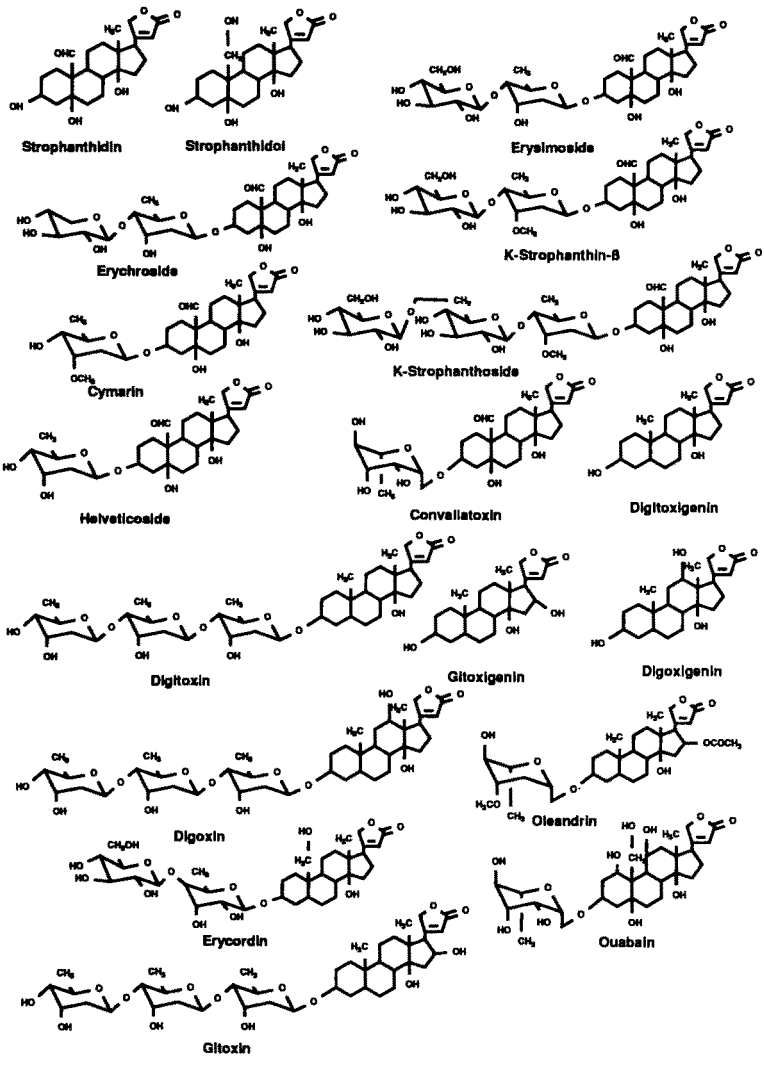

FIG. 1. Structures of cardenolides used in this study.

a paired t test, or a Waller-Duncan K-ratio t test on the transformed proportion was used to assess significance of differences between treatments and controls, between insect species, or among treatments, respectively.

Deterrent activities of cardenolides were compared by calculating an oviposition deterrent index:

$$ODI = 100 \text{ (control} - \text{treated)/(control} + \text{treated)}$$

An ODI of zero means that equal numbers of eggs were laid on treated and control plants. A positive ODI indicates more eggs were laid on control than on treated plants, whereas a negative ODI is obtained when more eggs were laid on treated plants.

RESULTS

In response to erysimoside or erychroside at 0.5×10^{-4} M (4 ml/cabbage plant) *P. napi oleracea* laid significantly fewer eggs on treated than on control plants ($P = 0.0045$ and 0.0001, respectively). Based on the ODIs, these two compounds had similar deterrent effects on the insect species (Figure 2). No significant differences were found between treatments and controls when erycordin, helveticoside, digitoxin, ouabain, or strophanthidin were tested on *P. napi oleracea*. The ODIs of these compounds were not significantly different from each other, but were significantly different from those of erysimoside and erychroside (Figure 2). These cardenolides were previously tested on *P. rapae* (Renwick et al., 1989; Sachdev-Gupta et al., 1990), and only the erysimoside

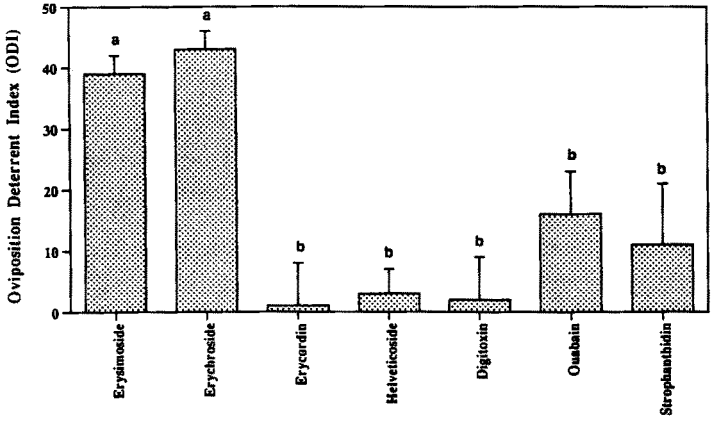

FIG. 2. Oviposition deterrent index (ODI) of seven cardenolides at a concentration of 0.5×10^{-4} M for *P. napi oleracea* on cabbage plants. ODI = 100 (control − treated)/(control + treated). Four milliliters from a cardenolide solution were used in each replication. The solvent (methanol) was used as control in all cases. Replicated eight times. A replication consisted of one bioassay cage with eight pairs of butterflies. Bars (±SE) with the same letters are not significantly different according to a Waller-Duncan *K*-ratio *t* test ($K = 100$).

and erychroside were active as oviposition deterrents. Therefore, similar patterns in responses to these compounds were observed for the two *Pieris* species, although the ODIs of erysimoside, erychroside, erycordin, helveticoside, and digitoxin for *P. napi oleracea* were somewhat lower than for *P. rapae*. However, ODIs of ouabain and strophanthidin were somewhat higher for *P. napi oleracea* than for *P. rapae*.

As shown in Figure 3, all cardenolides tested at a concentration of 0.5×10^{-4} M had some deterrent activity on *P. rapae*, with ODIs ranging from 9.0 (digoxigenin) to 61.1 (K-strophanthoside). However, *P. napi oleracea* was less sensitive to all these compounds, based on the lower ODIs (Figure 3). Cymarin was the most deterrent compound to *P. napi oleracea* (ODI = 43.2), in which case the difference between treatment and control was highly significant ($P = 0.0001$). A negative ODI of -12.8 was obtained for *P. napi oleracea* in response to digoxigenin, although the treatment and the control values were not significantly different ($P = 0.1896$). When the two *Pieris* species were compared, significant differences between ODIs for K-strophanthin-β, K-strophanthoside, or oleandrin were obtained. ODIs were not significantly different between species for strophanthidol, gitoxin, gitoxigenin, digoxigenin, digoxin, convalla-

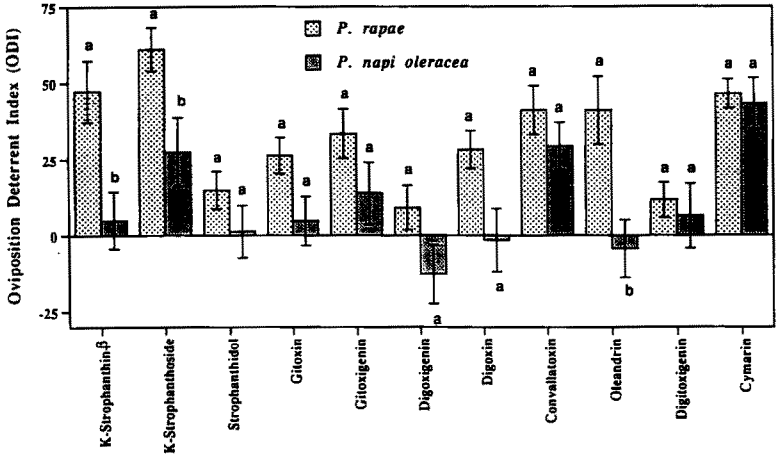

FIG. 3. Oviposition deterrent index (ODI) of 11 cardenolides at a concentration of 0.5×10^{-4} M for *P. rapae* and *P. napi oleracea* on cabbage plants. ODI = 100 (control − treated)/(control + treated). Four milliliters from a cardenolide solution were used in each replication. The solvent (methanol) was used as control in all cases. Replicated eight times. A replication consisted of one bioassay cage with eight pairs of butterflies. Bars (±SE) with the same letters in each series are not significantly different according to a paired *t* test ($P < 0.05$).

toxin, digitoxigenin, and cymarin (Figure 3). Interestingly, *P. rapae* was deterred equally by K-strophanthin-β and cymarin (K-strophanthin-α), whereas *P. napi oleracea* was only deterred by the cymarin.

P. rapae was not significantly deterred by K-strophanthin-β at a concentration of 0.031×10^{-4} M (difference between treatment and control, $P = 0.1429$) or 0.125×10^{-4} M ($P = 0.1383$), although ODIs of 34.9 and 24.2, respectively, were obtained (Figure 4). The responses of *P. rapae* and *P. napi oleracea* to K-strophanthin-β were significantly different at 0.5×10^{-4} M, but not at higher concentrations (Figure 4). Nevertheless, K-strophanthin-β at 2.0×10^{-4} M and 8.0×10^{-4} M was significantly deterrent to both *Pieris* species, giving ODIs of 49.0 (difference between treatment and control, $P = 0.0003$) and 74.2 ($P = 0.0173$) for *P. rapae*, and 31.1 ($P = 0.0268$) and 44.9 ($P = 0.0358$) for *P. napi oleracea*, respectively.

Deterrent activity of oleandrin to *P. rapae* gradually increased as concentrations were increased from 0.125×10^{-4} M to 2.0×10^{-4} M, but leveled off when the concentration was further increased to 8.0×10^{-4} M (Figure 5). Significant differences were obtained between the number of eggs laid by *P. rapae* on control or treated plants for oleandrin at 0.5×10^{-4} M or higher ($P \leq 0.0266$). No deterrent effect on *P. napi oleracea* was found at any of the

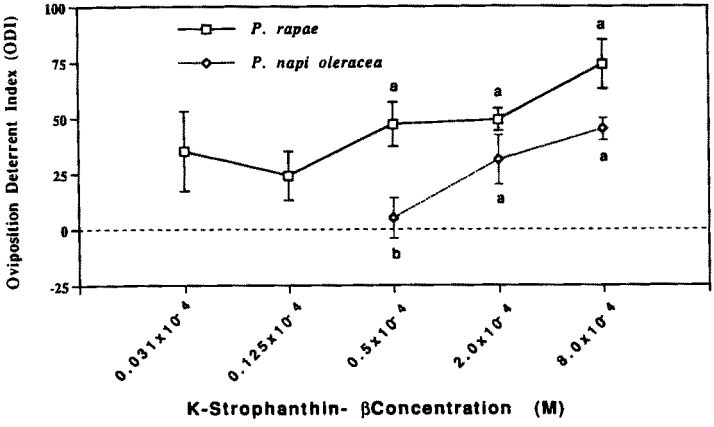

FIG. 4. Oviposition deterrent index (ODI) of K-strophanthin-β at different concentrations for *P. rapae* and *P. napi oleracea* on cabbage plants. ODI = 100 (control − treated)/(control + treated). Four milliliters from each concentration were used in each replication. The solvent (methanol) was used as control in all cases. Replicated eight times for concentrations of 0.5×10^{-4} M and 2.0×10^{-4} M, and four times for the others. A replication consisted of one bioassay cage with eight pairs of butterflies. Means (±SE) with the same letters at each concentration are not significantly different according to a paired *t* test ($P < 0.05$).

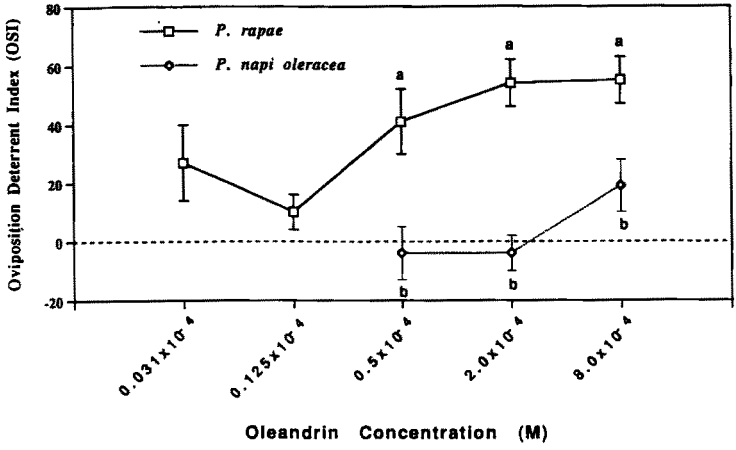

FIG. 5. Oviposition deterrent index (ODI) of oleandrin at different concentrations for *P. rapae* and *P. napi oleracea* on cabbage plants. ODI = 100 (control − treated)/(control + treated). Four milliliters from each concentration were used in each replication. The solvent (methanol) was used as control in all cases. Replicated eight times for concentrations of 0.5×10^{-4} M and 8.0×10^{-4} M, and four times for the others. A replication consisted of one bioassay cage with eight pairs of butterflies. Means (±SE) with the same letters at each concentration are not significantly different according to a paired *t* test ($P < 0.05$).

concentrations tested, although an ODI of 18.7 was obtained at 8.0×10^{-4} M ($P = 0.1054$). The responses of the two *Pieris* species to oleandrin concentrations of 0.5×10^{-4} M or higher were significantly different from each other (Figure 5).

The lowest concentration of cymarin used in this study (0.031×10^{-4} M) was significantly deterrent to *P. rapae* (between treatment and control, $P = 0.0097$), giving an ODI of 29.0 (Figure 6). The ODI increased to 51.7 when 0.125×10^{-4} M cymarin was applied to the test plants. The deterrent activity leveled off when concentration was further increased. Significant differences between the numbers of eggs laid by *P. rapae* on treated and control plants also occurred for concentrations from 0.125×10^{-4} M to 8.0×10^{-4} M. *P. napi oleracea* was significantly deterred by cymarin at all the concentrations with the exception of the lowest (0.031×10^{-4} M, $P = 0.0918$), although an ODI of 30.2 was obtained for this concentration. No significant difference in response of either *Pieris* species occurred as the concentration of cymarin was changed (Figure 6).

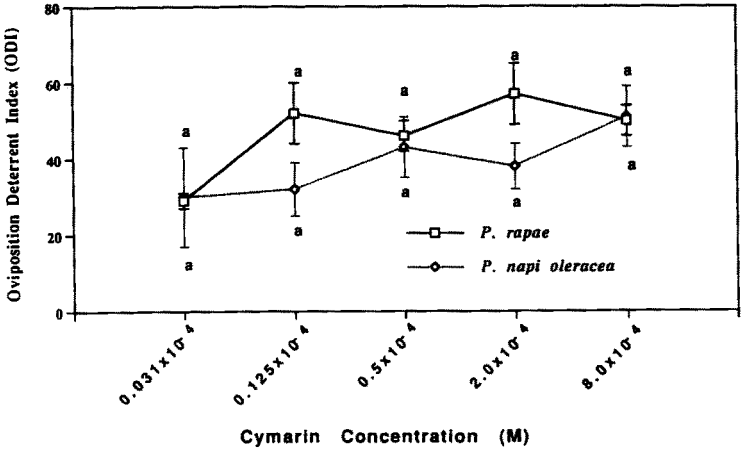

FIG. 6. Oviposition deterrent index (ODI) of cymarin at different concentrations for *P. rapae* and *P. napi oleracea* on cabbage plants. ODI = 100 (control − treated)/(control + treated). Four milliliters from each concentration were used in each replication. The solvent (methanol) was used as control in all cases. Replicated four times for concentrations of 0.031×10^{-4} M and 0.125×10^{-4} M, and eight times for the others. A replication consisted of one bioassay cage with eight pairs of butterflies. Means (±SE) with the same letters at each concentration are not significantly different according to a paired *t* test ($P < 0.05$).

DISCUSSION

The results clearly indicate that different cardenolides differ in their oviposition deterrent activity for both *P. rapae* and *P. napi oleracea*. At a concentration of 0.5×10^{-4} M, erysimoside and erychroside were deterrent to *P. napi oleracea*, but erycordin was inactive (Figure 2). Similar responses of *P. rapae* to these compounds were reported in a previous study (Sachdev-Gupta et al., 1990). The aglycone in both the active glycosides is strophanthidin, whereas the aldehydic group of strophanthidin is reduced to the corresponding alcohol in the aglycone of the inactive member of the group. Thus, distinct structural requirements for oviposition deterrent activity are suggested for both *Pieris* species. However, the two species may differ in their sensitivities to the deterrent cardenolides. The ODIs for responses of *P. napi oleracea* to the two active cardenolides were lower than for *P. rapae*, despite the fact that the concentrations of erysimoside (0.14 mg/plant) and erychroside (0.13 mg/plant) used for *P. napi oleracea* in this study were higher than those used by Sachdev-Gupta et al. (1990) (0.1 mg/plant) for *P. rapae*. This observation is consistent with

the finding that *P. napi oleracea* was less sensitive than *P. rapae* to these deterrents in extracts of *Erysimum cheiranthoides* (Huang et al., 1993a).

Some structure–activity relationship is also apparent for other groups of cardenolides. For example, the response of *P. rapae* to K-strophanthoside, K-strophanthin-β, cymarin, and convallatoxin resulted in higher ODIs than for the other cardenolides (Figure 3). As in the case of erysimoside and erychroside, these compounds are strophanthidin-based glycosides. However, the glucose and cymarose glycones seem to be necessary for activity. In strophanthidin and strophanthidol, the sugars are lacking. Helveticoside also has the strophanthidin aglycone, but the glycone consists of digitoxose alone, and the glycoside has little or no deterrent effect (Figure 3) (Renwick et al., 1989; Sachdev-Gupta et al., 1990). In tests with *P. napi oleracea*, higher ODIs were also obtained for the strophanthidin-based cymarin, convallatoxin, and K-strophanthoside (Figure 3). However, this species was much less sensitive to K-strophanthin-β than to cymarin at 0.5×10^{-4} M. The only difference between these two compounds is that K-strophanthin-β contains an additional glucose molecule, but when one more glucose is added (K-strophanthoside), the deterrent effect on *P. napi oleracea* is somewhat higher (Figure 3). Thus the number and type of sugar substitutions can have profound effects on activity, but the pattern is not yet clear. The digitoxigenin-based cardenolides tested in this study had little or no deterrent activity for either *Pieris* species (Figures 2 and 3).

Many similarities between the responses of *P. rapae* and *P. napi oleracea* to cardenolides are apparent, but distinct differences also occur. For *P. napi oleracea*, the deterrent activity of erysimoside, erychroside, erycordin, helveticoside, digitoxin, ouabain, and strophanthidin (Figure 2) showed a pattern similar to that obtained for *P. rapae* (Renwick et al., 1989; Sachdev-Gupta et al., 1990). Cymarin was equally deterrent to both species at a concentration of 0.5×10^{-4} M (Figure 3), and no significant difference in response to a series of cymarin concentrations was obtained (Figure 6). However, despite these similarities, the responses of the two species to some compounds were significantly different ($P < 0.05$). For example, K-strophanthin-β and oleandrin at 0.5×10^{-4} M were strongly deterrent to *P. rapae* but were inactive as deterrents to *P. napi oleracea*. This difference in response of the two species was still apparent at higher concentrations of oleandrin (Figure 5). Furthermore, the response threshold concentrations of K-strophanthin-β and oleandrin for *P. rapae* were much lower than for *P. napi oleracea*.

The results suggest that *P. rapae* is deterred by more compounds and is more sensitive to the active compounds than is *P. napi oleracea*. In a related study of oviposition stimulant activity, 10 glucosinolates representing different structural groups were tested, and *P. rapae* and *P. napi oleracea* showed different levels of response to most of the compounds (Huang and Renwick, 1994). Furthermore, the response threshold concentration of sinigrin was much lower

for *P. napi oleracea* than for *P. rapae*. Thus *P. napi oleracea* appears to be more sensitive to low concentrations of stimulants but is less sensitive to deterrents. Such differences in response to oviposition stimulants and deterrents undoubtedly play important roles in the differential acceptance of potential host plants by the two *Pieris* species. This point has been clearly demonstrated in the cases of *Erysimum cheiranthoides* (Huang et al., 1993a), *Iberis amara* (Huang et al., 1993b), and *Barbarea vulgaris* (Huang et al., 1994). Our findings might explain why the indigenous *P. napi oleracea* is able to utilize more plant species and is better adapted to native weeds than is the naturalized *P. rapae*.

Previous studies by Renwick et al. (1989) demonstrated the deterrent effect of cymarin on *P. rapae* at doses of 0.01 mg or 0.1 mg/plant, resulting in ODIs of 26.4 and 56.4, respectively. The cymarin concentrations of 0.031×10^{-4} M and 0.5×10^{-4} M tested in this study (4 ml/plant) were similar to those doses, and the ODIs obtained were consistent with the previous results (Figure 6). However, when concentrations were further increased fourfold and 16-fold in the present study, no increase in deterrent activity was obtained. Thus, although both *Pieris* species are readily deterred by cymarin, they are relatively insensitive to changes in concentration of this compound. Similarly, the responses of *P. rapae* to increased concentrations of K-strophanthin-β and oleandrin strengthened within certain ranges, but tended to level off at the higher concentrations. The same type of pattern was obtained for *P. napi oleracea* in response to K-strophanthin-β.

The lack of increased deterrent effects on these butterflies at higher concentrations of individual cardenolides would suggest that plant defense against herbivory cannot always be achieved by producing more of a single deterrent. The biosynthesis of multiple deterrents may be expected to provide a more effective line of defense (Jermy and Szentesi, 1978; Jermy, 1983). Furthermore, the activity of deterrents may be governed to some degree by the levels of stimulant in the plant (Hugentobler and Renwick, unpublished). Thus, the acceptability of a plant is likely to depend on the balance of positive and negative chemical cues perceived by the insect at the leaf surface (Renwick and Huang, 1994).

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ATTRACTION OF REPRODUCTIVE HONEY BEE SWARMS TO ARTIFICIAL NESTS BY NASONOV PHEROMONE

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Abstract—A crossover experimental design was established to test the attractancy of Nasonov pheromone to reproductive swarms of honey bees. Nineteen swarms were attracted to artificial nest cavities containing a slow-release blend of the Nasonov components citral, geraniol, and nerolic + geranic acids, and only four swarms were attracted to pheromone-free artificial nests. The results indicate that Nasonov pheromone plays a key role in the attraction of honey bee swarms to nest cavities.

Key Words—Honey bee, *Apis mellifera*, Hymenoptera, Apidae, pheromone, attractant, Nasonov, swarms, citral, geraniol, nerolic acid, geranic acid.

INTRODUCTION

Honey bee (*Apis mellifera*) colonies reproduce by a fission of the colony. In this process a reproductive swarm, consisting of approximately half the adult worker population and a queen, leaves the parent colony and flies to a new nesting site. After a swarm leaves the hive, it usually clusters for several hours to days in a nearby location before moving to a permanent nest site. Successful completion of the swarming process involves coordination of the activities of thousands of individuals beginning several days before swarm departure and terminating upon successful occupation of the new nest site (Free, 1987; Gary, 1992). During this time scout bees start searching for potential nest sites.

Nasonov pheromone is a key communication element in the scouting and nest-seeking behavior of honey bees. Scouts release Nasonov pheromone at the entrance of potential nest sites as well as on the surface of clustered swarms (Lindauer, 1951). Nasonov pheromone is also known to be crucial to maintain-

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ing the swarm cluster (Morse and Boch, 1971; Free et al., 1981a). Artificial nest cavities containing Nasonov pheromone-releasing lures attract honey bee swarms (Schmidt et al., 1989). Two hypotheses can explain the attraction of swarms to Nasonov-containing nest cavities: (1) the artificial nest cavities are sufficiently attractive to bees that swarms select them irrespective of pheromone; and (2) the Nasonov pheromone enhances cavity attractiveness. Reported here is an experiment designed to test these hypotheses and to determine the role that Nasonov pheromone plays in the attraction of honey bee swarms to nest cavities.

METHODS AND MATERIALS

Artificial nests composed of brown molded, reinforced wood pulp were obtained from Western Pulp Products of Corvallis, Oregon. The artificial nest cavities were inverted truncated cones measuring 40 cm top diameter, 25 cm bottom diameter, 40 cm high, each with a 3-cm-diameter hole at the bottom (Schmidt et al., 1989). The internal volume was 31 liters. Synthetic Nasonov pheromone lures consisted of two closed 400 μl polyethylene micro tubes (Bio-Rad, Richmond, California), each containing 100 μl of a mixture containing equal parts of citral (Sigma Chemical Co., St. Louis, Missouri), geraniol (Aldrich Chemical Co., Milwaukee, Wisconsin), and nerolic + geranic acids (Scentry, Inc., Buckeye, Arizona). Tubes were wrapped in light-protective black, porous paper (Schmidt and Thoenes, 1987). The pheromone packets were attached with thumb tacks to the inside the nest cavities just above the entrance hole.

Twenty experimental stations containing four artificial nests were established on March 21 and 22, 1989, in and around Tucson, Arizona. The nests were attached at heights of 2–4 m to the trunks and main branches of mesquite or other suitable trees. Most stations were at least 3 km apart. Ten stations were assigned randomly to contain a pheromone lure inside each nest, and the remaining 10 stations received no lures. Thereafter, until May 26, 1989, when the swarming season was nearly complete, the treatments were reversed at weekly intervals by detaching and exchanging pheromone tubes. This design controlled for potential location and temporal effects. Swarm attraction was determined by the presence of a swarm inside a nest cavity when opened. Occupied nests were replaced with new nests. The experiment was repeated from March 13, to May 3, 1990, with one additional station added.

RESULTS

Natural honey bee swarms discovered and selected artificial nests containing synthetic Nasonov pheromone more frequently than pheromone-free nests (Table 1). In 1989, six of a total of seven swarms were found in nest cavities

TABLE 1. ATTRACTION OF HONEY BEE SWARMS TO ARTIFICIAL NESTS

Year	Number of swarms in nest cavities containing		Prob.[a]
	Nasonov pheromone	No pheromone	
1989	6	1	0.06
1990	13	3	0.01
Total	19	4	0.002

[a] One-tailed binomial test.

containing Nasonov pheromone. In 1990, 13 of 16 swarms were found in nests containing Nasonov pheromone. Overall, 83% of swarms selected nest cavities containing pheromone. Clearly Nasonov pheromone was a significant component in the nest selection behavior of swarms.

DISCUSSION

Progress has been made in understanding bee communication via dance language and behavior during the swarming process (Lindauer, 1961), and specific criteria relating to nest cavity suitability for swarms have been discovered (Seeley, 1977; Seeley and Morse, 1978; Schmidt and Thoenes, 1987). The role of pheromones during this process was suggested (Lindauer, 1951; Free et al., 1981a), and some experimental results have been consistent with this assumption (Free et al., 1981b; Lesher and Morse, 1983; Schmidt et al., 1989). Swarms were shown in side-by-side choice experiments to select artificial nests containing Nasonov pheromone over those without pheromone, and the preference was proportional to the release rate of pheromone (Schmidt and Thoenes, 1990). Swarms similarly exhibited preference for nests emanating a more complete pheromonal blend over those with a reduced blend (Schmidt and Thoenes, 1992); however, until this report, definitive evidence was lacking to demonstrate the powerful and independent role of Nasonov pheromone.

Nasonov pheromone is of fundamental importance to honey bees during reproductive swarming and the process of nest site location. The pheromone mediates cohesiveness of swarm clusters (Morse and Boch, 1971; Free et al., 1981a) and possibly of swarming bees in flight (Lindauer, 1951) and serves to identify a nest entrance or a lost queen (Mautz et al., 1972). This experiment suggests that Nasonov pheromone also acts as a long-range attractant for nest-seeking scouts, and possibly moving swarms, and that this independent function likely is crucial for the recruitment of nest mates to a cavity after its discovery by one or more scouts. Nasonov pheromone can be regarded as a practical tool

to attract honey bee scouts and swarms to artificial nests for research and control purposes.

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BIOCATALYTIC SYNTHESIS OF (*S*)-2-TRIDECANYL ACETATE AND (*S*)-2-PENTADECANYL ACETATE, AGGREGATION PHEROMONE COMPONENTS OF *Drosophila mulleri* and *D. busckii*, BY ENANTIOSELECTIVE HYDROLYSIS WITH LIPASE

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Abstract—The two chiral pheromone acetates, (*S*)-2-tridecanyl acetate and (*S*)-2-pentadecanyl acetate, were synthesized with an enantiomeric excess (e.e.) of almost 100% by *Pseudomonas cepacia* lipase-catalyzed hydrolysis of their corresponding racemic acetates in an acetone–water solvent system.

Key Words—*Drosophila mulleri*, *D. busckii*, Diptera, Drosophilidae, aggregation pheromone, (*S*)-2-tridecanyl acetate, (*S*)-2-pentadecanyl acetate, lipase, hydrolysis, enantioselectivity, chirality.

INTRODUCTION

Lipases have been widely utilized over the past five years as a routine reagent for organic synthesis, mainly because of their ability to accept and transform enantioselectively not only natural substrates but also organic xenobiotics (Klibanov, 1990; Halgas, 1992). The enzymatic hydrolysis of the acetates of racemic alkan-2- and -3-ols was carried out by using lipase PS (lipase from *Pseudomonas cepacia*, Amano PS) in the presence of organic media and showed that the

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enantioselectivity could be enhanced in all of the organic–water solvent systems tested, particularly in an acetone–water system (Naoshima et al., 1992, 1993).

(*S*)-2-Tridecanyl acetate **(1)**, an aklan-2-yl acetate, is an aggregation pheromone component of *Drosophila mulleri* (Bartelt et al., 1989) and was previously synthesized by several methods (Gopalan and Jacobs, 1990; Enders and Plant, 1991; Hintze and Hoppe, 1992). The present work is a simplified, short-step synthesis of (*S*)-2-tridecanyl acetate **(1)** with almost 100% e.e. based upon the enzymatic hydrolysis of racemic acetate with lipase PS. The same enzymatic strategy also yielded (*S*)-2-pentadecanyl acetate **(2)** with almost 100% e.e., which is an aggregation pheromone component of *D. busckii* (Schaner et al., 1989).

METHODS AND MATERIALS

IR spectra were determined on a Fourier transform Perkin-Elmer 1720 IR spectrometer. ^{1}H NMR spectra were obtained on a Fourier transform Hitachi R-1500 (60 MHz) spectrometer or a Bruker AMX-R400 spectrometer in $CDCl_3$ solutions, using Me_4Si as an internal standard. Column chromatography was performed with 70–230 mesh silica gel (Merck Kieselgel 60 Art 7734). All solvent systems were expressed in ratios by volume (v/v). Gas chromatography was carried out on a Hitachi G-3000 chromatograph equipped with a SE-30 25-m × 0.25-mm capillary column (GL Sciences, Tokyo, Japan), using He as carrier gas. Optical rotations were measured with a Horiba SEPA-200 high-sensitivity polarimeter. Lipase from *Pseudomonas cepacia* was used (Amano Lipase PS, Amano Pharmaceutical Co., Nagoya, Japan).

Determination of Enantiomeric Purity. The enantiomeric purity of acetates (*S*)-**1** and (*S*)-**2** is based on that of the alcohols prepared by treating the acetates with KOH in methanol. Enantiomeric excesses (e.e.) of the alcohols were determined by a GC analysis of the diastereomeric esters prepared by treating the alcohols with (*S*)-2-acetoxypropionyl chloride (Slessor et al., 1985; Millar et al., 1991). The diastereomers derived from racemic alcohols were each separated into two equal peaks (column temperature, 200° C). For (±)-2-tridecanol: R_t 10.8 and 11.5 min. For (±)-2-pentadecanol: R_t 12.5 and 13.4 min.

(±)-2-Tridecanyl Acetate [(±) − 1]. To a stirred solution of (±)-2-tridecanol (5 g, 25 mmol) and 4-pyrrolidinopyridine (0.8 g) in dry CH_2Cl_2 (70 ml) was added acetic anhydride (5.1 g, 50 mmol) at room temperature. After being stirred for 5 hr, the mixture was poured into ice-water and extracted with CH_2Cl_2. The extract was washed successively with 10% HCl and water, dried over Na_2SO_4, and concentrated. The crude product obtained was purified by column chromatography on silica gel (110 g) with hexane–ether (20 : 1) to give (±)-**1** as a colorless liquid (5.76 g, 95%). The IR and ^{1}H NMR spectra were identical with those reported for (*S*)-**1** (Enders and Plant, 1991).

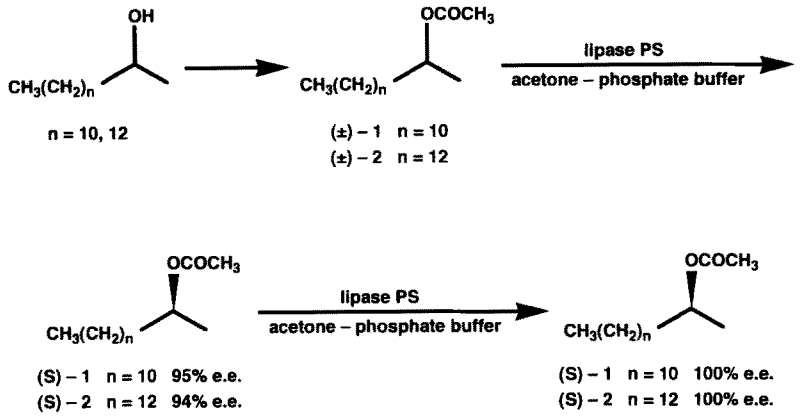
(±)-2-Pentadecanyl Acetate [(±)-2]. (±)-2-Pentadecanol (5 g, 21.9 mmol) was treated with acetic anhydride (4.47 g, 43.8 mmol) in dry CH_2Cl_2 (70 ml) in the presence of 4-pyrrolidinopyridine (0.8 g). The usual workup of the reaction mixture and subsequent purification by column chromatography on silica gel (110 g) with hexane–ether (20 : 1) gave (±)-**2** as a colorless liquid (5.63 g, 95%). Compound **2** was identified by comparison of its IR and ^{1}H NMR spectra with those of (±)-**1.**

(S)-*2-Tridecanyl Acetate [(S)-1].* A mixture of (±)-**1** (4 g, 16.5 mmol), lipase PS (1.6 g), acetone (48 ml), and 0.1 M phosphate buffer (pH 8.0, 72 ml) was stirred for 145 hr at 30°C. GC analysis showed that conversion was about 52%. After filtration through Celite, the filtrate was extracted with ether and the extract was washed with brine, dried, and concentrated. Purification by column chromatography on silica gel (70 g) with hexane–ether (25 : 1) gave (*S*)-**1** (1.76 g, 44%) with 95% e.e., $[\alpha]_D^{20} = +4.48°$ (c3.58, pentane). This acetate (1 g, 4.13 mmol) was added to a mixture of lipase PS (0.4 g), acetone (12 ml), and 0.1 M phosphate buffer (pH 8.0, 18 ml). The mixture was stirred for 115 hr at room temperature; GC showed a conversion of 17%. Column chromatography as already described gave (*S*)-**1** (0.77 g, 77%) with almost 100% e.e., $[\alpha]_D^{20} = +4.75°$ (c2.46, pentane) {literature value: $[\alpha]_D^{23} = +4.6°$ (c0.57, hexane), Bartelt et al., 1989}. The IR and ^{1}H NMR spectra were identical with those of racemic **1.**

(S)-*2-Pentadecanyl Acetate [(S)-2].* A mixture of (±)-**2** (4 g, 14.8 mmol), lipase PS (1.6 g), acetone (48 ml), and 0.1 M phosphate buffer (pH 8.0, 72 ml) was stirred for 155 hr at 30°C. GC showed a conversion of 55%. Column chromatography on silica gel (70 g) with hexane–ether (25 : 1) gave (*S*)-**2** (1.64 g, 41%) with 94% e.e., $[\alpha]_D^{20} = +4.61°$ (c3.57, pentane). This acetate (1 g, 3.7 mmol) was submitted to a second hydrolysis with lipase PS (0.4 g) in a mixture of acetone (12 ml) and 0.1 M phosphate buffer (pH 8.0, 18 ml). After being stirred for 150 hr (20% conversion), the mixture was worked up in the usual way. Column chromatography gave (*S*)-**2** (0.72 g, 72%) with almost 100% e.e., $[\alpha]_D^{20} = +4.99°$ (c2.55, pentane). The IR and ^{1}H NMR spectra were identical with those of racemic 2.

RESULTS AND DISCUSSION

As shown in Scheme 1, the key feature of the present synthesis is that the *S* configuration of the chiral pheromones **1** and **2** is established by the enantioselective hydrolysis of (±)-acetates **1** and **2** with lipase PS in an acetone–water solvent system. (±)-2-Tridecanol was converted by treatment with acetic anhydride in the presence of 4-pyrrolidinopyridine into acetate **1,** and the latter was hydrolyzed with lipase PS in an acetone–phosphate buffer. The enzymatic



SCHEME 1.

hydrolysis resulted in 52% conversion, and the unreacted acetate (95% e.e.) was resubmitted to lipase hydrolysis. (*S*)-Pheromone acetate **1** thus obtained showed an enantiomeric purity of almost 100%. Similarly, the acetate **2** derived from (±)-2-pentadecanol was initially hydrolyzed with lipase PS (55% conversion), and (*S*)-acetate **2** of 94% e.e. was obtained. The acetate was submitted to the second hydrolysis with lipase PS to give (*S*)-pheromone acetate **2** of almost 100% e.e.

Recently, Gopalan and Jacobs (1990) reported a four-step synthesis of (*S*)-**1** with 98% e.e. via the baker's yeast reduction of a ketosulfone (21% overall yield). Enders and Plant (1991) synthesized (*S*)-**1** with 93.5% e.e. in six steps from propiophenone (48% overall yield). More recently, the pheromone **1** with 98% e.e. was prepared in six steps with a 29% overall yield by the enantioselective lithiation and methylation of dodecyl carbamate (Hintze and Hoppe, 1992). A six-step synthesis of (*S*)-**1** in ca. 50% overall yield was also described starting from ethyl (*S*)-lactate (Bartelt et al., 1989). (*S*)-Pheromone **2** was prepared from ethyl (*S*)-lactate in the same manner as reported for (*S*)-**1** (Bartelt et al., 1989; Mori, 1992); there was no description of the optical rotation of (*S*)-**2** (Schaner et al., 1989).

In conclusion, we have prepared the two chiral pheromone acetates (*S*)-**1** and (*S*)-**2** with high enantiomeric excesses in three steps from (±)-2-tridecanol (32% overall yield) and from (±)-2-pentadecanol (28% overall yield), respectively, by biochemical transformation with lipase. The present method would be expected to facilitate the synthesis of optically active natural products such as chiral pheromone esters and alcohols.

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OXIME ETHER ANALOGS OF SEX PHEROMONE COMPONENTS OF TURNIP MOTH (*Agrotis segetum* SCHIFFERMÜLLER)

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Abstract—Oxime ether analogs of sex pheromone components of the turnip moth (*Agrotis segetum* Schiff.) were synthesized by the acidolytic opening of cyclic enol ethers with *O*-alkyl hydroxylamine hydrochlorides. The compounds varying in chain lengths and in the position of the C=N double bond were studied by electrophysiological single sensillum recordings (electrosensillography: ESG). The ESG data indicate in general reduced receptor interaction of all analogs investigated in comparison with natural pheromone components of the turnip moth. The data also show that the grade of decrease of receptor interaction depends on specific structural changes within the molecule. The results demonstrate high complementary pheromone–receptor relationships, predominantly depending on the position of the unsaturated group in the chain, whereas analogs with other structural changes are still recognized as a pheromone-like compound by the receptor.

Key Words—Oxime ether, NMR data, pheromone mimics, ESG studies, structure–response relationships, turnip moth, *Agrotis segetum* Schiff., Lepidoptera, Noctuidae.

INTRODUCTION

Derivatization of natural pheromone structures enables study of the properties of a receptor structure via electrophysiological valuation of response data of the receptor site to the synthesized compounds using electroantennographic (EAG)

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or electrosensillographic (ESG) techniques. Results of synthesis and biological activity of structurally modified lepidopteran pheromone components have been reported by several authors (e.g., Bestmann et al., 1986; Camps et al., 1984; Prestwich et al., 1990; Sun and Prestwich, 1990). Analogs of natural pheromone structures of the sex pheromone of *Agrotis segetum* Schiff. (Lepidoptera: Noctuidae) have been synthesized and studied for their biological significance, e.g., by Sun et al. (1992), Jönsson et al. (1991), and Bengtsson et al. (1990).

The male moth of *Agrotis segetum* is attracted by a specific female sex pheromone bouquet, consisting mainly of (Z)-5-decenyl acetate **(1a)** (Z5–10:OAc), (Z)-7-dodecenyl acetate (Z7–12:OAc), (Z)-9-tetradecenyl acetate (Z9–14:OAc), and decyl acetate at a ratio of about 1:5:2.5:0.6 (Arn et al., 1992; Hansson and Baker, 1991). Furthermore, three physiologically different pheromone-sensitive cell types were found occurring in the male antenna, which interact with single components of the sex pheromone bouquet. The authors mentioned above showed that, in wild populations of *Agrotis segetum* from Sweden, about 66% of the sensillum cells are specialized to Z5–10:OAc, and that the males, when placed in a wind tunnel, prefer a pheromone bouquet in the ratio mentioned above.

The male olfactory receptor structures in general respond to the Z-isomers of the different compounds, whereas in only one published case has E5–12:OAc been determined as a component of an *Agrotis segetum* pheromone composition (Arn, 1980; see also summarized bibliography in Arn et al., 1992).

Preliminary ESG studies (unpublished data) revealed that the male receptor structure responded in general to the E/Z isomeric mixtures as well as to the E-isomers of the main pheromone components, but with somewhat decreased sensitivity. This makes it possible to use the E/Z isomeric mixtures, resulting from the synthesis procedures used for the ESG studies. The investigations cited above dealt with partially fluorinated analogs of Z5–10:OAc obtained by selective regiochemical introduction of fluorine into the methyl and methylene position of the compound in order to probe hydrophobicity requirements of the receptor site (Sun et al., 1992), and alkyl substitution in the terminal chain of Z5–10:OAc (Jönsson et al., 1991). Azomethine analogs of natural *Agrotis segetum* pheromones have not yet been analyzed for their electrophysiological activity. In the present work, we followed a bioisosteric concept to probe the influence of the replacement of the C=C double bond with an azomethine group as well as changing of the position of the unsaturated group within the chain.

METHODS AND MATERIALS

Synthesis of Oxime Ether Analogs of Pheromones

We started with the corresponding hydroxy compound (E/Z)-5-decen-1-ol **(1b)**, where the C=C double bond should be exchanged by the C=N group. One of the simple azomethine analogs is excluded, because it exists in a cyclic

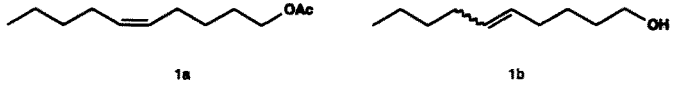
structure as 2-butylaminotetrahydropyran (Potekhin et al., 1976; Potekhin and Zhdanov, 1979; Glacet and Gaumeton, 1955). Therefore we used the corresponding oxime ethers as model substances, where the sequence $-[CH_2-CH=CH-CH_2]-$ is substituted by $-[O-N=CH-CH_2]-$ or $-[CH_2-CH=N-O]-$ (Scheme 1).

The synthesis of the oxime ethers **2** started from 5-hydroxypentanal oxime occurring exclusively in the acyclic form (Potekhin et al., 1976; Potekhin and Zhdanov, 1979), but the alkylation to **2** always yielded mixtures of the oxime ethers and nitrones. However, we found, in support of Gerecs and Windholz (1958), that 2H-3,4-dihydropyran or 2,3-dihydrofuran yields *O*-alkyl-5-hydroxypentanal oximes **(2a–d)** as well as *O*-alkyl-4-hydroxybutanaloximes **(3a–e)** by regioselective opening of the enol ether ring using *O*-alkyl hydroxylamine hydrochloride. The reaction requires a weak acid medium (pH 3–4) and produces oxime ethers **2** and **3** with a yield between 75 and 93% (details see Table 1) (Scheme 2).

The ^{13}C NMR spectra (Table 2) shows a signal doubling for most of the C atoms. This proves the occurrence of a mixture of *E* and *Z*-oxime ethers. The high-field shifted signals belong to the *Z*-configuration by means of the steric compression shift, which is also congruent with the ^{13}C shifting calculated with the increment scheme (Kleinpeter and Borsdorf, 1981). The same signal doubling has also been observed in the ^1H NMR spectra. Two doublets for **2a** (J = 11.8 Hz) were found for the methyl group at 3.78 and 3.72 ppm; for the olefinic proton the triplet of the *E*-isomer at 7.30 ppm (J = 6 Hz); for the *Z*-isomer at 6.57 ppm (J = 6 Hz).

The *E*- and *Z*-isomers of selected compounds occurring at a mixture ratio of about 3 : 1 were separated by high-pressure liquid chromatography using a Si-60-column (see experimental part) and tested subsequently for their purity by their ^1H NMR spectra. **(*E*)-2a** (retention time: 4.38 min) shows a triplet only at ~7.3 ppm, and **(*Z*)-2a** (retention time: 5.19 min) results in a triplet at ~6.6 ppm. A similar separation procedure was successful for the separation of the **(*E*)-2b** (4.34 min) and **(*Z*)-2b** (5.21 min) isomers. More details on the separation and structural elucidation will be published elsewhere.

To prove the dependence of the pheromone receptor response to the orientation of the oxime ether group within the chain, the isomeric compound **4** was synthesized by condensation of valeraldehyde with *O*-[3-hydroxypropyl]hydroxylamine.



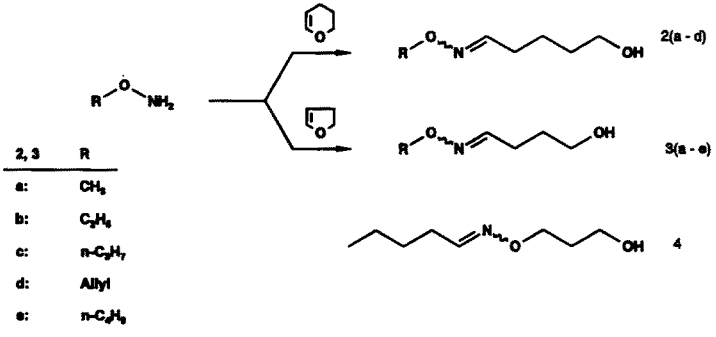
1a 1b

SCHEME 1.

TABLE 1. PREPARED *O*-ALKYL 5-HYDROXYPENTANAL OXIMES **2** AND *O*-ALKYL 4-HYDROXYBUTANAL OXIMES **3**[a,b]

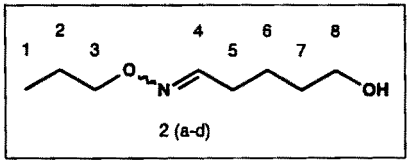
	R	Formula	Molecular peak,[c] intensity (%)	Yield (%)	bp (°C/torr)	n_D^{20}
2a	CH_3	$C_6H_{13}NO_2$ (131.2)	131 (2.1)	87	67–68/0.7	1.4523
2b	C_2H_5	$C_7H_{15}NO_2$ (145.2)	145 (0.4)	78	77–78/0.8	1.4517
2c	n-C_3H_7	$C_8H_{17}NO_2$ (159.2)	159 (0.4)	86	87–88/0.5	1.4516
2d	Allyl	$C_8H_{15}NO_2$ (157.2)	157 (0.3)	95	84–86/0.6	1.4675
3a	CH_3	$C_5H_{11}NO_2$ (117.1)	117 (0.3)	75	46–47/0.4	1.4510
3b	C_2H_5	$C_6H_{13}NO_2$ (131.2)	131 (1.1)	87	53–55/0.3	1.4465
3c	n-C_3H_7	$C_7H_{15}NO_2$ (145.2)	145 (6.6)	73	61–62/0.3	1.4472
3d	Allyl	$C_7H_{13}NO_2$ (143.2)	143 (4.9)	82	66–67/0.3	1.4474
3e	n-C_4H_9	$C_8H_{17}NO_2$ (159.2)	159 (0.4)	93	75–77/0.4	1.4513

[a] Satisfactory analytical data (±0.4% for C, H, N) were reported for all new compounds.
[b] IR spectra (film): ν_{OH} 3360–3396, ν_{CH} 2920–2944, $\nu_{C=N}$ 1630–1635, ν_{C-O} 1035–1062 cm^{-1}.
[c] The main peaks are $N{=}CH{-}(CH_2)_n{-}OH]^+$ (n = **3**, **4**) and R^+ or RO^+, respectively, depending on the nature of R.

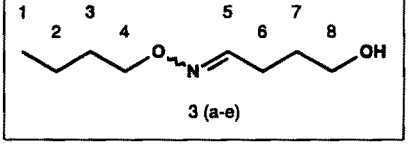


SCHEME 2.

TABLE 2. ^{13}C NMR SPECTRA OF *O*-ALKYL 5-HYDROXYPENTANAL OXIMES **2**, *O*-ALKYL 4-HYDROXYBUTANAL OXIMES **3**, AND *O*-[3-HYDROXYPROPYL] PENTANAL OXIME **4**



2 (a-d)



3 (a-e)

	C1	C2	C3	C4	C5	C6	C7	C8
2a			61.05	150.65	25.14	22.48	32.11	60.65
				151.48	28.88	22.92	32.36	
2b		14.44	68.14	150.15	25.21	22.53	32.14	60.67
		14.59	68.54	151.15	29.03	22.99	32.38	
2c	10.18	21.95	74.11	150.08	25.04	22.32	31.97	60.40
		22.08	74.43	151.66	28.81	22.79	32.22	
2d	116.41	134.80	73.72	150.86	25.26	22.45	32.07	60.64
	116.93	134.90	74.04	151.66	28.94	22.91	32.34	
a					25.40	19.70	33.60	62.70
b					29.80	23.40	33.90	
3a[c]				60.80	150.17	21.68	28.38	60.45
					150.94	25.41	28.77	60.75
3b			13.85	68.24	149.84	21.81	28.47	60.57
			13.98	68.66	150.63	25.58	28.90	60.86
3c[c]		9.72	21.80	74.41	149.65	21.69	28.52	60.61
		9.75		74.78	150.51	25.59	28.93	60.89
3d		116.79	133.56	73.65	150.44	21.86	28.37	60.52
		117.01	133.70	73.89	151.17	25.48	28.77	60.83
3e	13.30	18.53	30.51	72.59	149.52	21.80	28.52	60.57
	13.31	18.57	30.62	72.99	150.40	25.58	28.93	60.85
4	13.73	22.17	25.37	28.78	151.35	70.37	32.02	59.16
	13.94	22.44	28.25	29.15			32.30	59.70

[a] Calculated values for the *Z* isomer.
[b] Calculated values for the *E* isomer (Kleinpeter and Borsdorf 1981).
[c] The underlined values could not be clearly assigned and may be interchangeable.

Preparations and Spectra

Synthesis of O*-alkyl ω-Hydroxyalkanal Oximes (2, 3).* Freshly distilled 2,3-dihydrofuran (3.51 g, 50 mmol) or 2H-3,4-dihydropyran (4.21 g, 50 mmol) was added dropwise with stirring to *O*-alkyl hydroxylamine hydrochloride (50 mmol) in water (30 ml). Each reaction mixture was stirred for 2 hr at room temperature. Subsequently, $NaHCO_3$ (4.20 g, 50 mmol) was added, and the reaction mixture was extracted after saturation with NaCl four times with ether.

After drying with Na_2SO_4, the ether was removed, and the colorless residue was distilled (details see Table 1).

O-[3-Hydroxypropyl] Pentanal Oxime (4). To *O*-[3-hydroxypropyl]hydroxylamine (4.55 g, 50 mmol; bp 71.5–73°C/1 torr, n_D^{20} 1.4593, prepared from 3-bromopropanal and *N*-ethoxycarbonyl hydroxylamine, followed by alkaline hydrolysis (for a similar procedure see Zeeh and Metzger, 1971)), was added pentanal (freshly distilled under argon), with stirring. The temperature reached about 50°C. After 1 hr at this temperature, the mixture was cooled, small pieces of solid KOH were added and distilled: 5.67 g (71%) colorless oil, bp 70–72°C/0.6 torr, n_D^{20} 1.4462, NMR data see Table 2.

^{1}H NMR spectra were obtained with a Tesla BS 567 instrument (100 MHz, tetramethylsilane as internal standard). ^{13}C NMR spectra were recorded with an Brucker AXR 300 (75 MHz, HMDS as internal standard). All spectra were measured in $CDCl_3$. IR spectra were taken with an M 80 instrument (Fa. Zeiss) in thin layer. Mass spectra were determined with a Hewlett Packard 5988 at 70 eV. The HPLC separation was carried out with a Knauer HPL 64 instrument at a Si-60 column (solvent: *n*-hexane–EtOH 97:3 (v/v), flow rate: 6 ml/min, detection: 217 nm).

Electrophysiological Method

Single sensillum recordings were carried out on freshly prepared male antennae of juvenile *Agrotis segetum* moths according to standard procedures (see e.g., Kaissling and Thorson, 1980). The pheromone analogs were applied to filter paper strips in the order of increasing concentration levels via a defined (1 liter/min) and filtered airstream, using consistent measurement standards. The electrodes used were Ag–AgCl wires inserted into glass capillaries filled with a specific Ringer solution (Beadle-Ephrussi-Ringer). The electrode tips were additionally filled with a solution of polyvinylpyrrolidone in Ringer solution and sealed with vaseline in order to prevent drying out of the sensillum hairs.

The amplitudes of the slow (AC coupled) receptor potentials of the receptor neurons in the sensilla trichodea of the male antenna caused by this stimulation were recorded and measured subsequently for a statistical analysis (standard deviation).

RESULTS AND DISCUSSION

Table 3 summarizes the arithmetical means of the standardized ESG data, together with their standard deviations and their number of replicates. The results show that in no case did a pheromone analog reach the response values of the natural component of the *Agrotis* pheromone (Z5–10:OAc), and that most of them caused a clearly reduced ESG response or had no electrophysiological

TABLE 3. ARITHMETICAL MEANS OF STANDARDIZED ESG VALUES (ESG/ESG_{max}) OF ANTENNAL PREPARATIONS AT DIFFERENT CONCENTRATIONS

Sample[a]	Amount (μg), mean (SD)					
	0.002	0.02	0.2	2.0	20	200
***E*-2a** (5)	0.33 (0.09)	0.31 (0.18)	0.31 (0.20)	0.41 (0.18)	0.39 (0.27)	0.36 (0.24)
***Z*-2a** (5)	0.48 (0.21)	0.47 (0.24)	0.53 (0.24)	0.60 (0.13)	0.60 (0.14)	0.56 (0.09)
***E*-2b** (5)	0.29 (0.25)	0.31 (0.24)	0.32 (0.28)	0.34 (0.28)	0.42 (0.23)	0.32 (0.08)
***Z*-2b** (5)	0.34 (0.30)	0.33 (0.28)	0.36 (0.29)	0.34 (0.28)	0.42 (0.30)	0.41 (0.24)
2c (10)	0.48 (0.21)	0.53 (0.23)	0.55 (0.21)	0.58 (0.22)	0.57 (0.29)	0.61 (0.18)
2d (8)	0.51 (0.19)	0.47 (0.19)	0.51 (0.18)	0.49 (0.21)	0.58 (0.18)	0.59 (0.15)
3a (4)	0.38 (0.18)	0.23 (0.16)	0.28 (0.18)	0.38 (0.08)	0.46 (0.05)	0.45 (0.01)
3b (4)	0.22 (0.08)	0.18 (0.02)	0.30 (0.09)	0.27 (0.07)	0.30 (0.06)	0.27 (0.23)
3c (5)	0.18 (0.05)	0.17 (0.09)	0.17 (0.09)	0.21 (0.07)	0.16 (0.03)	0.27 (0.24)
3d (4)	0.15 (0.03)	0.14 (0.04)	0.17 (0.02)	0.15 (0.02)	0.18 (0.02)	0.13 (0.04)
3e (6)	0.49 (0.24)	0.36 (0.22)	0.47 (0.26)	0.46 (0.25)	0.44 (0.24)	0.41 (0.19)
4 (4)	0.49 (0.26)	0.50 (0.31)	0.43 (0.26)	0.48 (0.31)	0.43 (0.25)	0.51 (0.21)
Z5–10:OAc (10)	0.65 (0.21)	0.58 (0.23)	0.59 (0.22)	0.72 (0.24)	0.79 (0.15)	0.79 (0.15)

[a]Number of replicates is given in parentheses.

effect. Only at higher concentrations (>2.0 μg), did three of the analogs reach response values between about 50 and 60% **(Z-2a, 2c, 2d).** At concentrations of ≥20 μg, two further clusters of dose–response values are recognizable (Figure 1). With the exception of compounds **3b, 3c,** and **3d,** with ESG response values below 30% (also at higher concentrations), a second main group of compounds, comprising the rest of the studied analogs, yielded dose–response values between about 30 and 60%. Surprisingly, these results demonstrate in general that the decenyl acetate receptor also responds to pheromone analogs with a large degree of molecular change. Obviously, the loss of receptor response depends on some essential structural properties of the molecule, whereas other changes, such as the introduction of an oxime ether group and/or additional olefinic groups **(2d)** are still recognized and tolerated by the receptor structure as more or less a pheromone-like compound.

The smallest ESG values were measured for compounds **3b–d.** Analogs showing a change in the position of their C=N double bond within the chain (i.e., a shortening or elongation of the chain length between the double bond and the functional groups) obviously can not be recognized by the receptor site as a pheromone structure.

Although not researched in the present study, the comparatively higher ESG response values of **3a** could be explained by the higher volatility caused by the reduced chain length, and to a lesser degree the same may be true also for **4.** For the given experimental (ESG) technique, a comparatively higher amount of substance per time unit is transported by the airstream to the antenna

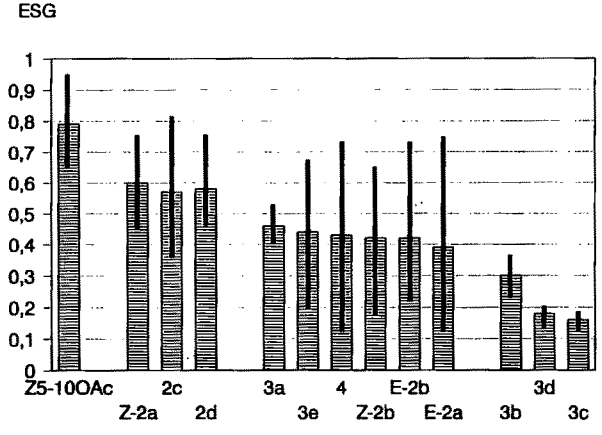


FIG. 1 Comparison of the standardized dose–response value (ESG/ESG_{max}) at 20 μg active compound on the filter paper strip.

preparation and thus causes the higher ESG signals (for details see Bengtsson et al., 1990).

Compounds *Z*-**2a**, **2c**, and **2d** (the last with a second double bond in the terminal position) show the highest ESG response among the analogs studied. However, all of these compounds have an unchanged chain length between the unsaturated and the functional groups in relation to the natural pheromone. Compounds **3a–e** [with a shifting of the double bond from position (*E*/*Z*)-5 to (*E*/*Z*)-4] and **4**, show in no case a response value over 65%.

The electrophysiological response values of the *E*- and *Z*-isomers demonstrate that the receptor prefers the *Z*-isomer of **2a**, whereas with **2b**, no distinction between the isomers was found. Further experiments are required to clarify the reason for this different reaction.

The distinct decrease of the electrophysiological activity of all pheromone analogs analyzed indicates again that the pheromone–receptor interaction is a highly complementary process. Single cell studies on *Agrotis segetum* demonstrate that, in general, chain-shortened (Bengtsson et al., 1990) or chain-elongated (Liljefors et al., 1985) analogs of pheromone structures result in a lower electrophysiological activity of the compounds in comparison with natural pheromones. Bengtsson et al. (1990) interpreted their results in terms of a receptor interaction model (see also Liljefors et al. 1987), suggesting an interaction of the terminal alkyl chain with a hydrophobic "pocket" of the receptor site extending over the two methylene groups closest to the terminal methyl group of the pheromone. Prestwich et al. (1990) reported electrophysiological studies on perfluorinated moth pheromones. The authors showed that replacement of terminal alkyl groups with perfluoroalkyl groups in pheromone components yields electrophysiologically active compounds with reduced ESG response and suggested that the binding of the fluorinated analogs to the receptor is reduced as a result of the less favorable interaction between the polar perfluoroalkyl compounds and the hydrophobic protein binding site of the receptor structure. Furthermore, Jönsson et al. (1991) showed that the replacement of the terminal methyl group of *Z*5–10:OAc with larger and branched alkyl groups reduces the biological activity of these analogs dramatically and suggested a highly steric selectivity of the receptor with regard to the terminal alkyl group.

The results described in the present paper support, in general, receptor models, which suggest a predominantly steric conditioned selectivity of the receptor site. The ESG responses of the sterically changed analogs (especially **3b–d**) are apparently more reduced than those with the same molecular constitution, such as natural structures, along with any replacement of C atoms with heteroatoms within their chain (e.g., **2c**, **2d**).

The clearly graduated distribution of the dose–response profiles of the oxime ether analogs (Figure 1) supports also pheromone–receptor models discussed by Bestmann and coworkers (e.g., Bestmann et al., 1979; Bestmann et al., 1986).

According to such models, the majority of the moth pheromone components can occur in different conformations, especially the long-chained, nonbranched and free rotating pheromone molecules. Their interaction with a putative receptor is characterized by a flexible and a step-wise adaptation to the pheromone receptor structure. Such models could also explain rather easily why the receptor also still responds to highly modified pheromone analogs with weak ESG signals, whereas responses at the optimum level strictly depend on some essential steric properties, which are required by the specific receptor structure (e.g., position of the functional groups within the molecule, chain length of the terminal alkyl group, polarity of the terminal rest, etc.). The triggering of a useful and specific behavioral pattern apparently requires the complete repertoire of constitutional and configurative properties of the natural pheromone structure being highly complementary to the receptor structure.

CONCLUSIONS

Oxime ether analogs **(2, 3)** of the natural pheromone component **(1a)** of the turnip moth interact with the receptor site to a rather reduced degree. Single sensillum recordings support that the recognition of a pheromone structure by the receptor is a flexible adaptation of the pheromone molecule to the receptor site, but, on the other hand, the optimum receptor potential level (possibly corresponding with a specific behavioral pattern) exclusively depends on the occurrence of a molecule with the complete structural properties of the corresponding natural pheromone.

Apparently, several constitutional and configurative properties of the molecule are of special significance to the recognition process. The chain length of the terminal alkyl group, i.e., the position of the unsaturated group within the chain, seems to be one of the essential molecular properties required for an optimal receptor response.

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COMPOSITION OF LARVAL SECRETION OF *Chrysomela lapponica* (COLEOPTERA, CHRYSOMELIDAE) AND ITS DEPENDENCE ON HOST PLANT

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Abstract—The defensive secretion of *Chrysomela lapponica* larvae, which is produced by nine pairs of exocrine dorsal glands, has been chemically analyzed. The *C. lapponica* larvae were kept in the laboratory on leaves of either birch (*Betula pendula*), alder (*Alnus glutinosa*), or willow (*Salix fragilis*). Larvae developed normally on birch and willow, whereas those on alder died within a few days. GC-MS analyses of the secretion of larvae on birch and willow revealed that the composition of this secretion differs distinctly from the known ones of several other *Chrysomela* species feeding exclusively on Salicaceae. In the exocrine secretion of larvae on birch, 69 compounds were identified, which included the main components isobutyric acid, 2-methylbutyric acid, and esters of the two. Several of the esters have not been reported previously from nature. The alcoholic components of the esters may be hydrolysis products of *Betula* glycosides. Most components of the secretion of larvae feeding on birch were also found in the secretion of larvae feeding on willow. In addition, major amounts of benzoic acid and salicylalcohol were present in the secretion of the larvae feeding on willow. *C. lapponica* obviously acquires salicylalcohol by hydrolysis of salicin from willow leaves. However, in contrast to other *Chrysomela* species, *C. lapponica* larvae oxidize only traces of salicylalcohol to salicylaldehyde. The repellent activity of single authentic compounds of the secretion of larvae feeding on birch and willow, respectively, was tested in laboratory bioassays with ants (*Myrmica sabuleti*). Biosynthetic pathways to some identified compounds are suggested and discussed under evolutionary and functional aspects.

Key Words—Coleoptera, Chrysomelidae, *Chrysomela lapponica*, larval secretion, defense.

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INTRODUCTION

It is well known from several chrysomelid taxa that larvae defend themselves against enemies by oozing exocrine secretion from dorsal glands when attacked. The species of the subtribes Chrysomelina and Phyllodectina (Chrysomelinae, Chrysomelini) possess nine pairs of dorsal exocrine glands located in the meso- and metathorax and in the abdominal segments 1–7. Iridoid monoterpenes have been identified as main components of their larval glandular secretions for many of these species (review: Pasteels et al., 1988a,b). These compounds are produced de novo by the larvae via the acetate–mevalonate pathway (Lorenz et al., 1993). On the other hand, a few chrysomeline species use direct host plant precursors for production of main components of their larval secretions. *Gastrolina depressa* feeds on Juglandaceae and contains juglone in its larval secretion, which is obviously derived from the host plant (Matsuda and Sugawara, 1980). Several *Chrysomela* species and *Phratora vitellinae*, which feed on Salicaceae, use salicin of their host plants, hydrolyze this phenolglycoside, and oxidize the resulting aglycone by a specific enzyme to salicylaldehyde, which is discharged by the exocrine larval glands (Pasteels et al., 1982, 1984, 1990; Wain, 1943). In contrast to these *Chrysomela* species feeding exclusively on Salicaceae, *C. interrupta* larvae, which feed on alder (Betulaceae), contain 2-phenylethyl isobutyrate and 2-phenylethyl 2-methylbutyrate as the main components of their exocrine secretions (Blum et al., 1972). These compounds or possible precursors are unknown from alder leaves. Nevertheless, the glycosides of 2-phenylethylalcohol are believed to be common constituents of the green parts of plants (Stahl-Biskup et al. 1993).

According to Brown (1956), the nearctic *C. interrupta* and its sibling species, which feed on Betulaceae and/or Salicaceae, have often been confused with the European *C. lapponica*, for which birch and alder (both Betulaceae) as well as willow and poplar (Salicaceae) are cited as host plants (Brown, 1956; Mohr, 1966). The chemistry of the larval secretion of *C. lapponica* has not been studied before, whereas the morphology of the larval dorsal glands of this species has been known for a long time (Garb, 1915). Since several other *Chrysomela* species have specialized on willows and use mainly one particular allelochemical of their host plant (salicin), *C. lapponica* is an appropriate species to examine how a switch from one host plant family to another one with different characteristic plant allelochemicals may influence the composition of larval secretion. Summaries of known allelochemicals of Salicaceae and Betulaceae are given by Hegnauer (1964, 1973), Merkx and Baerheim Svendson (1990), Palo (1984), and Thieme (1971). Will *C. lapponica* larvae use the different plant allelochemicals for the production of their exocrine secretion compounds? Does the larval secretion of *C. lapponica* differ from the known one of its closely

related species *C. interrupta*? In order to answer these questions we conducted the study presented here.

METHODS AND MATERIALS

Larvae of *C. lapponica* (L1, L2) feeding on *Betula pendula* were collected in June 1992 near Selb, Bavaria. The larvae were kept in the laboratory in climate chambers (20°C, light–dark cycle: 16 hr/8 hr) on *B. pendula*, *Alnus glutinosa*, and *Salix fragilis*. Small twigs of the host plants placed in water-filled vials were offered to the larvae. After one to two weeks, the exocrine secretion of the dorsal glands was analyzed by GC-MS. Living larvae were placed under a stereomicroscope, disturbed with forceps, and the emerging secretion was directly collected with a microsyringe. This secretion was immediately analyzed by the following GC-MS systems:

System I. A VG 70/250 S mass spectrometer coupled to a Hewlett-Packard HP 5890 A gas chromatograph with splitless injection (injector temperature 250°C) or on-column injection, equipped with a 30-m × 0.32-mm Rt_x-5 (Restek) fused-silica column programmed from 50°C to 300°C at 5°C/min. EI (70 eV) and CI mass spectra (70 eV, isobutane) were recorded. The same column and conditions were used for GC analyses. Enantiomer separations were performed using this GC-MS system equipped with a 50-m fused silica capillary coated with a 1:1 mixture of heptakis(2,6-di-*O*-methyl-3-*O*-pentyl)-β-cyclodextrine and OV-1701 (König et al. 1992).

System II. EI mass spectra (70 eV) were obtained using a Carlo Erba Vega Series 2 gas chromatograph with splitless injection (injector temperature 220°C) coupled to a Finnigan MAT Ion Trap Detector ITD 800. A 12.5-m × 0.32-mm FS-OV-1701 (Chrompack, Frankfurt, Germany) column programmed from 60°C to 280°C at 10°C/min was used.

Helium was used as the carrier gas in both systems.

The structures of the identified compounds were confirmed by comparison with synthetic samples. Esters of isobutyric acid and 2-methylbutyric acid were synthesized by conventional methods (Tietze and Eicher, 1991). Mono- and diesters were separated by column chromatography, if necessary. (*S*)-2-Methylbutyric acid was prepared by hydrolysis of commercially available (*S*)-2-methylbutyric anhydride. (*S*)-1,2-Propanediol was obtained from ethyl (*S*)-lactate. (*R*)-1,3-Hexandiol was synthesized by reduction of ethyl 3-oxohexanoate with baker's yeast yielding ethyl (*R*)-3-hydroxyhexanoate, followed by reduction with lithium aluminum hydride (Dillon et al., 1991). (*R*,*E*)-8-Hydroxylinalool [(*R*,*E*)-2,6-dimethyl-2,7-octadiene-1,6-diol] was obtained by catalytic selenium dioxide oxidation (Umbreit and Sharpless, 1977) of (*R*)-(−)-linalool (ICN, Meckenheim, Germany).

Microreductions were performed to obtain further information on the nature of the secretions. Collected larval secretions were injected into 200 μl of absolute diethyl ether and 5 mg prewashed $LiAlH_4$ added. After stirring for 3 hr, ice was added and the aqueous phase extracted three times with diethyl ether. After filtration of the combined organic phases over prewashed anhydrous Na_2SO_4, the solvent was reduced to a volume suitable for GC-MS analyses, or treated with 100 μl MSTFA (*N*-Methyl-trimethylsilyltrifluormethylacetamide) for 3 hr. Careful removal of solvent and excess reagent furnished a modified extract suitable for GC-MS analyses.

In order to examine the biological significance of the compounds identified, the repellent activity of single synthetic secretion components against ants was tested in laboratory bioassays with *Myrmica sabuleti*, which is known as generalized entomophagous predator (Hölldobler and Wilson, 1990). One microliter of a pure, fluid, synthetic component (purity: 99%) was placed on a small piece of glass (5 mm × 10 mm), which was deposited in one arm of a T-shaped tube of Plexiglas (T axis: 8 cm long; each T arm: 7.5 cm long; inner size of tube: 1 × 1 × 1 cm, openings of the T tube at the base of the T axis and at both ends of the T arms). When testing the repellent activity of the solids benzoic acid (Roth AG, Basel, Switzerland) and salicylalcohol (Sigma GmbH, Deisenhofer, Germany), 1 mg was placed onto the small piece of glass. The repellent activity of the natural larval secretion was examined by collecting the secretion of one larva with a small piece of filterpaper (5 × 10 mm) and by placing it into a T arm.

M. sabuleti workers of a laboratory colony were released at the base of the T tube. The ants that ran to the T arm with the synthetic component and natural secretion (test side) or to the opposite control side were counted. Untreated pieces of glass (synthetic components) or filter paper (natural secretions) were located in the control T arm. Reactions of 20 ants were tested for each component. After monitoring the reaction of one individual, the tube was cleaned before testing the next one. Only those ants were counted that immediately ran to the openings of one of the T arms. Ants that left the T tube at the base where they were released were discarded. The sign test for paired observations was used for statistical analysis (Lorenz, 1988).

RESULTS

C. lapponica larvae developed normally on birch or willow, whereas larvae that were fed with alder leaves died within a week. Thus, only the secretions of larvae feeding on birch or willow (referred to below as birch larvae and willow larvae) were investigated. The total ion current chromatogram of the secretion of both types of larvae is shown in Figure 1. A list of the identified compounds is given in Table 1.

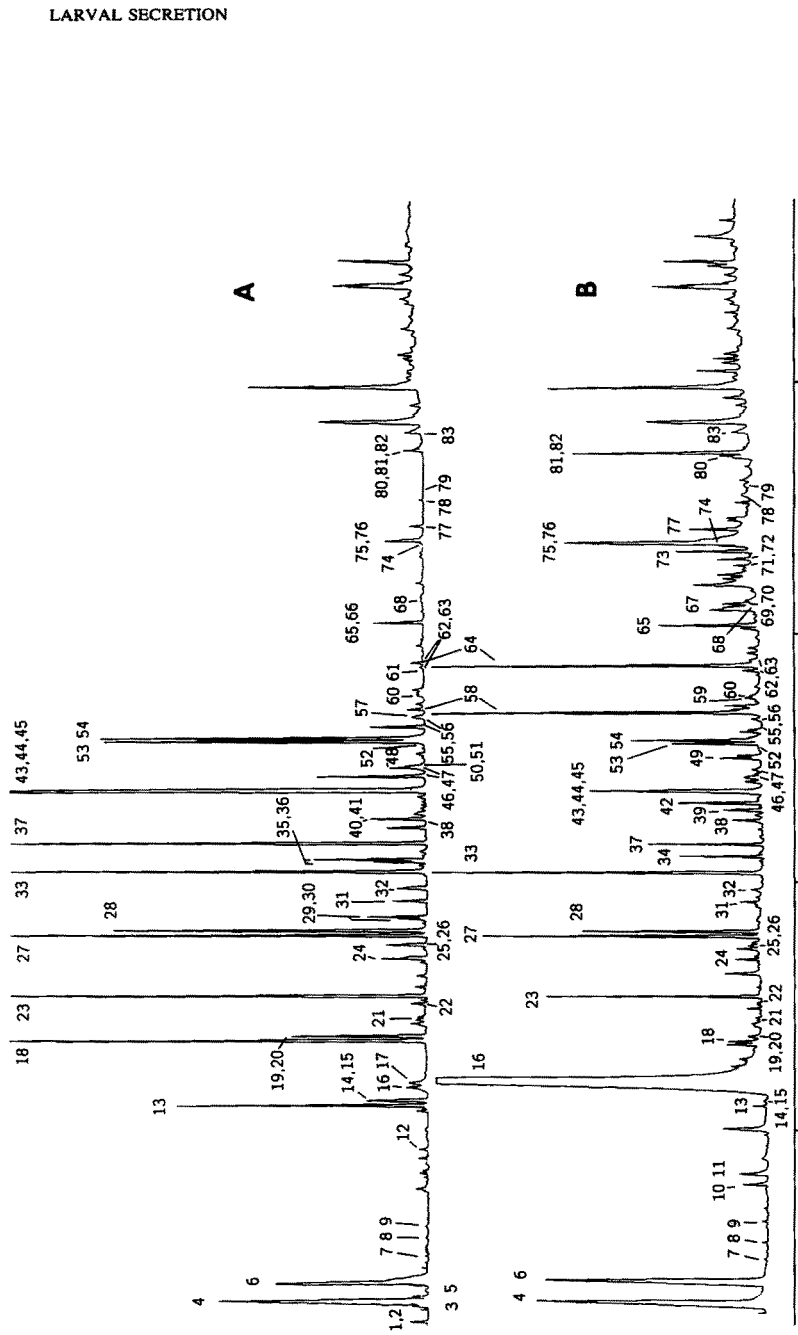

FIG. 1. Total ion current chromatograms (TIC) of exocrine larval secretion of *Chrysomela lapponica* larvae feeding on birch, *Betula pendula* (A) or willow, *Salix fragilis* (B). Both chromatograms were obtained with GC-MS system I (see text); numbering of compounds according to Table 1.

TABLE 1. COMPOUNDS IDENTIFIED FROM EXOCRINE SECRETION OF *Chrysomela lapponica* LARVAE FEEDING ON BIRCH (*Betula pendula*) OR WILLOW (*Salix fragilis*)[a]

	Compound	*Betula*	*Salix*
1	2-Methylbutanal	+	
2	3-Methylbutanal	+	
3	Ethyl isobutyrate	+	
4	Isobutyric acid	+++	+++
5	Ethyl 2-methylbutyrate	+	
6	2-Methylbutyric acid	+++	+++
7	2-Methylbutyl isobutyrate	+	+
8	Phenylacetaldehyde	+	+
9	2-Methylbutyl 2-methylbutyrate	+	+
10	Salicylaldehyde		+
11	Benzyl alcohol		+
12	Linalool oxide	+	+
13	(*Z*)-3-Hexenyl isobutyrate	++	+
14	Hexyl isobutyrate	++	+
15	5-Hexenyl isobutyrate	++	+
16	Benzoic acid		+++
17	6-Ethenyl-2,6,6-trimethyltetrahydropyran-3-ol	+	
18	(*Z*)-3-Hexenyl 2-methylbutyrate	++	+
19	Hexyl 2-methylbutyrate	++	+
20	5-Hexenyl 2-methylbutyrate	++	+
21	1,2-Ethandiyl diisobutyrate	++	+
22	1,2-Propandiyl diisobutyrate	+	+
23	Benzyl isobutyrate	+++	+++
24	1,2-Ethandiyl 1-isobutyrate 2-methylbutyrate	+	
25	1,2-Propandiyl 1-isobutyrate 2-(2-methylbutyrate)	+	+
26	1,2-Propandiyl 2-isobutyrate 1-(2-methylbutyrate)	+	+
27	Benzyl 2-methylbutyrate	+++	+++
28	2-Phenylethyl isobutyrate	+++	+++
29	*cis*-2-Ethenyl-2,6,6-trimethyltetrahydropyran-3-yl isobutyrate	++	
30	*trans*-2-Ethenyl-2,6,6-trimethyltetrahydropyran-3-yl isobutyrate	++	
31	1,2-Ethandiyl bis(2-methylbutyrate)	+	+
32	1,2-Propandiyl bis(2-methylbutyrate)	+	+
33	2-Phenylethyl 2-methylbutyrate	+++	+++
34	Geranyl isobutyrate		++
35	*cis*-2-Ethenyl-2,6,6-trimethyltetrahydropyran-3-yl 2-methylbutyrate	++	
36	*trans*-2-Ethenyl-2,6,6-trimethyltetrahydropyran-3-yl 2-methylbutyrate	++	
37	1,3-Hexandiyl diisobutyrate	+++	++
38	(*Z*)-3-Hexenyl benzoate	+	++
39	Hexyl benzoate		+
40	(*Z*)-8-Isobutyryloxylinalool	++	
41	1,4-Hexandiyl diisobutyrate	+	
42	Geranyl 2-methylbutyrate		++

TABLE 1. CONTINUED

	Compound	*Betula*	*Salix*
43	1,3-Hexandiyl 1-isobutyrate 3-(2-methylbutyrate)	+++	++
44	1,3-Hexandiyl 3-isobutyrate 1-(2-methylbutyrate)	+++	++
45	(*E*)-8-Isobutyryloxylinalool	+++	+++
46	*cis*-1,4-Cyclohexandiyl diisobutyrate	+	+
47	*trans*-1,4-Cyclohexandiyl diisobutyrate	+	+
48	(*Z*)-8-(2-Methylbutyryloxy)linalool	++	
49	1,2-Ethandiyl 1-benzoate 2-isobutyrate		+
50	1,4-Hexandiyl 1-isobutyrate 4-(2-methylbutyrate)	+	
51	1,4-Hexandiyl 4-isobutyrate 1-(2-methylbutyrate)	+	
52	2-(4-Hydroxyphenyl)ethyl isobutyrate	+	+
53	1,3-Hexandiyl bis(2-methylbutyrate)	+++	++
54	(*E*)-8-(2-Methylbutyryloxy)linalool	+++	+++
55	*cis*-1,4-Cyclohexandiyl isobutyrate 2-methylbutyrate	+	+
56	*trans*-1,4-Cyclohexandiyl isobutyrate 2-methylbutyrate	+	+
57	1,4-Hexandiyl bis(2-methylbutyrate)	+	
58	Benzyl benzoate	+	+++
59	1,2-Ethandiyl benzoate 2-methylbutyrate		+
60	2-(4-Hydroxyphenyl)ethyl 2-methylbutyrate	+	+
61	Rhododendryl isobutyrate	+	
62	*cis*-1,4-Cyclohexandiyl bis(2-methylbutyrate)	+	+
63	*trans*-1,4-Cyclohexandiyl bis(2-methylbutyrate)	+	+
64	2-Phenylethyl benzoate	+	+++
65	Methyl palmitate	++	++
66	Rhododendryl 2-methylbutyrate	+	
67	Geranyl benzoate		++
68	Ethyl palmitate	+	+
69	1,3-Hexandiyl 1-benzoate 3-isobutyrate		++
70	1,3-Hexandiyl 3-benzoate 1-isobutyrate		++
71	1,3-Hexandiyl 3-benzoate 1-(2-methylbutyrate)		++
72	1,3-Hexandiyl 1-benzoate 3-(2-methylbutyrate)		++
73	(*E*)-8-Benzoyloxylinalool		++
74	Methyl linoleate	++	++
75	Methyl linolenate	++	+
76	Methyl oleate	+	+
77	Methyl stearate	+	+
78	Ethyl linolenate	+	+
79	Ethyl stearate	+	+
80	Methyl eicosadienoate	++	+
81	Methyl eicosatrienoate	++	+
82	Methyl eicosenoate	++	+
83	Methyl eicosanoate	++	+
84	Salicylalcohol		+++

[a] + (trace components), ++ (minor components), +++ (major components).

Isobutyric and 2-methylbutyric acids could be easily identified by their mass spectra. Most of the other compounds exhibited mass spectra with prominent ions at m/z = 43, 71, and 89, or at m/z = 57, 85, and 103. In some spectra both ion groups occurred (Figure 2). These spectra indicated the presence of mono- and diesters of the mentioned acids in the secretion. Molecular weights were obtained by CI-MS. Reduction of the secretion, followed by silylation led to the identification of the following alcohols in the derivatized extracts: (*E*)- and (*Z*)-8-hydroxylinalool, 1,3-hexandiol, benzylalcohol, and 2-phenylethanol were major components, while ethandiol, 1,2-propandiol, 1,4-hexandiol, hexanol, (*Z*)-3-hexenol, 5-hexenol, *cis*- and *trans*-1,4-cyclohexandiol, pyranoid *cis*- and *trans*-linalool oxide (2,2,6-trimethyl-6-vinyltetrahydropyran-3-ol), 4-(4-hydroxyphenyl)-butan-2-ol (rhododendrol), and fatty alcohols occurred in smaller amounts. The naturally occurring compounds represent, therefore, mono- and diesters of these alcohols with isobutyric and 2-methylbutyric acids except for the fatty alcohols. These were formed during the derivatization procedure by reduction of methyl and ethyl esters of fatty acids present in the secretion. Unsymmetrical diols such as 1,3-hexandiol occurred as four different types of diesters (Table 1 and Figure 3).

Generally, only primary and secondary alcohols are esterified, while tertiary alcohol functions remain free. For example, 8-hydroxylinalool is esterified at the primary hydroxy group at C-8 only, and no diester could be detected in the natural secretion. While small amounts of the furanoid linalool oxide with a free alcohol group were present, no respective ester could be identified. The esters and the acids were the predominant constituents of the secretion, but small amounts of free alcohols and some aldehydes were also present. Thus, the secretion was made up by a complex mixture of more than 60 compounds. Unambiguous proof of the structures was obtained by comparison with synthetic samples (see Methods and Materials). Some compounds, which represent also esters of the mentioned acids, remained unidentified (see below).

Most of the compounds identified from the secretion of birch larvae were also present in willow larvae (Figure 1A and B, and Table 1). The major difference was the occurrence of large amounts of benzoic acid and salicylalcohol in the secretion of willow larvae. Benzoic acid esters could be readily identified in the secretion by their diagnostic ions at m/z = 77 and 105. This acid was esterified with the alcohols present in birch larvae esters, except for the linalool oxides, esters of which were absent in willow larvae. In addition, also esters of geraniol occurred in willow larvae. Benzyl benzoate and 2-phenylethyl benzoate became major components in their secretion.

Salicylalcohol could not be identified by GC-MS in extracts investigated on a 30-m fused silica column coated with an apolar phenylmethylsilicone phase using splitless injection or on-column injection. This may be due to the very polar character of the molecule, being unable to dissolve in the apolar silicone.

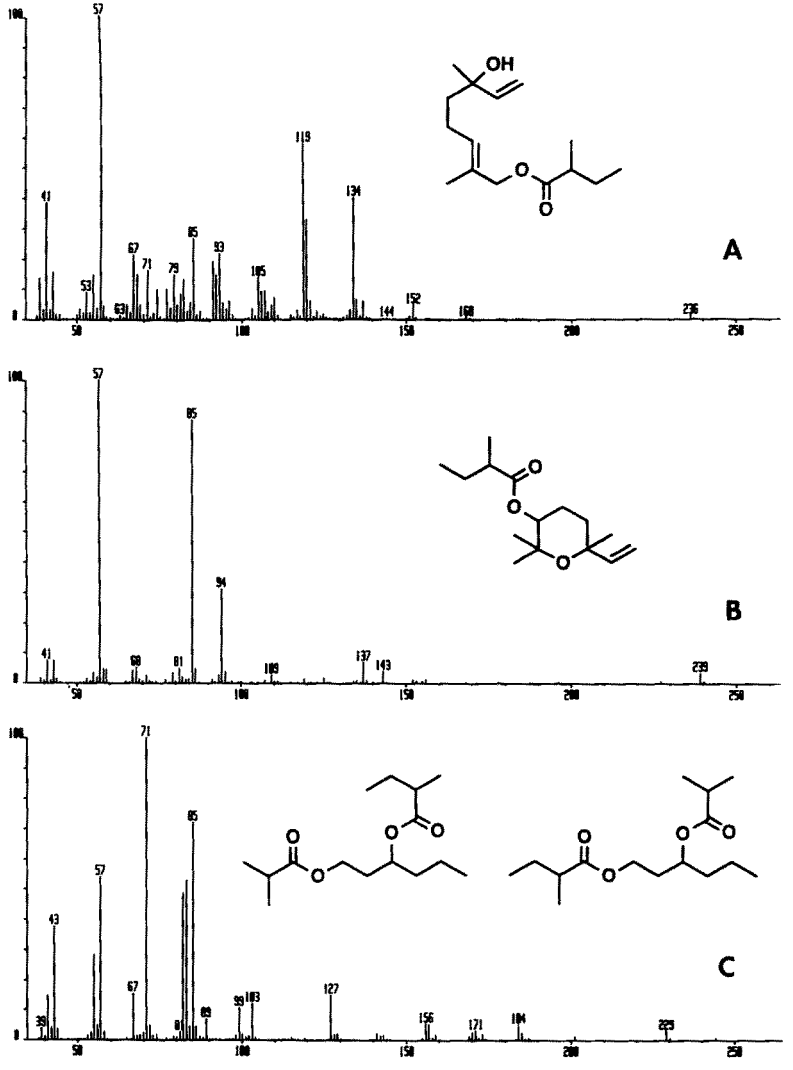

FIG. 2. Mass spectra of compounds 54 (A), 35 or 36 (B), and a mixture of 43 and 44 (C). Compounds 43 and 44 were not separated by GC-MS. Careful analyses of all scans of the respective peak nevertheless showed differences in ion intensities between early and late scans, which could be attributed to slightly different retention volumes of the two compounds. A synthetic mixture of 43 and 44 showed the same behavior.

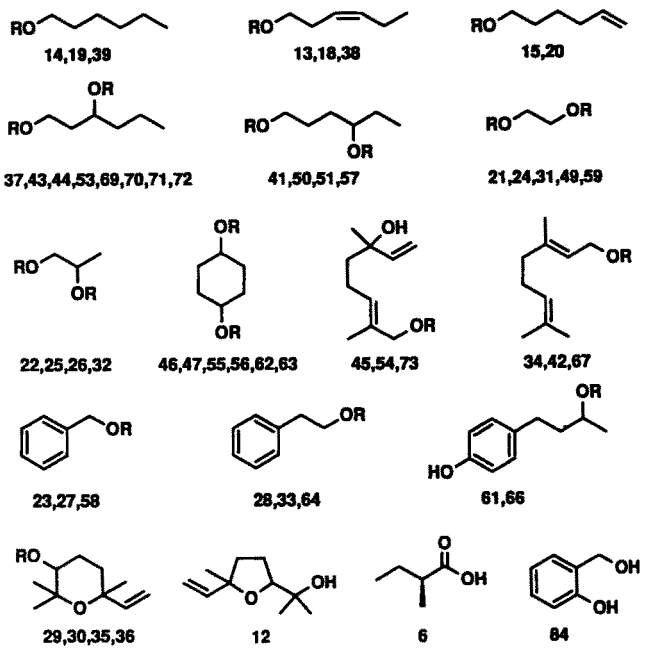

FIG. 3. Compounds identified from the exocrine secretion of *Chrysomela lapponica* larvae. R may be isobutyryl, 2-methylbutyryl, or benzoyl, depending on the actual composition of the compounds (compare Table 1).

Even relatively large amounts of pure compound eluted as a broad "hill" only. By using a 12.5-m fused silica column coated with medium polar OV-1701, the presence of this compound could be clearly demonstrated (Figure 4). Surprisingly, no ester of salicylalcohol but small amounts of salicylaldehyde, a major component of the larval secretion of other *Chrysomela* species feeding exclusively on Salicaceae, could be identified.

The absolute configuration of some representative compounds of the secretion of birch larvae was elucidated by using a GC-MS system equipped with a fused silica capillary coated with a modified cyclodextrine (see Methods and Materials) as chiral stationary phase. The proof of the identity of a given peak by its mass spectrum was necessary because of the high number of compounds present in the secretions. Comparison of racemates and synthetic enantiomers revealed the results given in Table 2. (*S*)-2-Methylbutyric acid was present as pure enantiomer, while the 8-hydroxylinalyl esters and the 1,3-hexandiyl diisobutyrate surprisingly were present as nonracemic mixtures (7:3) of enantiomers. The main enantiomer of 1,2-propandiyl diisobutyrate exhibited the (*S*) config-

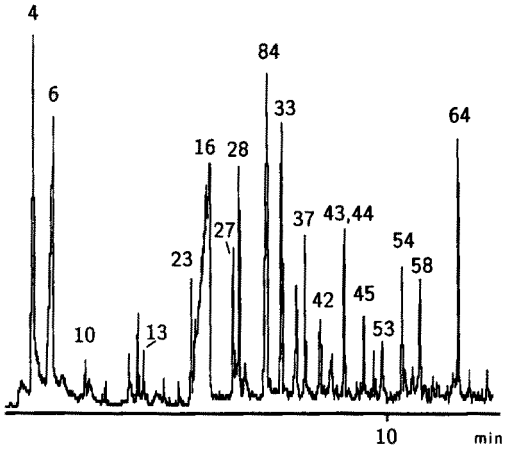

FIG. 4. Total ion current chromatogram of exocrine larval secretion of *Chrysomela lapponica* larvae feeding on *Salix fragilis*; this analysis was performed with GC-MS system II (see text); numbering of compounds according to Table 1.

TABLE 2. RELATIVE PROPORTIONS OF ENANTIOMERS (%) OF SELECTED COMPOUNDS PRESENT IN LARVAL SECRETION OF *Chrysomela lapponica*

Compound	*S*	*R*
2-Methylbutyric acid	>95	
1,2-Propandiyl diisobutyrate	+	–
1,3-Hexandiyl diisobutyrate	70	30
(*E*)-8-Isobutyryloxylinalool	30	70
(*E*)-8-(2-Methylbutyryloxy)linalool	30	70

uration, while the low abundance of this compound did not allow determination of whether the *R*-enantiomer was also present (Figure 5).

The total ion current chromatograms of both the secretion of birch larvae and willow larvae showed several late eluting peaks. The structures of these compounds are still unknown. However, mass spectra with fragments at m/z = 43, 57, 71, and 85 indicated these compounds to be polyesters, too.

Chiral compounds were tested as racemates only. Bioassays with ants were conducted with the compounds and secretions listed in Table 3. The exocrine secretion of larvae on both birch and willow significantly repelled ants. With the exception of benzoic acid and salicylalcohol, each compound tested revealed a repellent activity against the ants.

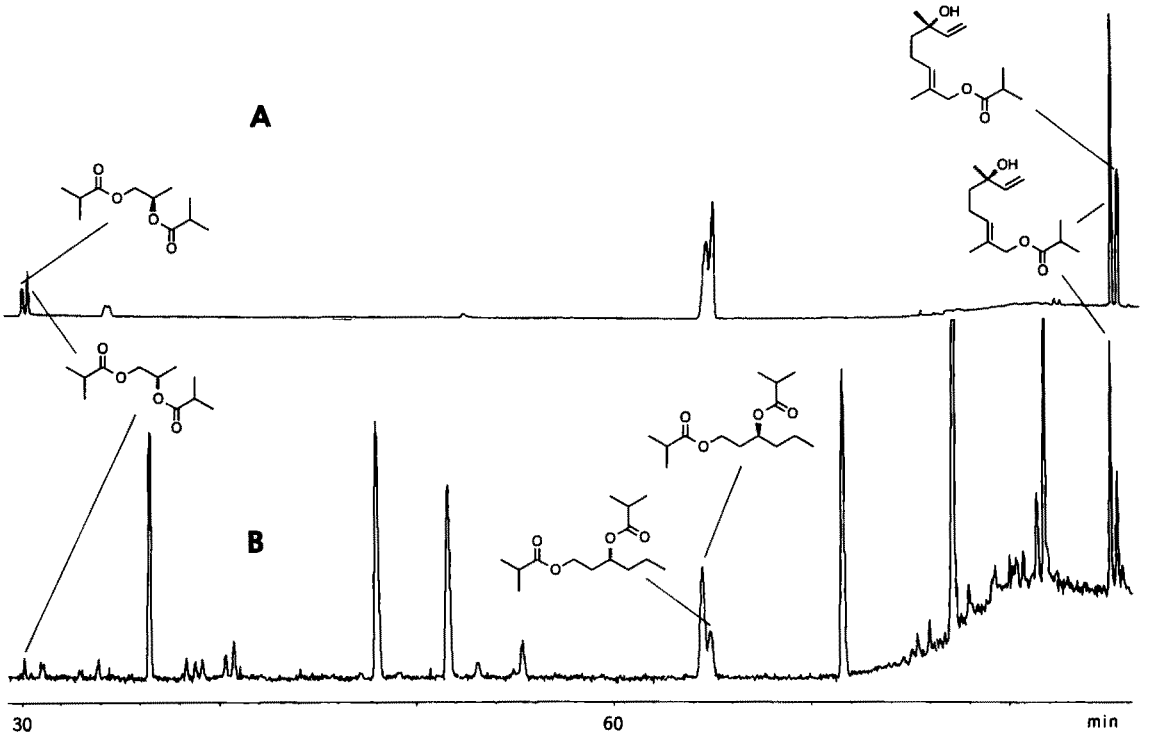

FIG. 5. Enantiomer separation of compounds 22, 37, and 45 on a 50-m heptakis(2,6-di-*O*-methyl-3-*O*-pentyl)-β-cyclodextrine chiral phase (TIC). (A) Mixture of synthetic references. (B) Secretion of larvae feeding on birch. Programmed from 60°C to 100°C at 30°C/min, 20 min isothermal, then at 30°C/min to 115°C, 60 min isothermal, then at 5°C/min to 170°C.

TABLE 3. REPELLENT ACTIVITY OF SECRETION OF *Chrysomela lapponica* LARVAE FEEDING ON BIRCH (*Betula pendula*) OR WILLOW (*Salix fragilis*) AND SYNTHETIC MAIN COMPONENTS OF SECRETIONS AGAINST *Myrmica sabuleti*[a]

Compound/secretion	% ants		Significance
	At test side	At control side	
Secretion of larvae on birch	15	85	**
Secretion of larvae on willow	10	90	***
Isobutyric acid	10	90	***
2-Methylbutyric acid	10	90	***
Hexyl isobutyrate	25	75	*
1-Hexenyl isobutyrate	10	90	***
(*Z*)-3-Hexenyl isobutyrate	5	95	***
1,3-Hexandiyl diisobutyrate	5	95	**
Benzyl isobutyrate	0	100	***
Benzyl 2-methylbutyrate	10	90	***
2-Phenylethyl isobutyrate	5	95	***
2-Phenylethyl 2-methylbutyrate	15	85	**
(*E*)-8-Isobutyryloxylinalool	15	85	***
Benzoic acid	60	40	NS
Salicylalcohol	40	60	NS
Salicylaldehyde[b]	5	95	***

[a] 100% ants: $N = 20$; for details see text. Statistical analysis: two-sided sign test for paired observations. $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$; NS, not significant.
[b] This compound is present only in traces in the secretion of *C. lapponica* larvae feeding on willow.

DISCUSSION

The larval secretions of *C. lapponica* feeding on willow or birch contain many compounds that have not been reported from nature before. The diesters of the aliphatic diols represent new natural products. To our knowledge, this class of compounds has not been reported from any other insect source before. The related, nonesterified 1,3-nonandiol is a component of the rectal gland secretions of fruit flies (Kitching et al., 1986, Nishida et al. 1990). The esters of (*E*)- and (*Z*)-8-hydroxylinalool and the pyranoid linalool oxides are also new natural products. Free 8-hydroxylinalool has been reported from scentless plant bugs (Aldrich et al., 1990), while the linalool oxides have been identified in hairpencils of danaine butterflies (Schulz et al., 1988, 1993).

The alcoholic components of several of the detected esters may be derived from plant glycosides by hydrolyzation or directly from plant alcohols. Glucosides of (*E*)- and (*Z*)-8-hydroxylinalool and rhododendrol are known as constituents of *Betula* leaves (Klischies and Zenk, 1978; Tschesche et al., 1977).

In *Salix fragilis*, glycosides of 3-hexenol, benzylalcohol, 2-phenylethanol, linalool, and other terpene alcohols have been identified from fresh leaves after enzymatic hydrolysis with β-glucosidase (Merkx and Baerheim Svendsen, 1990). Linalool and 2-phenylethanol are also major constituents of the larval secretion of *Gonioctena viminalis* feeding upon *Salix* spp. (Dettner and Schwinger, 1987). Glycosides of (Z)-3-hexenol, hexanol, benzyl alcohol, and 2-phenylethanol as well as linalool and geraniol are believed to be common plant constituents (Merkx and Baerheim Svendsen 1990, Stahl-Biskup et al. 1993). Glycosides of 1,4-cyclohexandiol, 1,2-propandiol, and 1,2-ethandiol have not been reported from any plant source, but 1,2-cyclohexandiol occurs glycosidically bound in Salicaceae. Nevertheless, we could not identify diesters of 1,2-cyclohexandiol with certainty in the secretion.

Free C_6 alcohols are common plant constituents (Visser et al., 1979; Visser, 1986). For production of the diesters, C_6 diols are necessary, which are not known as constituents of willow or birch leaves. These diols are most probably biosynthetically derived from 3-hexenol. The occurrence of 1,3- and 1,4-hexandiol should exclude a normal acetate pathway to these compounds, because such a mechanism leads exclusively to the 1,3-product. We hypothesize that oxidation of 3-hexenol with a monooxygenase gives 3,4-epoxyhexanol, which could be enzymatically isomerized to 3- and 4-oxohexanols. Reduction would yield the 1,3- and 1,4-hexandiols (Figure 6). The observed enantiomer composition can be explained by a not very enantioselective enzymatic reduction of the ketone intermediates. Whether this transformation is performed by the host plant or by larvae is unknown. We were not able to identify esters of 3,4-epoxyhexanol, the key intermediate of the hypothesized pathway, in the secre-

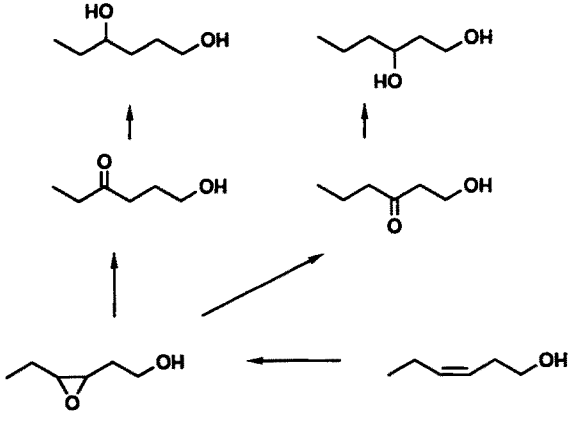

FIG. 6. Proposed biosynthetic pathway leading to 1,3- and 1,4-hexandiol.

tion of *C. lapponica* larvae. Nevertheless, esters of the postulated intermediates have been identified in different organisms. 3,4-Epoxyhexyl acetate is a floral scent component of *Jasminum sambac* (Kaiser, 1986). Esters of 3-oxohexanol have been identified in the scent organs of *Heliconius* butterflies (Schulz, unpublished results).

Another monoxygenase transformation leading to compounds present in the larval secretion seems to take place exclusively in the plants. The linalool oxides and 8-hydroxylinalool are biosynthesized from linalool. While allylic oxidation of linalool yields 8-hydroxylinalool, monooxygenase oxidation yields the relatively unstable 6,7-epoxylinalool, which is known to cyclize easily to the furanoid and pyranoid linalool oxides. The absence of the linalool oxide esters in the secretion of willow larvae points to the plant origin of the linalool oxides in birch larvae and, thus, to the occurrence of monooxygenase transformations in the host plants rather than in the larval secretion.

Although many precursors of the secretion constituents are obviously present in the host plant, we cannot exclude larval de novo synthesis of them. Alcoholic components of some esters are very likely derived from amino acids: phenylethanol from phenylalanine and hydroxyphenylethanol from tyrosine. 8-Hydroxylinalool may be plant-derived or biosynthesized de novo by *C. lapponica* via the acetate–mevalonate pathway, but the presence of its glycoside, betulalboside, in *Betula* leaves points to plant origin. Betulalbosides have been identified in several plants (Stahl-Biskup et al., 1993) and may thus also be present in willow, which would explain its presence in the secretion of willow larvae.

The aliphatic carboxylic acids detected in the secretion of *C. lapponica* larvae may be derived from amino acids (Blum, 1987). Isobutyric acid and 2-methylbutyric acid, which are also known as constituents of the exocrine glandular secretion of the lepidopteran larvae *Papilio aegeus*, have been proved to be biosynthesized in this species from valine and isoleucine, respectively (Seligman and Doy, 1973). Most probably these compounds are biosynthesized de novo by *C. lapponica* larvae.

A striking difference between the secretion of *Chrysomela lapponica* larvae feeding on willow and birch is the presence of benzoic acid and salicylalcohol as main components in the secretion of willow larvae. Salicylalcohol is obviously derived from salicin from the host plant (*Salix fragilis*) by hydrolysis. In contrast to other *Chrysomela* spp. feeding on Salicaceae, *C. lapponica* larvae only hydrolyze salicin, but hardly oxidize the resulting salicylalcohol to salicylaldehyde. The occurrence of large amounts of free and esterified benzoic acid is characteristic for Salicaceae (Hegnauer, 1973), and thus the benzoic acid may be obtained from the host plant. Our results strongly suggest that *C. lapponica* larvae liberate plant allelochemicals from the glandular secretions.

The results of the present study prompt the hypothesis that *C. lapponica*

larvae produce the main components of their exocrine glandular secretions on a metabolic pathway that has not yet been described for the biosynthesis of chrysomelid larval secretions. The known pathways are:

1. De novo biosynthesis of iridoid monoterpenes via the acetate–mevalonate pathway (Lorenz et al., 1993).

2. Hydrolysis of plant glycosides by a β-glucosidase and oxidation of the resulting aglycone by a specific oxidase (proved for the production of salicylaldehyde, suggested for juglone) (Pasteels et al. 1983, 1984; Matsuda and Sugawara, 1980).

For major compounds in the larval secretion of *C. lapponica*, we suggest the following pathway: De novo synthesis of isobutyric and 2-methylbutyric acid from amino acids, followed by esterification with alcohols derived from the host plant (obtained most probably by hydrolysis of plant glycosides) or with de novo synthesized alcohols. This hypothesis needs to be proved by precise biogenetic studies with labelled precursors.

The de novo synthesis of iridoid monoterpenes in chrysomelid larval secretions is considered a widespread ancestral character by Pasteels et al. (1990), whereas the use of plant allelochemicals as direct precursors for the production of larval secretions is regarded as a derived character occurring in a few chrysomelid taxa. If further studies will confirm the pathway of biogenesis suggested above for the larval secretion of *C. lapponica*, this species would connect de novo synthesis of glandular components (production of carboxylic acids from amino acids) with the use of direct plant precursors for the production of carboxylic acid esters (see, e.g., 8-hydroxylinalyl esters or rhododendryl esters). The presence of carboxylic acid esters as larval allomones is, up to now, only known in two *Chrysomela* species, *C. lapponica* and *C. interrupta*, whereas in seven other *Chrysomela* species salicylaldehyde is a major component. Larvae of *Linaeidea aenea*, a species feeding on alder, produce iridoid monoterpenes in their defensive glands (Sugawara et al., 1979). This species is closely related to the genus *Chrysomela*. Formerly, *Linaeidea* had been considered as a subgenus of the current genus *Chrysomela* (Hennig, 1938; Seeno and Wilcox, 1982). Thus, within this group of closely related species both ancestral (monoterpenes) and derived (salicylaldehyde) characters occur when following the trait of evolution outlined by Pasteels et al. (1990).

How does the chemical composition of the larval secretion of *C. lapponica* fit into this evolutionary sequence? Up to now, it has been impossible to judge whether a de novo synthesis of carboxylic acids from amino acids in the larval exocrine glands is an ancestral or a derived character. The use of amino acids for the production of larval secretion components could either have been reduced in other *Chrysomela* species or have evolved alternatively to the development of a specific oxidase of salicylalcohol. Pasteels et al. (1989) suggested the use of amino acids for the production of exocrine glandular secretions in chrysome-

line adults (glands at the pronotum and elytra) as an ancestral character because of its widespread occurrence. For a careful evolutionary consideration of larval secretion components of *C. lapponica*, further knowledge on their biosynthesis and the enzymes involved will be necessary.

The bioassays with ants revealed that the repellent activity of the larval secretion is independent of whether larvae feed on birch or willow. Since the larval secretion was not analyzed quantitatively, the synthetic components were not tested in natural concentrations. Therefore, these bioassays provide only preliminary information on the actual defensive efficiency of single compounds of the natural secretion. Nevertheless, the bioassays show that benzoic acid and salicylalcohol, major components of the secretion of willow larvae, display no repellent effect against ants. In contrast, larvae of other *Chrysomela* species are able to convert high amounts of salicylalcohol to salicylaldehyde and, thus, to a significant repellent against ants. In contrast, larvae of other *Chrysomela* species are able to convert high amounts of salicylalcohol to salicylaldehyde and, thus, to a significant repellent against ants (Table 3). While salicylalcohol is inactive as a repellent against ants, Pasteels et al. (1983) demonstrated that it acts as a significant feeding deterrent against *Myrmica rubra*. In *Plagiodera versicolora*, which is also a chrysomelid *Salix* feeder, neither salicylalcohol nor salicylaldehyde is present in the larval secretion, but iridoid monoterpenes, which also significantly repel ants (Meinwald et al., 1977), are present; this species excretes salicin and salicylalcohol with the feces (Pasteels et al., 1990). Further detailed investigations on the metabolism of plant components within the gut, fat body, and hemolymph of chrysomeline larvae could elucidate which physiological parameters "decide" about the discharge of plant allelochemicals by the alimentary tract or exocrine glands.

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ATTRACTANT PHEROMONE FOR NEARCTIC STINK BUG, *Euschistus obscurus* (HETEROPTERA: PENTATOMIDAE): INSIGHT INTO A NEOTROPICAL RELATIVE[1]

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Abstract—Volatiles from *Euschistus obscurus* males were found to be attractive to conspecific females in bioassays. A new type of olfactometer assembled from commercially available glassware was used to bioassay aeration extracts of the Nearctic stink bug, *E. obscurus*. Fractionated extracts suggest that pheromonal activity is associated with late-eluting, male-specific compounds. Research on *E. obscurus* offered a means to indirectly study the pheromone of the Neotropical pest species, *E. heros*, without importing this soybean pest into the United States.

Key Words—*Euschistus heros*, behavior, olfactometer, attraction, volatiles.

INTRODUCTION

Soybean, *Glycine max* (L.) Merril, in the United States and around the world is damaged by complexes of stink bugs. In the United States, the most important members of the complex are *Nezara viridula* (L.), *Acrosternum hilare* (Say), *Euschistus servus* (Say), and other *Euschistus* spp. (Turnipseed, 1973; Russin et al., 1987). Several species of *Euschistus* have been reported as sporadic pests of deciduous fruits, cotton, and seed alfalfa (Borden et al., 1952; Hoffmann et al., 1987; Toscano and Stern, 1976). In Central and South America, other

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Euschistus spp. are important pests (Panizzi and Slansky, 1985). For example, the Neotropical pentatomid, *E. heros* (F.), is a major pest of soybean in Brazil (Panizzi and Rossi, 1991).

A chemical attractant for *Euschistus* species would be a valuable means to monitor levels of these pests (Aldrich et al., 1991). In addition, attractants might be useful for concentrating the stink bugs in early maturing trap crops where they could be economically destroyed by limited insecticide application (McPherson and Newson, 1984). The identification and attractiveness of a major pheromone component for Nearctic *Euschistus* spp. was reported by Aldrich et al. (1991). Methyl (2*E*,4*Z*)-decadienoate is the major male-specific volatile of five *Euschistus* spp. In an analysis of a single individual for a sixth species, *E. obscurus* (Palisot), this ester was a relatively minor male-specific component, with the major component being tentatively identified as methyl 2,6-dimethyltetradecanoate (Aldrich et al., 1991). It has now been determined that the putative identification was incorrect (Aldrich et al., 1993). Our aerations of *E. heros* indicate that males of this species may produce the same major pheromone as *E. obscurus*. This study of *E. obscurus* was undertaken primarily as a means to elucidate the chemical communication system of its Neotropical relative, *E. heros*. To do this, a bioassay was developed to quantify female *E. obscurus* responses to the odor of live male *E. obscurus* and to extracts of the pheromone isolated from males.

METHODS AND MATERIALS

Insects. E. obscurus was obtained from a laboratory colony from Dr. W. Jones (USDA-ARS-SPA, Weslaco, Texas) and reared on sunflower seeds, soybeans, and green beans at 28°C and 65% relative humidity on a 16:8-hr light–dark photoperiodic regime. To prevent olfactory interactions between the sexes, males were separated from females after the imaginal molt and cuticular hardening, but before sexual maturity (ca. 48–72 hr).

Volatiles Extracts. Samples were collected from groups of 40 virgin adult male or female *E. obscurus* of known age by coaxing the bugs into a 1-liter glass column and trapping volatiles from the air drawn by vacuum (100 ml/min) over the bugs through 30 mg of activated charcoal for 24 hr. Trapped volatiles were washed from the filter in 150 μl of CH_2Cl_2 and stored at -20°C (Aldrich et al., 1987). Extraction of *E. heros*, in the Brazilian laboratory, followed the same methodology described for *E. obscurus*.

Fractionated Samples. Fractions were isolated in glass capillary tubes jacketed in Dry Ice as they eluted from a DB-1 column (15 m × 0.53 mm ID) in a Varian 3700 GC equipped with a thermal conductivity detector (Aldrich et al., 1987), isothermally at 150°C for 6 min.

Samples were analyzed by gas chromatography (GC) on a bonded methyl silicone column (0.25-μm film, 30 m long, 0.25 mm ID; DB-1, J & W Scientific, Folsom, California) using a Varian 3500 GC with helium as carrier (40 cm/sec), and a temperature program from 50°C for 2 min to 235°C at 15°C/min with a 2-min final hold.

Olfactometer. A two-choice olfactometer was used to test the biological activity of live males, male aeration extracts, and trapped fractions (Figure 1). The olfactometer consisted of a release chamber that was a 500-ml three-neck, round-bottom flask (all 204/40 joints, Kontes, Vineland, New Jersey 08360-2841). Two 250-ml rotary evaporator trap adapters (24/40 joints) were attached to the side arms (the treatment and control chambers). Charcoal filters (20/40 mesh) were attached to the side arms using 24/40 to 14/35 adapters to 130-mm × 10-mm ID, charcoal-filled columns. The middle neck of the flask was connected to the house vacuum through an adapter (24/40 to 10/30, reducing/enlarging joints) adjusted with a valve to a flow of 0.75 ml/sec. The apparatus was positioned horizontally on a countertop in a room (24 ± 1.5°C) with bright fluorescent lights (Aldrich et al., 1990).

Bioassay. Observations through the day showed no obvious variation in the level of responsiveness of females to males. The bioassays were therefore run throughout the light period between 9:00 AM and 4:00 PM and were allowed to run for 20 min for all computed data.

The bioassay procedure used by Borges et al. (1987) was followed with some modification as follows: the natural stimulus was provided by two sexually mature virgin males (>14 days old), released into the treatment chamber, and

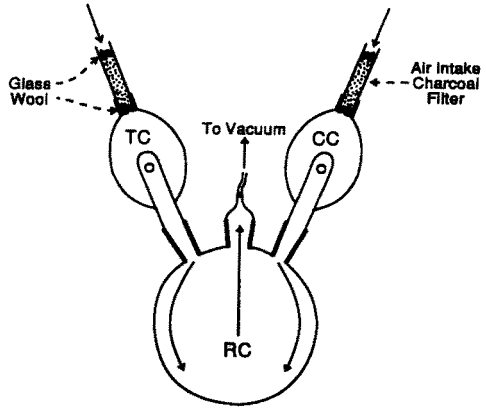

FIG. 1. Olfactometer apparatus used in the bioassays. TC = treatment chamber, CC = control chamber, RC = release chamber. Air moves from the treatment/control chamber to the release chamber (arrows). Dimensions quoted in text.

10 sexually mature females were placed into the release chamber. Prior to testing, the insects were allowed to acclimatize for a short period (ca. 5 min) in the release chamber before attaching the side arms. The olfactometer sections were then connected, and the behavioral responses of females were recorded. Females that entered a ball of a rotary evaporator trap were counted as making a choice; data are reported as percent response. Sixteen replicates were performed using 10 females per replicate and blank as a control.

Effects of male aeration extracts on the behavioral responses of females was determined by recording female responses to 0.3, 1.3, and 2.7 individual equivalents (IE) applied to a strip of filter paper (2.0 × 0.5 cm) with 1, 5, or 10 μl microcap (Drummond), respectively (1 IE = 3.75 μl of extract). The solvent was allowed to evaporate for 20 sec and then the filter paper strips were placed in the ball of the rotary evaporator trap for the treatment side of the olfactometer. As a control, the same dose of solvent on a filter paper strip was placed in the control chamber, allowed to evaporate, and the filter paper was tested simultaneously with the male aeration extract. Sixteen replicates were performed using 10 females per replicate.

Effects of the fractionated male aeration extract on the behavioral responses of females were determined by recording female responses to a series of trapped fractions. The first "fraction" assayed, the total fraction, was a 6-min elution to monitor activity of all volatiles submitted to the GC program. Fraction 1 was the trapped fraction eluting from injection until 2.5 min, and fraction 2 consisted of the trapped volatiles from 2.5 to 6.0 min. The bioassay of the trapped fractions followed the same methods as for male aeration extracts. Four replicates were performed using 10 females per replicate and solvent as a control.

Statistical Analysis. Statistical analyses were performed on a microcomputer using StatXact-Turbo, statistical software for exact nonparametric inference (CYTEL software Corp., Cambridge, Massachusetts 02139). Each control versus treatment combination was analyzed as a paired difference using the Wilcoxon signed rank test. The difference was treatment minus control for each replicate. The *P* values for the alternate hypothesis (Ha: the difference between the treatment and control is not zero) are reported on the figures.

RESULTS

GC analysis showed that the *E. obscurus* male aeration extracts contained a high concentration of two volatile compounds and relatively low concentrations of others (Figure 2). Chemical analysis disclosed that three of the volatiles of *E. obscurus* also are released by *E. heros* males (Figure 2) (Aldrich et al., 1993). For both *E. obscurus* and *E. heros*, aeration extracts of females totally lacked compounds **1, 2,** and **3**.

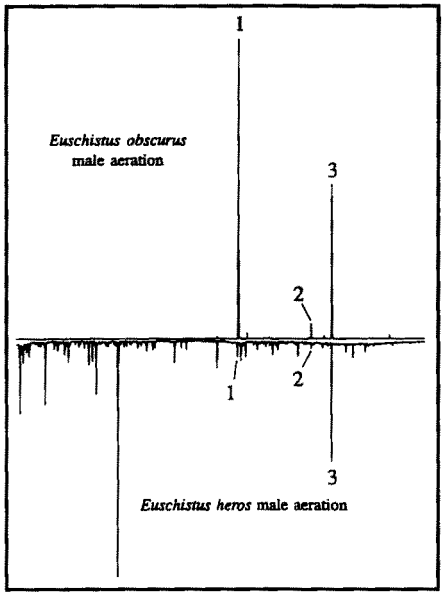

FIG. 2. Gas chromatograms from 40 male *Euschistus obscurus* and 40 male *Euschistus heros*. Numbers indicate matching compounds verified by GC-MS (Aldrich et al., 1993).

There was no significant attraction of sexes to extracts of the same sex; i.e., female insects were not attracted to extracts of females, and male insects were not attracted to extracts of males. Although live females were not tested, there was no significant difference in the attraction of males to extracts of females as compared to controls (Figure 3). However, it was observed that males always showed a higher upwind response than females. On the other hand, the attraction of females to live males and extracts of live males at 0.3 and 1.3 IE was significantly greater than controls. At higher concentrations (2.7 IE) the attraction of females to extracts of males was not significantly different from controls (Figure 4).

Results of bioassays for fractions were not significant at the 95% level. Responses to the total fraction ($P = 0.25$) and fraction 2 ($P = 0.125$) were comparable, whereas response to fraction 1 was low ($P = 0.75$).

DISCUSSION

The major male-specific compound of *E. heros* (ignoring chirality) is also abundantly produced by *E. obscurus* males (Aldrich et al., 1993). Thus, studying the Nearctic *E. obscurus* offered a means to identify and study the pheromone

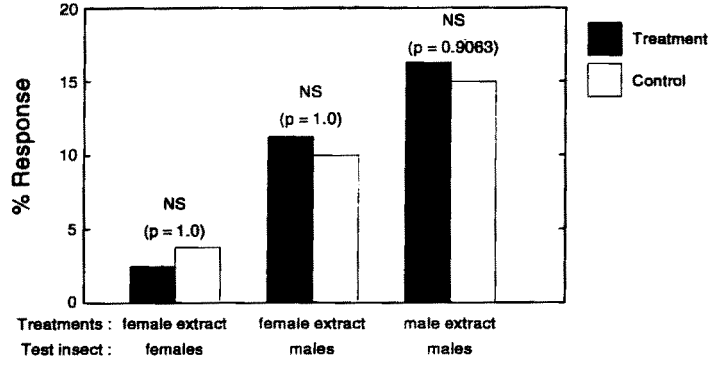

FIG. 3. Percentages of females responding to 1.3 IE dose of female aeration extract and males responding to 1.3 IE dose of female aeration extract and 1.3 IE dose of male aeration extract.

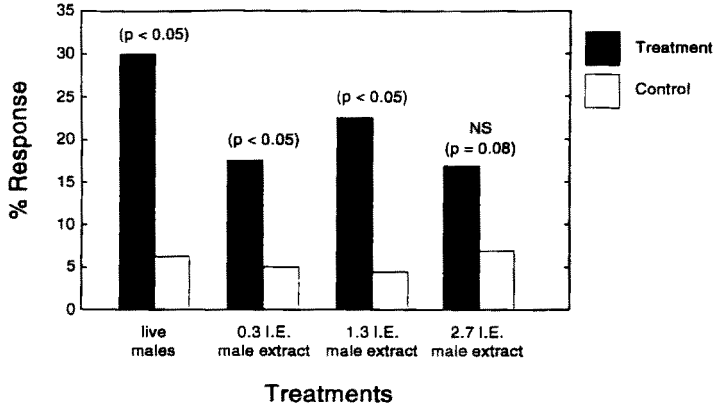

FIG. 4. Percentages of females responding to live males and to 0.3, 1.3, and 2.7 IE of male pheromone extract applied to a filter paper.

of the important Neotropical pest, *E. heros*, without actually importing this pest species into the United States. Moreover, *E. obscurus* males produce about 10 times more pheromone per day than do *E. heros* males, making isolation of sufficient pheromone for analysis more practical.

Euschistus obscurus males release an attractant pheromone, and aeration extracts of males at 0.3 and 1.3 IE were as active as live males in laboratory bioassay. At the higher pheromone concentration tested (2.7 IE) attraction was reduced. Although bioassay results for fractionated pheromone samples were

not significant at the 95% level, the high activity of fraction 2 suggests that the main pheromone activity is associated with late eluting compounds.

The higher upwind responses shown by *E. obscurus* males during this study may be a function of the recolonization process characteristic of many heteropteran species. For example, males *P. maculiventris* often search for food first, and then call females with pheromone (Aldrich et al., 1984). This behavior might be related to the periodic migration of the adults of many heteropterans (e.g., Evans, 1982).

A global mapping of the sex pheromones for complexes of economically important pentatomids would be useful for biological control and integrated pest management programs. Many tachinid flies use heteropteran pheromones as host-finding kairomones (Aldrich, 1988) and are important biocontrol agents (Todd, 1989). If tachinids discriminate between host pheromone blends, it is important that these parasites be imported from regions where the host has a compatible pheromone blend.

Further chemical and behavioral research must be carried out in Brazil for *E. heros*, including testing individual stereoisomers that are now being synthesized (K. Mori, personal communication).

Acknowledgments—We are grateful to EMBRAPA/CENARGEN, CNPq, and to the U.S. Department of Agriculture for their financial support of the first author during his stay in the United States. We would like to thank Mr. Kenneth R. Wilzer Jr., USDA-ARS-INHL Beltsville, for his help with the computer, Dr. Mary Camp for her help with the statistical analysis.

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IDENTIFICATION OF MALE-SPECIFIC VOLATILES FROM NEARCTIC AND NEOTROPICAL STINK BUGS (HETEROPTERA: PENTATOMIDAE)

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Abstract—Males of the Central American stink bug species, *Euschistus obscurus*, produce an attractant pheromone composed of a blend of compounds characteristic of North American *Euschistus* spp. and the South American soybean pest, *E. heros*. The range of *E. obscurus* extends into the southern United States, the species is easy to rear, and males produce an exceptionally large quantity of pheromone (>0.5 μg/day/male). These factors made *E. obscurus* useful for characterizing the novel pheromone components of *E. heros* without importing this pest species into the United States. *Euschistus obscurus* males produce methyl (2*E*,4*Z*)-decadienoate (61%) in abundance, which is characteristic of North American species, and methyl 2,6,10-trimethyltridecanoate (27%), the main male-specific ester of *E. heros*. The chirality of *Euschistus* spp. methyl-branched esters, and field activity of synthetic formulations, remain to be determined.

Key Words—Heteroptera, Pentatomidae, pheromone, attractant, *Euschistus*, soybean, methyl 2,6,10-trimethyltridecanoate.

INTRODUCTION

Methyl (2*E*,4*Z*)-decadienoate is the major male-specific volatile of five Nearctic stink bugs (Heteroptera: Pentatomidae): *Euschistus conspersus*, *E. tristigmus*,

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E. servus, *E. politus*, and *E. ictericus* (Aldrich et al., 1991). Females, males, and nymphs of the first four of these species were significantly attracted to this ester in the field. Tests in Maryland also demonstrated that parasitic tachinid flies use the unsaturated methyl-ester as a host-finding kairomone (Aldrich et al., 1991). In a sixth species, *E. obscurus*, whose northern range extends into Texas and Florida (Froeschner, 1988), methyl (2*E*,4*Z*)-decadienoate was reported to be a relatively minor male-specific component, with the major component being tentatively identified as methyl 2,6-dimethyltetradecanoate (Aldrich et al., 1991).

Establishment of a prolific laboratory colony of *E. obscurus* has enabled us to reinvestigate the tentative identification of methyl 2,6-dimethyltetradecanoate from *E. obscurus*, which was based on analysis of a single field-collected male. We recently found that the main volatile from males of the South American soybean pest, *E. heros*, is identical to the ester tentatively identified in *E. obscurus* (ignoring chirality), giving added significance to structural verification of the novel *E. obscurus* pheromone component.

We correct here our earlier misidentification of the major male-specific volatile from *E. obscurus* (Aldrich et al., 1991) and provide more detailed information on the presence of other male-specific volatiles for this species and for *E. heros*.

METHODS AND MATERIALS

Insects. Euschistus heros used in the study were obtained from a colony started from adults collected near the Centro Nacional de Recuros Geneticos e Biotechnologic, Brasilia D.F., Brazil, and *E. obscurus* was obtained from a laboratory colony of Dr. Walker Jones (USDA-ARS, Weslaco, Texas). *Euschistus heros* was reared in Brazil on fresh green beans, raw peanuts, and water at 26 ± 1°C with a 16:8-hr light–dark photoperiod. *Euschistus obscurus* was reared in the Beltsville laboratory under similar conditions except that sunflower seeds were used instead of peanuts. The sunflower seeds were glued onto sheets of brown wrapping paper with wallpaper paste, and cut into 10-cm squares that were discarded after depletion by the insects.

Extractions. Airborne extracts were prepared from *Euschistus* spp. in the respective laboratories by confining 20–50 insects in a glass column (ca. 1 liter), drawing air for 24 hr by vacuum (100 ml/min) through ca. 30 mg of activated charcoal inside a Swinney Luer-lock filter holder (13 mm; Thomas Scientific, Philadelphia, Pennsylvania), and extracting the filter with 100–200 μl of CH_2Cl_2 or heptane (Aldrich et al., 1989, 1991).

Chemical Analyses. Brazilian samples were analyzed initially by gas chromatography (GC) on a bonded methyl silicone column (0.25 μm film, 30 m ×

0.25 mm ID; DB-1, J&W Scientific, Folsom, California) in a Varian 3700 GC with hydrogen as carrier (40 cm/sec), a temperature program from 45°C for 2 min to 230°C at 15°/min, a flame ionization detector (FID), and a Shimadzu C-R3A recorder. Samples of *E. obscurus* volatiles, and heptane extracts of *E. heros* brought to the United States, were analyzed on a DB-1 column (0.25 μm film, 30 m × 0.25 mm ID) in a Varian 3500 GC with helium as carrier (50 cm/sec), a temperature program from 50°C for 2 min to 235°C at 15°/min, and an FID. Data were recorded using the Varian GC Star Workstation software on a Gateway 2000 386/25 computer. GC traces presented in figures begin at 2.5 min (at left) so as to exclude the solvent peak and normalize peaks to the most abundant natural product. Inverted GC traces in figures were created using Corel Draw version 3.0 software.

Electron impact mass spectra (MS) were obtained using either a Finnigan 4510 GC-MS equipped with an INCOS Data System, at 70 eV, and a 30-m DB-1 column, programmed from 60°C for 2 min to 250°C at 5°/min, or a Hewlett Packard 5971 GC-MS instrument at 70 eV, with a HP-5 column (0.11 μm film; 25 m × 0.2 mm ID), programmed from 50°C for 2 min to 250°C at 15°/min.

Hydrogenation/Hydrogenolysis Reaction. High-temperature treatment of primary alcohols with lithium aluminum hydride plus platinum on alumina has been successfully applied to produce a mixture of the corresponding hydrocarbon and chain-shortened hydrocarbon (Bierl-Leonhardt and DeVilbiss, 1983; Aldrich et al., 1986). To test whether this reaction would be suitable for structure determination of the acid moieties of methyl esters, the procedure was applied to a 5-μl solution of methyl dodecanoate (2.7 μg/μl hexane). Dodecane and undecane were obtained (ca. 4 : 1 respectively), with complete disappearance of the parent ester. Therefore, the reaction was carried out using an aeration sample of 30 male *E. obscurus*, comparable to that shown in Figure 1 below, as follows. $LiAlH_4$ and 5% Pt on Al_2O_3 (Aesar, Seabrook, New Hampshire) (14 mg + 16 mg, respectively) were pulverized together, and 3 mg of the powder was transferred into a melting point capillary tube (caution: ignition hazard). Fifty microliters of the aeration extract was concentrated in a conical vial, then pentane was used to transfer the residue to the capillary containing the catalytic mixture. The solvent was evaporated with a slow stream of argon through a syringe needle, and the tube was then flame-sealed. After heating for 40 min at 250°C, the tube was allowed to cool to room temperature, opened, and the products were extracted with a few small portions of hexane (total ≤ 20 μl). This solution was filtered through a few grains of silica gel using an additional 5–10 μl of hexane, and the eluate was concentrated to 10–15 μl for GC and GC-MS analyses.

Standards. Methyl (2*E*,4*Z*)-decadienoate and ethyl (2*E*,4*Z*)-decadienoate

(pear ester) were obtained from Bedoukian Research Inc., Danbury, Connecticut. The pear ester was used as an internal standard in some aeration extracts.

2,6-Dimethyltetradecanoic acid was prepared by successive malonic ester condensations. The anion of diethyl methylmalonate was alkylated with 1-bromooctane in refluxing ethanol, then saponified with KOH–ethanol. After removal of ethanol, the salt was acidified (HCl) and the precipitated malonic acid recrystallized from hexane (two crops, 77%, mp 92–97°C). Decarboxylation (oil bath, 175°C) and distillation gave 2-methyldecanoic acid (bp 161–163°C/23 mm) in quantitative yield. The acid was reduced with $LiAlH_4$ in refluxing ether to give the alcohol (bp 129–132°C/23 mm, 87%), followed by conversion to the bromide with Ph_3PBr_2 in CH_2Cl_2 at 0–10°C. The bromide was separated by partition between hexane and 90% dimethyl sulfoxide, the hexane solution was passed through silica gel and the product distilled (bp 129–132°C/24 mm, 74%). A similar reaction of this bromide with the anion of diethyl malonate gave the chain-extended malonic acid (two crops from hexane, mp 51–54°C, 64%). Decarboxylation (oil bath, 180°C) and distillation (135–140°C/0.5 mm) gave a colorless oil that was crystallized from pentane to give colorless platelets (mp 19.5–21.5, 50% from the bromide). Reduction with $LiAlH_4$ in ether gave crude 4-methyldodecanol (two runs, combined yield of 56%, 99% pure by GC). Conversion to the bromide (bp 155–157°C/22 mm, 88.5%), reaction with the anion of methyl malonic ester as above, and saponification gave methyl 4-methyldodecyl malonic acid as a colorless oil (70%). The acid was purified through the bis(dicyclohexylamine) salt, mp 154–156°C dec. from acetone, with 71% recovery. Decarboxylation and distillation gave 2,6-dimethyltetradecanoic acid as a colorless oil (bp 208–211°C/22 mm) in 81% yield. Gas chromatography of the methyl ester (diazomethane) showed a purity of 98.9%, and mass spectra were consistent with the expected structure.

2,6,10-Trimethyldodecane was prepared by hydrogenation (Pd/C, 1 atm, ethanol) of farnesol (Bedoukian Research Inc.) followed by hydrogenation/hydrogenolysis of hexahydrofarnesol as described above.

2,6,10-Trimethyltridecane was synthesized by an organocopper coupling reaction (Posner, 1975) as follows. A solution of 472 mg of copper iodide in 10 ml of tetrahydrofuran (THF) was treated with 3 ml of 1.4 M methyllithium under argon for several min at −40°C, then the CuI/MeLi mixture was warmed slightly (−30°C), and ca. 75 mg (0.25 mmol) of hexahydrofarnesyl bromide (1-bromo-4,8,11-trimethyldodecane; prepared by reaction of hexahydrofarnesol and triphenylphosphine dibromide) in 0.5 ml THF was added. After stirring for 30 min at −30°C, the mixture was allowed to slowly warm to 15°C, then was partitioned between aqueous NH_4Cl and pentane. The pentane solution was rinsed with 2 N HCl, H_2O, and aqueous $NaHCO_3$, then was dried, and concentrated to provide a nearly quantitative yield of the title hydrocarbon.

RESULTS

Four compounds account for nearly 92% (by GC) of the total volatiles in aeration extracts ($N = 7$) of male *E. obscurus* that were uncontaminated by metathoracic scent gland secretion ($X \pm$ SEM; R_t = retention time): compound I (60.97% ± 1.45; 9.92 min), II (1.13% ± 0.12; 10.22 min), III (2.59% ± 0.14; 12.36 min), and IV (27.10% ± 1.89; 13.04 min) (Figure 1A). Gas chromatograms of aerations of *E. obscurus* females (not shown) either totally lacked compounds I–IV, or contained only metathoracic scent gland components if bugs were disturbed during loading or died during the aeration period (Figure 1A).

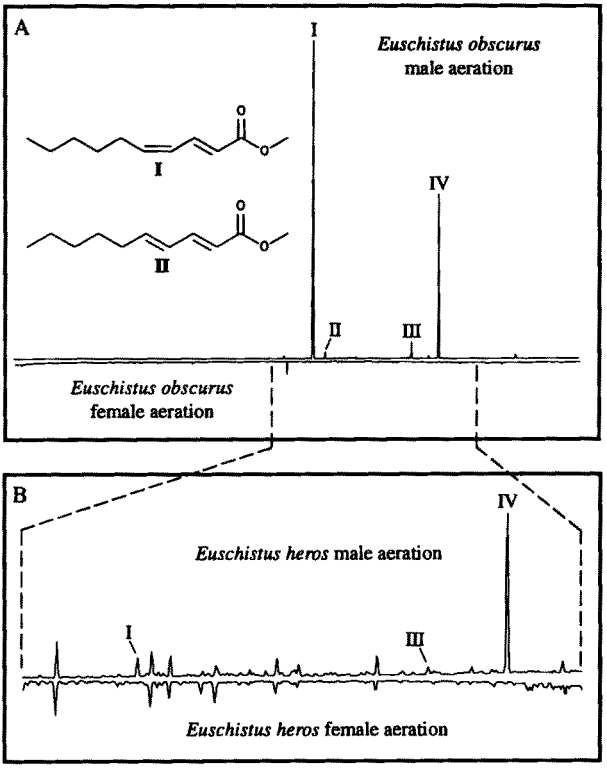

FIG. 1. (A) Typical gas chromatograms for aeration extracts of 40 sexually mature *Euschistus obscurus* males (top), and females (bottom). (B) Chromatograms of aeration extracts of 40 mature male (top), and female (bottom) *Euschistus heros*, expanded to show the region containing male-specific esters and attenuated ca. 10× that of the chromatograms shown for *E. obscurus*.

Aeration extracts of *E. heros* were prepared in Brazil using hexane that later proved to contain a substantial level of impurities. However, GC traces of the R_t = 9–14 min range for aeration extracts of *E. heros* males (Figure 1B) contained a major component having an R_t and EI-MS matching those of compound IV of *E. obscurus*, and minor components matching compounds I and III of *E. obscurus*. Compounds I, III, and IV were absent from comparably prepared aeration extracts of *E. heros* females (Figure 1B). Compound IV is released at a much lower rate by *E. heros* males (0.04 μg/male/day) than by *E. obscurus* males (0.48 μg/male/day).

Mass spectral (Baeckstrom et al., 1988) and gas chromatographic data verified the earlier report (Aldrich et al., 1991) of methyl (2*E*,4*Z*)-decadienoate (I) and methyl (2*E*,4*E*)-decadienoate (II) from male *E. obscurus* (Figure 1A). However, synthetic methyl 2,6-dimethyltetradecanoate (R_t = 13.68) did not coelute with compound IV, despite the close similarities of the MS of the natural product and synthetic standard (Figure 2A and B). The MS of III suggests that this compound is a chain-shortened analog of IV (Figure 2C).

Hydrogenation/hydrogenolysis of a pooled sample of four aeration extracts of *E. obscurus* males (N = 160) yielded ca. 1:5 ratio of nonane and decane, substantiating the presence of methyl decadienoates, plus a set of compounds eluting in the range expected for C_{14-16} hydrocarbons (Figure 3A). Synthetic 2,6,10-trimethyltridecane, and the compound derived from the natural product eluting at 10.84 min by GC-MS, coeluted on both the 30-m DB-1 and HP-5 GC columns. The identity of the 10.84-min unknown hydrocarbon as 2,6,10-trimethyltridecane was confirmed by mass spectrometry (Figure 3B and C). The 10.30-min compound derived from the aeration sample matched the MS of 4,8-dimethyltridecane (the expected chain-shortened hydrocarbon, M^+ = 212) retrieved from the computerized library of spectra. Thus, compound IV is methyl 2,6,10-trimethyltridecanoate.

Similarly, synthetic 2,6,10-trimethyldodecane, and the compound derived from the natural product eluting at 10.03 min by GC-MS, coeluted on both columns and produced virtually identical mass spectra (Figure 3D and E). Thus, compound III is methyl 2,6,10-trimethyldodecanoate.

DISCUSSION

Methyl (2*E*,4*Z*)-decadienoate and methyl 2,6,10-trimethyltridecanoate account for 88% of the total male-specific volatiles of *Euschistus obscurus*. Several minor methyl-esters are also released by males, including methyl 2,6,10-trimethyldodecanoate and methyl (2*E*,4*E*)-decadienoate. Determination that the noval volatiles from *E. obscurus* males are trimethyl-branched compounds corrects the earlier tentative report of dimethyl branching for these methyl-esters

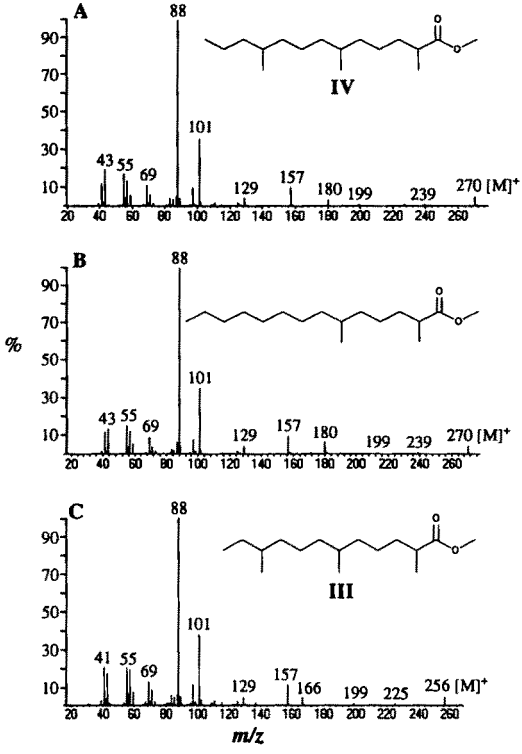

FIG. 2. Electron impact mass spectra of *Euschistus obscurus* (A) male-specific compound IV, (B) a standard of the compound tentatively identified earlier as the structure for IV (methyl 2,6-dimethyltetradecanoate), and (C) male-specific compound III.

(Aldrich et al., 1991). Live *E. obscurus* males and aeration extracts of males were attractive to conspecific females in laboratory bioassays (Borges and Aldrich, 1994).

Comparison of aerations from males of the Neotropical species, *E. heros*, to those of *E. obscurus*, indicates that only methyl 2,6,10-trimethyltridecanoate is a major component of the suspected pheromone for the South American species. In addition, sexually active *E. heros* males apparently release only about a tenth as much pheromone per day as do *E. obscurus* males. Thus, research on the relatively innocuous *E. obscurus* offered a means to characterize the main male-specific volatile from *E. heros* without importing this soybean pest into the United States (Borges and Aldrich, 1994). The chirality of methyl 2,6,10-trimethyltridecanoate is currently unknown, but synthesis of stereoisomers for

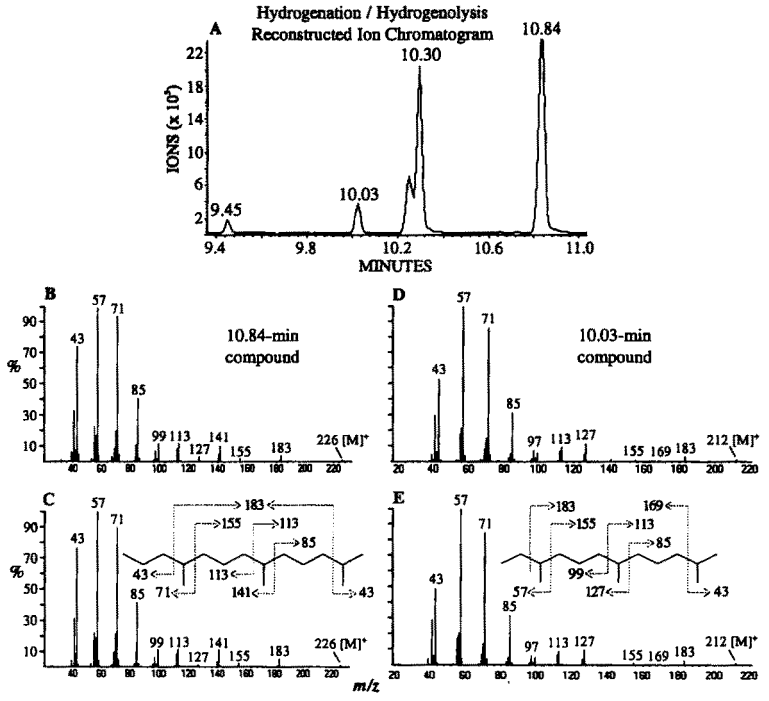

FIG. 3. (A) Reconstructed ion chromatogram for the region showing the hydrogenation/hydrogenolysis reaction products from *Euschistus obscurus* male-specific compounds III (R_t = 9.45 and 10.03 min) and IV (R_t = 10.30 and 10.84 min). Electron impact mass spectra of (B) the 10.84-min compound derived from natural product IV, (C) a standard of 2,6,10-trimethyltridecane, (D) the 10.03-min compound derived from natural product III, and (E) a standard of 2,6,10-trimethyldodecane.

further bioassay experiments with the South American pest species is underway (K. Mori, personal communication).

Prior to the present chemical analysis of a Neotropical *Euschistus* sp., it was suggested that the unusual chemistry of *E. obscurus* males may provide a clue as to the pheromonal pattern of South American *Euschistus* spp. (Aldrich et al., 1991). This speculation was supported by the lack of response of two Neotropical species (*E. taurulus* and *E. acutus*) to methyl (2*E*,4*Z*)-decadienoate in the field (Aldrich et al., 1991). The discovery that *E. heros* males produce predominantly methyl 2,6,10-trimethyltetradecanoate substantiates our earlier speculation, notwithstanding the correction herein for *E. obscurus* methyl-branched esters. Analyses of more Neotropical and Central American Nearctic *Euschistus* spp. are needed to determine if the most recent generic revisions are

consistent with the pheromonal chemistry of the group (Rolston, 1974, 1984). From a practical standpoint, we hope that identification of the suspected pheromone components of *E. heros* will lead to the use of semiochemicals for management of this pest.

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RESPONSES BY AMPHISBAENIAN *Blanus cinereus* TO CHEMICALS FROM PREY OR POTENTIALLY HARMFUL ANT SPECIES

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Abstract—We tested the ability of amphisbaenians (*Blanus cinereus*) to discriminate between odors of ant species selected as prey (*Pheidole pallidula*) and odors of potentially harmful ant species (*Messor barbarus*) that are avoided. Tongue-flick rate to swabs impregnated with ant odors, cologne, or deionized water differed among treatments, showing that amphisbaenians were able to discriminate ant species odors. Amphisbaenians showed an aggressive response and bit applicators bearing the odor of harmful ants, while the odor of prey ants did not elicit bites to swabs. The possible evolutionary advantage of identifying and avoiding harmful ants is discussed in relation to the fossoriality of amphisbaenians.

Key Words—Hymenoptera, Formicidae, Reptilia, Amphisbaenidae, *Blanus cinereus*, prey odor, harmful prey, tongue-flicking.

INTRODUCTION

In some reptile species potentially nutritive food is ignored throughout life or during some stage of the life cycle (Rissing, 1981; Vogel and Von Brockhusen-Holzer, 1984; Vitt and Cooper, 1988; Webb and Shine, 1993). This might be considered an adaptation if the prey species are toxic or damage the predator during capture (Heller, 1980).

Ants constitute the main sources of food for some reptiles, but some ants are avoided despite being an abundant and seasonally stable resource (Pianka,

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1986). This may reflect the variety of defensive mechanisms against potential predators developed by some ants, such as powerful jaws, poison apparatus, and ability to launch a mass attack (Blum, 1981; Sudd and Franks, 1987; Detrain and Pasteels, 1992).

Blanus cinereus is a fossorial amphisbaenian endemic to the Iberian Peninsula (Salvador, 1981). Its diet mainly consists of insect larvae and ants. However, ants of some genera commonly available in the habitat are not eaten (López et al., 1991). Amphisbaenians capture ants underground in their nests with consequent risk of being mass-attacked by the ant colony. Defensive behavior of ant species would encourage selection of less aggressive and more easily captured species (Rissing, 1981; Vitt and Cooper, 1988; Webb and Shine, 1993).

If attempting to capture harmful ants decreases an amphisbaenian's life expectancy, detection and avoidance of these ant species would be advantageous (Heller, 1980). Vision is reduced in amphisbaenians, but studies of tongue-flicking behavior suggest that the vomeronasal system is used by *B. cinereus* to detect odors of prey (López and Salvador, 1992, In press) and conspecifics (Cooper et al., 1994). In this experiment we tested the ability of *B. cinereus* to discriminate between odors of ant species selected as prey and odors of potentially harmful aggressive ant species that are avoided.

METHODS AND MATERIALS

Eighteen adult *B. cinereus* (snout–vent length $\bar{X} \pm SE = 147 \pm 8$ mm) were collected in March 1992 near Navacerrada (Madrid Province, Spain) and transported to the laboratory at El Ventorrillo Field Station, 5 km from the capture site. Animals were housed individually in 5-liter glass jars containing sand substratum from the capture area and fed mealworm pupae (*Tenebrio molitor*) and adult ants (*Pheidole pallidula*) twice weekly. To standardize hunger levels, they were not fed for five days prior to experiments. Humidity was maintained daily with a water spray. Amphisbaenians were held in captivity at least one month before testing to allow acclimation to laboratory conditions and the experimenter's presence.

Some ant species have a marked morphological and chemical dimorphism between smaller normal workers (minors) and the large-headed soldiers (majors) that defend the colony against other hostile ants or potential predators (Law et al., 1965; Sudd and Franks, 1987). Therefore, we tested responses to odors from ants belonging to two castes of two different species that are usually found in the same microhabitats as amphisbaenians (Acosta, 1980; Martínez Ibañez, 1984; López et al., 1991): *Pheidole pallidula* (soldiers and workers) is one of the habitual prey species of *B. cinereus* (López et al., 1991); *Messor barbarus*,

a very large and very aggressive species, is not. Captive *B. cinereus* sometimes readily consume larvae of *Messor* in the laboratory, as long as adult ants are not present. These larvae are never found in the diet of wild amphisbaenians (López et al., 1991). Adult *Messor* attack amphisbaenians in the laboratory, causing them to retreat (unpublished data). This suggests that amphisbaenians are vulnerable to attacks by *Messor* ants.

Comparisons of tongue-flick (TF) rate by amphisbaenians in response to stimuli arising from cotton applicators impregnated with (1) ant odors (workers or soldiers of *Pheidole*, or workers or soldiers of *Messor*), (2) cologne (pungency control), or (3) deionized water (odorless control) were made to test for differential responses to odors (Cooper and Burghardt, 1990). Water was used to gauge baseline TF rates in the experimental situation. We prepared stimuli by dipping the cotton tip (1 cm) of a wooden applicator (10 cm) in deionized water. Other stimuli were added by rolling the moistened cotton over the body surface of ants, or by dipping it in diluted cologne. A new stimulus was used in each trial. Every amphisbaenian responded to each stimulus once in a randomized block design, and order of presentation was counterbalanced. One trial was conducted per day for each animal. Trials were conducted between July 1 and 15, 1992, between 1000 and 1400 hr when amphisbaenians were fully active.

We simulated fossorial conditions for experiments by placing each individual in a different transparent plastic tube (30 × 1 cm), with sand in the lower half and the ends plugged with cotton (López and Salvador, 1992, In press; Cooper et al., 1994). The laboratory was darkened during trials, and observations were made using a 50-W red light. Room temperature was maintained at a constant 20°C. The amphisbaenians were placed in their tubes for 5 hr/day during the acclimation period where they behaved and fed normally. Each individual was placed in its trial tube for 10 min prior to testing for acclimatization in its novel environment.

To begin a trial, an experimenter slowly approached a tube, removed the cotton plug from one end, slowly moved the cotton swab to a position 2 cm anterior to the amphisbaenian's snout, and closed the tube. Total TFs and TFs directed to the swab were recorded for 60 sec beginning with the first TF. Latency to the first TF was computed as the period elapsed between closing the tube and the first TF.

To examine differences in number of TFs and latency to first TF between conditions, we used the nonparametric Friedman two-way ANOVA because variances were not homogeneous. Pairwise comparisons of means were planned using Wilcoxon signed rank-matched pairs tests (Siegel, 1956). Spearman rank correlations were conducted between latency to first TF and number of TFs (Sokal and Rohlf, 1981).

RESULTS

All amphisbaenians directed TFs to the swab in all conditions. There were significant differences among stimulus condition in total TFs ($\chi^2 = 75.71$; $P < 0.0001$) (Figure 1). Chemicals from soldier *Messor* ants elicited more TFs than from the other conditions ($P < 0.001$ in all cases). Chemicals from workers of *Messor* elicited a significantly higher response ($P < 0.01$) than cologne and other treatments. Responses for both castes of *Pheidole* were nearly identical ($P = 0.80$), and marginally lower ($P = 0.06$) than for cologne. Finally, the response to deionized water was significantly lower ($P < 0.001$) than for all other stimuli.

The number of TFs directed to swabs differed significantly among treatments ($\chi^2 = 64.99$; $P < 0.0001$) (Figure 1). The number of TFs directed to swabs with soldier and worker *Messor* ant stimuli were not significantly different ($P = 0.10$), but both were significantly higher than for other stimuli ($P < 0.01$ in all cases). Response to chemicals from both castes of *Pheidole* ants and cologne were not significantly different ($P > 0.1$ in all cases). The TF rate in response to deionized water was significantly lower than to all the other conditions ($P < 0.001$ in all cases).

Mean latency to first TF differed significantly among conditions ($\chi^2 = 28.55$; $P < 0.0001$) (Figure 2). Latency to the first TF in response to deionized water was significantly longer than to other stimuli ($P < 0.001$ in all cases). Latency to first TF did not differ between cologne and each of the castes of *Messor* or *Pheidole* ants ($P > 0.05$ in all cases). There was a weak but significant negative correlation between latency to first TF and total number of TFs in all trials combined ($r_s = -0.27$; $P = 0.004$), and between latency and number of TFs directed to the swab ($r_s = -0.27$; $P = 0.005$).

Four amphisbaenians bit the applicators with soldier *Messor* odor, and three different individuals bit them in response to worker *Messor* stimulus. Further-

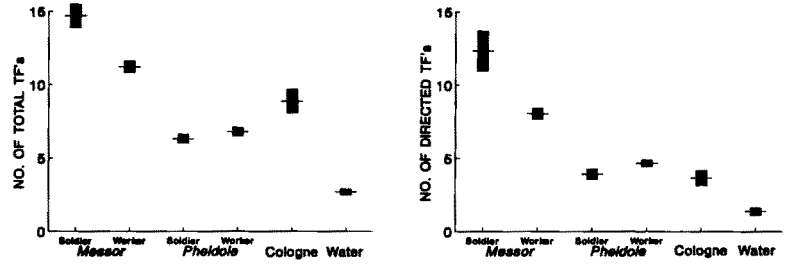

FIG. 1. Mean (± 1 SE) of total number of tongue-flicks (TF), and TFs directed to swabs in 60 sec by *Blanus cinereus* in response to several species and castes of ants, cologne, or deionized water stimuli presented on cotton-tipped applicators.

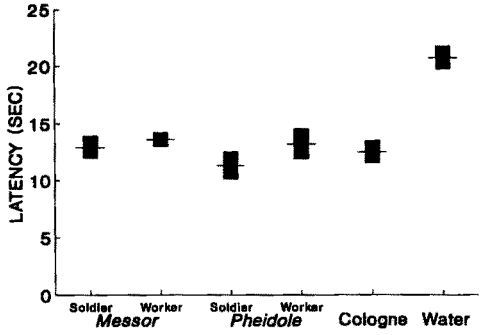

FIG. 2. Latency in seconds (mean ± 1 SE) to the first tongue-flick by *Blanus cinereus*, testing responses to several species and castes of ants, cologne, or deionized water odor stimuli.

more, one individual bit the swab with cologne. On the null assumption that the likelihood of biting was equal in all four conditions (pooling the castes within species), the binomial probability that seven of the eight bites would be in the *Messor* ant stimulus condition is 0.0024.

DISCUSSION

Detection and avoidance of harmful ants is one important task faced by amphisbaenians in underground environments. Individuals accidentally entering a *Messor* ant nest could be seriously damaged during a massed counterattack. Thus, natural selection should favor individuals that detect harmful ants early, thereby minimizing the risk of being attacked (Heller, 1980). Our results show that amphisbaenians are able to discriminate between ant species odors and to respond aggressively toward stimuli from dangerous ant species.

Some visually oriented epigeal lizards that can hardly perceive differences between harmful and harmless ant species show a general reluctance to take ants (Vogel and Von Brockhusen-Holzer, 1984). Other lizards may avoid ant species based on differences in ant foraging-group size and foraging method that are correlated with ant aggressive behavior (Rissing, 1981). However, for a blind fossorial reptile chemical discrimination seems to be the only means of detecting differences among ant species.

Ants produce secretions for offense, defense, and communication from a system of exocrine glands extending through the body (Cavill and Robertson, 1965; Attygalle and Morgan, 1985). Differences between groups of ants, species, or even castes might be correlated with chemical differences in the compounds that these glands secrete (Cavill and Robertson, 1965; Law et al., 1965;

Attygalle and Morgan, 1985; Ali et al., 1988, 1989). Thus, amphisbaenians may be able to discriminate among these chemical compounds and associate some of them with the presence of a specific ant.

Differential responses to chemical cues of several ant species have also been found in the ant trail-following behavior of a fossorial blindsnake (Webb and Shine, 1992). Young blindsnakes may use this ability to avoid dangerous ant species (Webb and Shine, 1993).

Amphisbaenians not only detected harmful ants but sometimes responded aggressively toward their odor stimuli, briefly biting the swabs before retreating immediately into the tube. The bites would thus appear to represent a defensive response to a perceived threat at close range. Amphisbaenians also bit the swabs impregnated with odor of conspecific males (Cooper et al., 1994), but did not flee after biting, reflecting aggressive motivation.

Responses to chemicals from *Pheidole* ants did not elicit bites to the swab (see also López and Salvador, 1992). Amphisbaenians may use several sensory cues to release attack to prey, and critical tactile or hearing stimuli needed may have been absent (Cooper, 1990; Graves and Halpern, 1990). However, tongue-flicking is actually used to discriminate between different live prey types (López and Salvador, In press).

This experiment shows that the amphisbaenian *B. cinereus* has developed the ability to discriminate, probably via the vomeronasal sense, chemical compounds of potentially dangerous ants from those of ants selected as prey. This capacity would be evolutionarily advantageous in aiding avoidance of the harmful species and most likely represents a component of the suite of adaptations associated with fossoriality.

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Journal of Chemical Ecology, Vol. 20, No. 5, 1994

CUTICULAR HYDROCARBONS OF *Aedes hendersoni* COCKERELL and *A. triseriatus* (SAY)[1]

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Abstract—Field-caught adult male and female *Aedes hendersoni* are difficult to distinguish from the sibling species *A. triseriatus*. We found that mosquitoes from the same sex of the sibling species can not be readily separated either by unique cuticular hydrocarbon components or by differences in percent composition of those components. Multivariate analysis of the cuticular hydrocarbon data does not provide good separation. Cuticular hydrocarbons were identified using gas chromatography electron-impact mass spectrometry and gas chromatography chemical-ionization mass spectrometry. Flame-ionization capillary gas chromatography was used for quantitative analysis of individual mosquitoes. Sixty-four hydrocarbons with chain lengths from C_{16} to greater than C_{46} were common to both species. Identified hydrocarbon components were *n*-alkanes, monomethylalkanes, dimethylalkanes, trimethylalkanes, and alkenes.

Key Words—Insecta, Culicidae, *Aedes hendersoni*, *Aedes triseriatus*, cuticular hydrocarbons.

INTRODUCTION

Aedes hendersoni Cockerell and *Aedes triseriatus* (Say) are sibling species of treehole breeding mosquitoes and are sympatric throughout much of their range, even being found in the same treeholes (Hedeen, 1963; Truman and Craig, 1968; Zavortink, 1972; Scholl and DeFoliart, 1977; Sinsko and Grimstad, 1977). *Aedes triseriatus* transmits LaCrosse (LAC) virus (Watts et al., 1972, 1973;

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[1] Diptera: Culicidae.

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Berry et al., 1975), and, in the laboratory, eastern equine encephalitis (Davis, 1940; Chamberlain and Sudia, 1961), dengue virus (Freier and Grimstad, 1983), and dog heartworm (Weinmann and Garcia, 1974; Rogers and Newson, 1979). Until recently, *A. hendersoni* was known as a poor vector of LAC virus (Watts et al., 1975). *Aedes hendersoni* has been shown, however, to transmit LAC virus in the laboratory when coinfected with *Plasmodium gallinaceum* (Paulson et al., 1992).

Because of this difference in vector competence, several researchers have looked for ways to readily taxonomically separate the two species. Larvae and young, unrubbed adults can be distinguished (Harmston, 1969; Grimstad et al., 1974; Truman and Craig, 1968), but this is difficult on older, field-caught adults. Certain behavioral characters can be used to separate the two species in natural habitat (Sinsko and Grimstad, 1977; Scholl and DeFoliart, 1977; Novak et al., 1981), but these characters are clearly not practical for routine characterization. Saul et al. (1977) found isozyme variants at the malate dehydrogenase (MDH) locus that distinguished the two species. Matthews and Munstermann (1983) found four additional loci to be species diagnostic (no major alleles shared) and five loci to be species distinguishing (major alleles shared, but frequencies differed).

The purpose of this study was to evaluate cuticular hydrocarbons as a possible means of accurately and quickly identifying adults of both sexes of *A. hendersoni* and *A. triseriatus*. Electrophoresis can only be done on specimens frozen in liquid nitrogen and the specimen is unrecoverable after use. Cytological studies require meiotic chromosomes from pupae (Taylor and Craig, 1985). In contrast, cuticular hydrocarbon analysis can be done on field-caught, laboratory-reared, or museum specimens without destroying the specimen for later taxonomic reference.

Cuticular hydrocarbons offer the possibility of separating the two species by the occurrence of unique components and/or by quantitative differences in components occurring in both species. For this study, cuticular hydrocarbons were characterized with gas chromatography electron-impact mass spectrometry (GC EI-MS) and gas chromatography chemical-ionization mass spectrometry (GC CI-MS). In addition, the hydrocarbon components of individual mosquitoes were studied quantitatively with flame-ionization gas chromatography (GC). Similar techniques were used in an attempt to separate laboratory colonies of sympatric *Anopheles gambiae* and *A. arabiensis* populations (Carlson and Service, 1979, 1980), in partially characterizing the *Anopheles maculipennis* complex (Phillips et al., 1990), and in distinguishing geographic populations of *Aedes albopictus* (Kruger et al., 1991; Kruger and Pappas, 1992). Cuticular hydrocarbon profiles have also been used to distinguish other Diptera: geographic populations of *Drosophila* (Luyten, 1983, cited by Jallon and David, 1987) and 11 species of *Tabanus* (Hoppe et al., 1990).

METHODS AND MATERIALS

Specimens. Specimens were hatched from field-collected eggs or raised from field-collected larvae. Eggs were hatched in 1 : 10,000 nutrient broth with initial aeration for 15 min. Rearing of larvae followed Kruger et al. (1991). Briefly, larvae were reared at 23°C ± 2°C under identical conditions using ground Tetramin as food and 16 : 8 hr light–dark. Third- and fourth-instar larvae were identified using the diagnostic characters of Lunt (1977). Identification of representative specimens was confirmed by S.R. Lunt. Pupae were sexed by size and removed to emergence cages. Non-blood-fed adults were frozen (0°C) at the age of 5–8 days.

Characterization of Cuticular Hydrocarbons. Larvae of *A. triseriatus* reared for GC EI-MS samples were collected from treeholes at eight sites in the central and eastern United States: Brown County, Nebraska; Filmore County, Minnesota; Waupaca County, Wisconsin; Vermilion County, Illinois; St. Joseph County, Indiana; Roscommon County, Michigan; Ashtabula County, Ohio; and Oneida County, New York. These collections were made between May 15 and June 4, 1989. Larvae of *A. hendersoni* reared for GC EI-MS samples were collected during the same time period from treeholes at eight sites in the following Nebraska counties: Scottsbluff, Morrill, Dawes, Frontier, Brown, Gosper, Harlan, and Howard. Treehole material was removed using a turkey baster, siphon, or spoon; placed in canning jars; and transported to the laboratory.

For GC EI-MS, each sample (number of replicates) consisted of 40 mosquitoes from one collection site: *A. triseriatus* males (2), females (6); *A. hendersoni* males (1), females (1). Details of preparation for GC EI-MS are given in Kruger et al. (1991) and briefly summarized here. Cuticular hydrocarbons were extracted with hexane (Hexane Optima, Fisher Scientific, St. Louis, Missouri) three consecutive times for 1 min each. The nonpolar components were separated by mini-column chromatography with Biosil-A (Bio-Rad Labs, Richmond, California) (Howard et al., 1978). Samples were evaporated to dryness under nitrogen and redissolved to a concentration of 10 insect-equivalents/μl. One microliter of each sample was injected into the GC EI-MS for structural analyses. Even-numbered *n*-alkane external standards were run to calculate equivalent chain lengths (ECLs) (Jackson and Blomquist, 1976). ECLs along with published mass spectral fragmentation patterns were used to identify cuticular hydrocarbon components (Jackson and Blomquist, 1976; Nelson, 1978; Pomonis et al., 1980).

Gas chromatography EI-MS analysis was conducted using a Hewlett-Packard 5790A GC connected to a Hewlett-Packard 5970 Mass Selective Detector (70 eV) (Hewlett Packard, San Fernando, California). A 10-m × 0.2-mm DB-1 bonded phase capillary column with head pressure of 3.5 kg/cm^2 was used with helium carrier gas. After a 2-min initial hold period, the oven tem-

perature was increased from 150°C to 320°C at 5°C/min with a final hold period of 40 min.

A. triseriatus adults used for the GC CI-MS were reared from eggs laid on balsa paddles in ovitraps (Novak and Peloquin, 1981). The traps were located in a wooded area in Nemaha County, Nebraska, and eggs were collected in July 1991. Rearing conditions and sample preparation were identical to that for GC EI-MS except that each sample consisted of 100 mosquitoes of the same sex. Chemical-ionization mass spectra were obtained on two samples of *A. triseriatus* females and one sample of *A. triseriatus* males.

Chemical-ionization mass spectra were obtained using a Hewlett Packard 5890 Series II gas chromatograph coupled to a Hewlett Packard 5971 Mass Selective Detector (MSD) with the G1072A chemical ionization accessory. The GC was equipped with a 30-m × 0.32-mm DB-5 capillary column (J & W Scientific, Folsom, California), and all analyses utilized temperature programming from 50 to 150°C at 20°C/min and from 150 to 320°C at 5°C/min. Each run had an initial hold period of 2 min and a final hold time of 20 min. The splitless injector port was set at 250°C and the GC/MSD interface was set at 280°C. Ultrapure helium was the carrier gas with a column head pressure of 3.5 kg/cm^2, and ultrapure methane was the ionizing gas. Mass spectra were scanned from m/z 100 to 650 and data were collected and analyzed on a Hewlett Packard Vectra 486/33T workstation using HP Chemstation software.

Quantitative Analysis of Cuticular Hydrocarbon Components. Individual *Aedes triseriatus* and *A. hendersoni* mosquitoes were used for quantitative analyses (seven replicates of each sex from each species). *Aedes triseriatus* adults used were reared from balsa paddles collected in Nemaha County, Nebraska, as described above. *Aedes hendersoni* were reared from larvae collected from tree holes in Morrill County, Nebraska, in July 1991. Preparation of single insects for GC followed the procedure described for GC EI-MS, except the three hexane rinses were reduced to 0.25 ml each. Equipment and operating conditions were identical to those used in Kruger et al. (1991) except here the GC was equipped with a 15-m × 0.25-mm DB-5 fused silica capillary column, 0.25-μm film thickness (Supelco, Bellefonte, Pennsylvania).

Gas chromatography data were expressed as percent composition and transformed to the arcsine of the square root (Sokal and Rohlf, 1981) prior to univariate statistical analysis. Hydrocarbon components for principal component analysis (PCA) were selected by two-way analysis of variance (ANOVA). The two-way ANOVA was used to select hydrocarbon components (percent composition $\geq 0.5\%$), which did not show significant interaction ($P \leq 0.05$) between species and sex, but did show significant variance ($P \leq 0.05$) between the two species. Transformed data were standardized to zero means and unit standard deviation prior to PCA of a correlation matrix. Analysis was performed with SYSTAT Version 5.0 (SYSTAT, 1992).

RESULTS

Characterization of Cuticular Hydrocarbon Components. Sixty-four cuticular hydrocarbon peaks are shared by both *A. hendersoni* and *A. triseriatus* (Table 1, Figure 1). Forty-two of these cuticular hydrocarbon peaks were identified as single hydrocarbons or mixtures of hydrocarbons (Table 1): 16 *n*-alkanes, 19 monomethylalkanes, 4 dimethylalkanes, and 2 trimethylalkanes. Three alkenes were detected by their characteristic EI-MS fragmentation pattern. Together they constituted approximately 0.1% relative abundance. No effort was made to identify the location or stereochemistry of the double bond. Twenty-two peaks were unidentified (Unk). Eleven of the Unk peaks (Table 1, peaks 54–64) occurred at retention times greater than the largest *n*-alkane standard (dotetracontane) available to us, and no ECLs could be derived from GC EI-MS data. Total carbon number of four of these peaks was established with GC CI-MS. Three of the four are mixtures of di- and trimethylalkanes (Table 1). Peaks 59–64 have carbon numbers >46, masses >650, and are beyond the capability of the CI-MS instrument available to us.

Sixteen *n*-alkanes from hexadecane to hentriacontane were identified (Table 1). The most abundant cuticular *n*-alkane in both sexes of both species was heptacosane. Thirteen peaks were identified as pure monomethylalkanes (Table 1). Four additional peaks were identified as mixtures of two or more isomeric monomethylalkanes (Table 1). Peak 40, a mixture of 13- and 11-methylnonacosane, was the most abundant monomethylalkane component in *A. hendersoni* and *A. triseriatus*.

Dimethylalkanes occurred at carbon chain lengths ≥ 29 carbons (Table 1). The dimethylalkanes included: peak 43, 5,19-dimethylnonacosane; peak 48, 5,17-dimethylhentriacontane; peak 49, 3,10-dimethylhentriacontane; and peak 54, 13,X-dimethyldotetracontane.

The peaks labeled Unk can be divided into two groups (Table 1). The unidentified peaks with retention times less than our largest *n*-alkane standard (Unknowns with peak numbers <54) represent $<2\%$ of the total hydrocarbon components. The peaks with retention times greater than our largest *n*-alkane standard (Unknowns with peak numbers 54–64) include eight unidentified peaks present in substantial abundance (33–45%). Two molecular ions were found for peak 56 with CI-MS and the numerous EI-MS fragments indicate a mixture of two carbon chain lengths of multiple-branched methylalkanes. Peak 57 was too small to generate an $(M - 1)^+$ ion with CI-MS. The remaining six peaks have masses >650 and were beyond the capability of the CI-MS instrument.

Percent composition data for females of both species is presented in Table 2 and for males in Table 3. Although the means of several peaks appear quite different, the standard deviations indicate great variability, precluding unambiguous separation of species. Accordingly, data were subjected to principal com-

TABLE 1. CUTICULAR HYDROCARBON PEAKS OF *Aedes triseriatus* AND *A. Hendersoni*

Peak no.	Hydrocarbon	CN[a]	ECL[b]	Diagnostic ions (*m/z*) EI-MS	CI-MS
1	*n*-C_{16}	16	16.00	226	[d]
2	Unk[c]		16.56		[d]
3	*n*-C_{17}	17	17.00	240	[d]
4	*n*-C_{18}	18	18.00	254	[d]
5	Unk		18.81		[d]
6	*n*-C_{19}	19	19.00	268	[d]
7	Unk		19.28		[d]
8	Unk		19.72		[d]
9	*n*-C_{20}	20	20.00	282	[d]
10	Unk		20.05		[d]
11	*n*-C_{21}	21	21.00	296	[d]
12	*n*-C_{22}	22	22.00	310	[d]
13	Unk		22.64		[d]
14	C_{23}:1	23	22.47	322	[d]
15	Unk		22.71		[d]
16	*n*-C_{23}	23	23.00	324	[d]
17	11-MeC_{23}	24	23.36	169, 197, 323	[d]
18	7-MeC_{23}	24	23.42	113, 253, 323	[d]
19	5-MeC_{23}	24	23.51	85, 281, 323	[d]
20	3-MeC_{23}	24	23.73	57, 309, 323	[d]
21	*n*-C_{24}	24	24.00	338	[d]
22	C_{25}:1	25	24.80	350	[d]
23	*n*-C_{25}	25	25.00	352	[d]
24	13- & 11-MeC_{25}	26	25.36	169, 197, 225, 351	[d]
25	7-MeC_{25}	26	25.43	113, 281, 351	[d]
26	5-MeC_{25}	26	25.52	85, 309, 351	[d]
27	3-MeC_{25}	26	25.74	57, 337, 351	[d]
28	*n*-C_{26}	26	26.00	366	[d]
29	Unk		26.47		[d]
30	Unk		26.66		[d]
31	C_{27}:1	27	26.74	378	[d]
32	*n*-C_{27}	27	27.00	380	[d]
33	MeC_{27} mix	28	27.43	113, 141, 197, 225, 281, 379	[d]
34	5-MeC_{27}	28	27.52	85, 337, 379	[d]
35	3-MeC_{27}	28	27.74	57, 365, 379	[d]
36	*n*-C_{28}	28	28.00	394	[d]
37	Unk		28.11		[d]
38	11-MeC_{28}	29	28.34	169, 267, 393	[d]
39	*n*-C_{29}	29	29.00	408	[d]
40	13- & 11-MeC_{29}	30	29.35	169, 197, 253, 281, 393	[d]

TABLE 1. CONTINUED

Peak no.	Hydrocarbon	CN[a]	ECL[b]	Diagnostic ions (*m/z*) EI-MS	CI-MS
41	5-MeC_{29}	30	29.52	85, 365, 393	[d]
42	Unk		29.75		[d]
43	5,19-diMeC_{29}	31	29.85	85, 169, 295, 379	[d]
44	*n*-C_{30}	30	30.00	422	[d]
45	11-MeC_{30}	31	30.33	169, 295, 421	[d]
46	*n*-C_{31}	31	31.00	436	[d]
47	13-MeC_{31}	32	31.32	197, 281, 421	[d]
48	5,17-diMeC_{31}	33	31.81	85, 197, 295, 407	[d]
49	3,10-diMeC_{31}	33	32.04	57, 169, 295, 323, 435	[d]
50	Unk		32.29		[d]
51	15- & 13-MeC_{33}	34	33.28	197, 225, 281, 309	[d]
52	Unk		33.60		[d]
53	Unk		33.77		[d]
54	13, X-diMeC_{42}	44	[e]	197, 253, 309, 337, 393, 449	617
55	13, 17, 21-triMeC_{42}	45	[e]	197, 267, 309, 337, 407, 463	631
56	Unk	45 & 46	[e]	169, 197, 267, 295, 323, 351, 393, 421, 477, 505	631 & 645
57	Unk				[d]
58	X, Y, Z-triMeC_{42}	45	[e]	141, 169, 183, 211, 239, 267, 295, 365, 393, 407, 421	631
59	Unk	[f]	[e]		[d]
60	Unk	[f]	[e]		[d]
61	Unk	[f]	[e]		[d]
62	Unk	[f]	[e]		[d]
63	Unk	[f]	[e]		[d]
64	Unk	[f]	[e]		[d]

[a] CN: carbon number.
[b] ECL: equivalent chain length.
[c] Unk: unknown structure.
[d] CI mass spectrum not obtained.
[e] ECL not available (see text).
[f] Larger than CI-MS capability; carbon number not available.

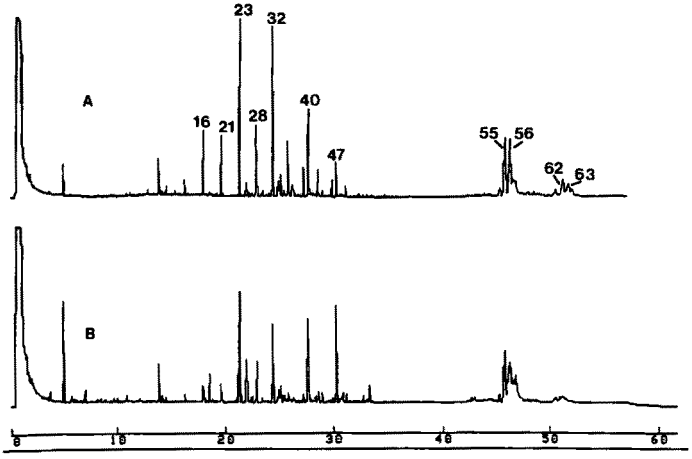

FIG. 1. Chromatograms of the cuticular hydrocarbons of *Aedes hendersoni* (A) and *A. triseriatus* (B). Labels indicate some major peaks as listed in Table 1.

ponent analysis to ascertain whether species separation could be attained with multivariate methods.

Multivariate Analysis. Eight components showed significant variance between the two species with no interaction between species and sex when submitted to two-way ANOVA. These eight components, peak numbers 23, 24, 34, 47, 49, 55, 61, and 62, were used for PCA. Figure 2 is a plot of the principal component scores for the first and second principal axes, representing 34 and 20% of the variance, respectively. Using a 95% confidence ellipse, the majority of *A. hendersoni* specimens lie within the 95% confidence ellipse of *A. triseriatus*.

DISCUSSION

The same cuticular hydrocarbons occur in *A. hendersoni* and in *A. triseriatus*, consisting of odd- and even-numbered normal, monomethyl-, dimethyl-, and trimethylalkanes, as well as alkenes, and high-molecular-weight multiple-branched methylalkane mixtures. Total carbon chain lengths range from 16 to more than 46 carbons. These results are similar to those found in *A. albopictus* (Kruger et al., 1991; Kruger and Pappas, 1992). Carlson and Service (1979, 1980) reported that the material sampled ". . . in *Anopheles gambiae* comprise a smoothly distributed array of peaks, centered about C_{27}, and have a small excess of paraffins with an even number of carbons . . . ." This result is at odds with all reported insect cuticular hydrocarbon profiles but matches closely that

TABLE 2. MEAN PERCENT (>0.5% in at Least One Species) AND STANDARD DEVIATION OF CUTICULAR HYDROCARBON COMPONENTS FOR SINGLE FEMALE MOSQUITOES

Peak no.	*A. hendersoni* (N = 7)		*A. triseriatus* N = 7)	
	Mean	SD	Mean	SD
3	0.5	0.8	0.5	0.3
6	0.5	0.3	0.7	0.5
8	0.1	0.1	0.1	0.1
9[a]	0.4	0.1	0.9	0.6
11	0.7	0.2	0.8	0.5
12[a]	1.0	0.2	1.3	0.8
13	0.1	0.2	0.8	1.0
16[a]	2.7	2.0	0.1	0.1
19	0.8	1.2	3.1	1.3
21	2.1	1.4	4.2	6.2
23[a]	7.0	4.8	1.5	1.3
24[a]	1.0	0.9	TR[b]	0.1
26	1.2	2.1	1.0	0.7
28	2.6	1.8	4.2	1.7
32	8.9	3.3	6.8	1.6
33	1.7	2.0	0.6	0.4
34[a]	1.0	0.2	1.4	0.6
36	1.5	0.9	2.3	1.0
37	0.8	0.6	1.0	0.5
38	0.6	0.2	0.2	0.1
39	1.9	0.9	3.1	0.9
40	7.4	3.2	4.6	1.8
41[a]	1.1	1.7	0.1	0.2
44	7.9	4.0	1.7	0.6
45[a]	0.8	0.3	3.0	4.0
46	0.7	0.3	1.0	0.4
47	2.4	1.0	4.6	1.7
48	0.4	0.7	1.2	0.9
49	0.8	0.2	1.2	0.3
50	0.1	0.1	0.6	0.4
52	0.5	0.2	0.3	0.1
53	0.1	0.1	1.0	0.3
54	1.1	0.5	0.9	0.3
55	9.6	2.3	10.0	1.3
56	11.0	3.3	16.0	2.9
57	2.8	2.1	10.0	4.3
58[a]	2.3	0.7	TR	TR
59[a]	1.4	0.3	0.7	0.4
60[a]	1.1	0.6	0.8	0.3
61	2.3	0.6	1.2	0.3
62	5.6	1.6	3.7	1.3
63[a]	4.6	1.9	1.1	0.3
64[a]	1.3	0.4	TR	TR

[a]Means in a row with this symbol are significantly different at P = 0.05.
[b]TR = trace, <0.1%.

TABLE 3. MEAN PERCENT (>0.5% in at Least One Species) AND STANDARD DEVIATION OF CUTICULAR HYDROCARBON COMPONENTS FOR SINGLE MALE MOSQUITOES

Peak no.	*A. hendersoni* (N = 7)		*A. triseriatus* (N = 7)	
	Mean	SD	Mean	SD
1	0.5	0.4	0.6	0.3
3	0.3	0.2	0.7	0.7
4	0.5	0.2	0.4	0.2
6	0.6	0.3	0.7	0.5
9	0.5	0.2	0.5	0.2
11[a]	0.6	0.1	0.9	0.5
12[a]	0.9	0.1	1.0	0.8
16	3.1	0.4	0.8	0.6
17[a]	2.9	2.2	0.2	0.4
20[a]	0.2	0.2	0.7	1.4
21[a]	3.2	1.6	0.8	0.3
23[a]	9.0	1.6	5.2	4.0
24	4.0	1.8	1.5	2.8
26	0.3	0.2	0.7	0.3
27[a]	0.8	1.2	0.6	0.4
28	4.2	2.6	3.6	4.2
32	9.3	1.2	8.1	2.4
33	0.6	0.1	0.6	0.2
34	0.7	0.1	1.3	0.2
35	0.5	0.2	0.5	0.1
36	2.8	1.3	2.3	2.9
37	1.0	0.5	0.8	0.7
38[a]	0.4	0.1	0.6	0.5
39[a]	2.6	0.8	2.4	2.6
40	5.8	1.3	5.3	2.3
43[a]	1.1	1.7	0.9	0.4
44	1.4	0.7	1.7	1.7
45	0.4	0.2	1.0	0.3
46	0.9	0.4	1.3	1.2
47	2.5	1.1	3.7	1.0
48	0.4	0.1	0.7	0.1
49[a]	0.7	0.2	1.1	0.3
51	0.4	0.3	0.9	0.5
52	0.5	0.3	0.5	0.4
54	1.2	0.4	1.2	0.4
55	8.9	3.3	11.0	3.3
56	9.2	2.6	13.0	4.2
57[a]	0.6	0.6	3.9	0.1
58[a]	1.9	1.0	5.3	4.1
59	1.1	0.7	0.8	0.7
60	1.1	0.8	0.9	0.6
61	1.8	1.2	1.1	1.0
62[a]	3.7	1.8	1.8	0.8
63[a]	2.4	0.5	1.9	1.1
64	0.7	0.3	0.3	0.6

[a] Means in a row with this symbol are significantly different at $P = 0.05$.

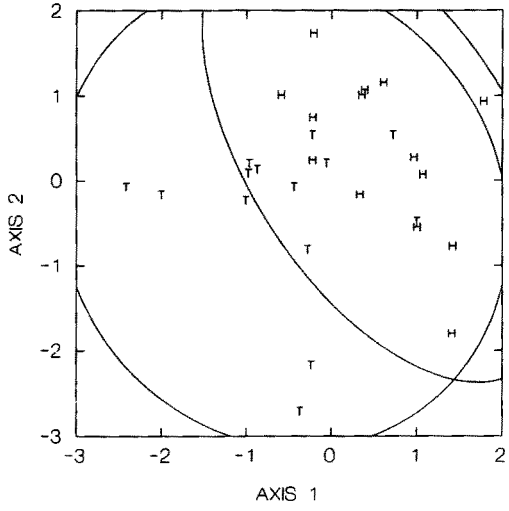

FIG. 2. Principal component analysis of *Aedes hendersoni* (H, 7♀, 7♂) and *A. triseriatus* (T, 7♀, 7♂). First principal axis represents 34% of the variance, second principal axis represents 20% of the variance. 95% confidence ellipses are shown.

of paraffin wax samples, leading us to suspect that their samples were contaminated. Their samples from *Anopheles arabiensis* showed slightly less contamination with odd-numbered *n*-alkanes in moderately greater abundance than even-numbered *n*-alkanes. In *Culex quinquefasciatus*, Chen et al. (1990) found *n*-alkanes of 23- to 33-carbon chain lengths, one branched or possibly unsaturated compound of 29-carbon chain length, and three broad peaks containing branched compounds of chain lengths 40–47 carbons. Although they have a smaller number of peaks, the published chromatograms of *Culex* species (Chen et al., 1990) show overall similarity to those presented in this paper and to our unpublished chromatograms of *A. albopictus* and *A. aegypti*. Phillips et al. (1990) published chromatograms of females of four members of the *Anopheles maculipennis* complex showing a large number of peaks. No identifications of hydrocarbon components were reported, however. Rosa-Freitas et al. (1992) studied cuticular hydrocarbons of three populations of *Anopheles darlingi*. As in Phillips et al. (1990), no identification of components was attempted, and no ECLs or Kovats indices were presented, leaving carbon chain lengths or total carbon numbers unknown for these species, also.

No cuticular hydrocarbon components of *A. hendersoni* and *A. triseriatus* were unique to either sex. A similar lack of qualitative sexual dimorphism was reported for *C. quinquefasciatus* (Chen et al., 1990). Although a lack of qualitative sexual dimorphism was also reported for *Anopheles gambiae* and *Anoph-*

eles arabiensis (Carlson and Service, 1979, 1980), their results are clearly questionable given the possible paraffin wax contamination noted above.

Carlson and Service (1979, 1980) attempted to use cuticular hydrocarbons for the quantitative separation of closely related species of mosquitoes. The separation of these anopheline species was based on ratios of three pairs of peaks. The first ratio utilized a peak of considerable size and was purported to be insect-derived *n*-hexacosane. As mentioned above, this is an unusual case of an insect having large amounts of even-numbered carbon chain alkanes and possibly resulted from contamination as noted above. Only one of the three ratios presented was at all consistent over the three preparations used: crude preparations of single mosquitoes, paraffins-only preparations of single mosquitoes, and paraffins-only extracts of five pooled individuals. The ratio technique has seen little use in subsequent insect hydrocarbon studies except by Chen et al. (1990).

We first tried one-way ANOVA as a means of identifying peaks significantly different between females and males of *A. hendersoni* and *A. triseriatus* (Tables 2 and 3). Although these one-way ANOVAs identified several peaks with significantly different means ($P < 0.05$), we feel that our data do not support practical separation of the species using individual hydrocarbon components alone due to the large standard deviations encountered for many of these components. Furthermore, most of the components with statistically significant, different means are small peaks with inherently large variation. Enlarging the sample size could decrease these variances but probably not enough to afford reliable separation of the species. This conclusion is based on our findings in an unpublished study of over 100 *A. triseriatus* females (Rumbaugh, Christen, and Pappas).

Since multivariate analysis had proved successful in separation of allopatric geographic populations of *A. albopictus* (Kruger et al., 1990; Kruger and Pappas, 1991), we turned to multivariate techniques for this study. Figure 2 shows that PCA separation of *A. hendersoni* and *A. triseriatus* is weak, at best. PCA works well in separation of groups when a large proportion of the variance is in three or fewer principal axes (Sneath and Sokal, 1973). Our inability to separate these two sibling species with PCA was in agreement with the relatively small proportion of variance (70%) in the first three principal axes (SYSTAT, 1992).

Phillips et al. (1990) used multivariate analysis of cuticular hydrocarbons to study species relationships in the *Anopheles maculipennis* complex. However, by using hexane-extracted samples without further treatment to limit the extract to nonpolar components, the six peaks selected for use in stepwise discriminant analysis may or may not have actually been hydrocarbons. Phillips et al. (1990) did utilize a statistical method to choose six peaks best suited to discriminating the species from the large number of peaks shown in their chromatograms.

No previous studies on mosquito cuticular hydrocarbons have used principal component analysis. However, Jallon and David (1987) utilized this multivariate technique to separate eight species of the *Drosophila melanogaster* subgroup. Several problems arise in comparing our work to theirs. Jallon and David (1987) mentioned using ANOVA, but did not indicate any criteria for selection of characters used in the PCA. Secondly, the positions of species on the plot of the first two principal components were indicated by a single symbol. Whether this single symbol is a centroid is unclear. Thirdly, no confidence ellipses were shown. The close proximity of some of the species on the PCA plot when compared to our work leads us to question whether intraspecific variation is adequately represented, and thus, whether species separation was really demonstrated by their work.

Interspecific and intraspecific geographic variation was addressed in this study only with respect to qualitative analysis of the cuticular hydrocarbons. As suggested by Phillips et al. (1990), sympatric sibling species may show greater variation in cuticular hydrocarbon composition than allopatric populations. Our multivariate studies were carried out on allopatric populations of sibling species and did indeed show little variation in hydrocarbon composition between either species or sex.

Future studies of mosquito cuticular hydrocarbons need to pay careful attention to several areas. The chemical details should include mass spectral analysis for identification of compounds, with no assumption that a peak with similar retention time or ECL between species, sexes, or life stages is exactly the same compound(s). We have seen in this, and other studies of ours, a great variety of methyl branch positions and broad peaks, especially those with carbon chain lengths >30 that were often highly variable mixtures of several isomers. Biological details should include careful attention to rearing conditions, laboratory versus wild populations; to variation of environmental factors such as temperature and diet; and to variation with seasonal changes. Analytical details need to be carefully considered also. Multivariate analytical techniques offer great promise in studying small differences in a suite of phenotypic characters. However, no assumption should be made that every cuticular hydrocarbon component has value in multivariate analysis, and the question asked should guide selection of variables. If the question asked is whether multivariate analysis of cuticular hydrocarbons can provide a tool for taxonomic separation, hydrocarbon components with statistically significant different means are most useful. If the question asked concerns interspecific or intraspecific semiochemical behavior, a wider suite of characters may prove useful. It is too early to unequivocally state that mosquito cuticular hydrocarbons will always be good inter- or intraspecific separators, or that, as in other Diptera (Blomquist et al., 1993), mosquitoes use hydrocarbons in chemical communication. It is certainly possible that differences

detectable by humans and differences detectable by mosquitoes are quantitatively very different.

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EFFECTS OF ORGANIC SOLVENTS ON USE OF TARBUSH BY SHEEP

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Abstract—Tarbush (*Flourensia cernua* DC) is a native perennial shrub prevalent in many parts of the Chihuahuan Desert. Ruminants have exhibited limited use of tarbush leaves and new leaders as a forage during the summer growing season. Efforts to increase use of tarbush by lambs through spraying with various organic solvents were unsuccessful, probably because of the highly variable response. However, complete immersion of tarbush in organic solvents (acetone and ethanol) increased ($P < 0.01$) tarbush use by ram lambs when compared to unaltered tarbush. Data suggest that removal of secondary compounds from the leaf surface of tarbush using organic solvents enhanced acceptability of tarbush to sheep.

Key Words—*Flourensia cernua*, leaf surface, organic solvents, sheep, herbivory, tarbush.

INTRODUCTION

Many arid rangeland shrubs are used sparingly or seasonally by livestock, if at all (Owen-Smith and Cooper, 1987). Tarbush (*Flourensia cernua* DC) is present throughout the Chihuahuan Desert of North America and has a resinous leaf surface. Tarbush is consumed in limited quantities by sheep and cattle during certain times of year (Nelson et al., 1970; Anderson and Holechek, 1983). Tarbush has a relatively high nutritional value (Nelson et al., 1970), but certain plant parts may be toxic to livestock (Mathews, 1944; Dollahite and Allen, 1975). Kingston et al. (1975) described two sesquiterpenes in tarbush. During short-term, forced use of tarbush by cattle, sheep, and goats, no obvious adverse effects were apparent (Anderson et al., 1991); however, degree of use of indi-

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vidual tarbush plants within the densely stocked paddocks was extremely variable.

Secondary plant metabolites may function as antiherbivory defense mechanisms in many plant species (Freeland, 1991). Various lipophilic compounds, e.g., mono- and sesquiterpenes, are present on the leaf surface of tarbush and are soluble in ethanol (Estell et al., 1994). Schwartz et al. (1980) reported that as volatile oils and terpenoid fractions increased in juniper, preference by deer decreased. Secondary compounds on the leaf surface of shrubs are at the plant–animal interface and, consequently, may dictate whether a plant is consumed or rejected by browsing ruminants. During forced use of tarbush, livestock exhibited a wide range of acceptance of individual plants, and degree of use of individual tarbush plants was related to epicuticular wax content (Estell et al., 1992). In addition, a few individual terpenes were related to individual plant use (R.E. Estell, unpublished data).

We hypothesized that removal of the surface resin and associated secondary chemicals might enhance tarbush acceptance by sheep. We applied organic solvents to tarbush in an attempt to remove surface secondary chemicals and to determine if the rate or extent of tarbush use by sheep could be altered. Five organic solvents were evaluated using two methods of application: spray and immersion.

METHODS AND MATERIALS

Experiment 1. Experiment 1 was designed to examine the effect of spraying with four organic solvents on the acceptability of tarbush to naive lambs. Our hypothesis was that spraying tarbush with organic solvents would rinse off surface chemicals and consequently enhance its acceptability to lambs. Ten tarbush plants were harvested from the Jornada Experimental Range (JER; located in southcentral New Mexico) the day prior to initiation of the study and transported to the laboratory. Five uniform branches were removed from each plant. Branches from one subset of five plants were placed in five bundles containing five branches each, one branch from each plant. This process was repeated for the other set of five plants, resulting in two sets of five "identical" bundles. Construction of bundles in this manner was an attempt to avoid confounding plant genetics and solvent treatment. Bundles were placed in 4-liter tin cans filled with water. Two bundles served as controls (unsprayed), and the other eight were sprayed (0.75 liters) with either 95% ethanol, methanol, ether, or acetone. Each treatment was applied to one bundle from each subset of plants.

Spraying took place the morning the study began. All solvents were reagent grade except acetone (histological grade). The hand sprayer (1 liter) used to apply treatments was thoroughly rinsed between solvents to prevent cross-con-

tamination. Branches were removed from cans, sprayed individually, and then wired into bundles. Branches were misted with the appropriate solvent (except controls) to allow for solubilization of surface compounds and then thoroughly sprayed before they had time to dry. Branches were sprayed from top to bottom (including underside as much as possible) to maximize drainage. After spraying at 0900 hr, all plant bundles in their water-filled tin cans were wired randomly to plywood sheets about 1 m apart on the perimeter of the fence.

Six ram lambs (Polypay × Rambouillet, 8 months of age, mean weight approximately 38.5 kg) with previous grazing experience limited to alfalfa pasture were placed in the pen (84 m^2) at 0900 hr. All plant material was removed from the pen before study initiation. Lambs were fasted for 24 hr prior to initiation of the experiment. Free access to water was available at all times. Percentage of use of each bundle was estimated (ocular estimation) at 1530 hr (6.5 hr after exposure) and at 0730 hr (22.5 hr after exposure) the following morning after removal of lambs. Ocular estimates of use were made to the nearest 5% use class by an experienced observer with no knowledge of the treatments applied. Percentage use data were collected at two times to assess differences in percentage use during the first few hours of exposure and over the entire period.

Experiment 2. Experiment 2 was designed to examine an alternative method (immersion) of applying solvent to tarbush. Our hypothesis was that removal of surface compounds would be more complete and consequently plants would be more acceptable to lambs when immersed in solvent rather than when sprayed with solvent. The lambs, facilities, and protocol used in experiment 1 were also used in experiment 2. Plants (eight plants, four branches of each plant per bundle) were harvested and placed in bundles as in experiment 1.

The major differences between experiments 1 and 2 were the washing technique and the solvents used. A shallow pan (approximately 30 × 60 × 8 cm) containing about 5 cm of solvent was used to wash branches; each branch was immersed individually in solvent, with constant agitation and rotation, for approximately 2 min while using pressure to immerse as much of the branch at once as possible without damaging the branch. The immersion process was conducted immediately prior to initiation of the study. Only three solvents (acetone, ethanol, and denatured ethanol) were compared to the control (no washing). Based on results of experiment 1, ether and methanol were eliminated from the comparison in experiment 2 because of similarities with other treatments. All four branches in each bundle were washed in the container, with replacement of solvent as necessary to maintain a constant fluid level. The pan was then emptied, rinsed with the solvent to be used next, and the process repeated for the four branches in the next bundle. Lambs were placed in the pen at approximately 0800 hr. Lambs were allowed to browse until 0730 hr the following morning. Visual estimates of percentage of use of each bundle were obtained

at 1430 hr (6.5 hr after exposure) and 0730 hr (23.5 hr after exposure) the following morning.

Statistical Analysis. Percentage use data were subjected to arcsine transformation (Steel and Torrie, 1960), and analysis of variance was conducted using GLM procedures of SAS (1989). Use at each time was analyzed using a randomized block design with block and treatment (organic solvent) in the model. Blocks consisted of plant bundles composed of the same plants (i.e., two blocks, each consisting of five bundles constructed from the same five plants in experiment 1 and four bundles from four plants in experiment 2). Means were separated using predicted difference (SAS, 1989) when significant F values ($P < 0.05$) were detected for overall model and treatment. Data presented in Tables 1 and 2 below were analyzed after arcsine transformation, but are presented as actual percentages of use.

RESULTS AND DISCUSSION

Experiment 1. No differences ($P > 0.05$) were noted for degree of use by lambs at either time (Table 1) when the four organic solvents were sprayed on tarbush; however, the variability between bundles within treatment was generally large. For the control, acetone, and ether treatments, one bundle exhibited relatively high use the following morning while the other bundle was used only slightly. The ethanol and methanol rinsed bundles exhibited fairly consistently low use. Very little use had occurred on any plants after 6.5 hr of exposure, suggesting that none of the solvent treatments dramatically altered plant palatability. Ethanol was chosen as a solvent because previous research (Goatcher

TABLE 1. PERCENTAGE USE BY RAM LAMBS OF TARBUSH SPRAYED WITH ORGANIC SOLVENTS

Treatment[a]	Use (%)[b]	
	6.5 hr	22.5 hr
Control[c]	8	43
Acetone[c]	8	45
Ether[c]	5	38
Methanol[c]	6	13
Ethanol[c]	3	13

[a] Treatment refers to the solvent used as spray treatment.
[b] Percent use based on ocular estimation after 6.5 and 22.5 hr of exposure.
[c] Least square means, $N = 2$; pooled standard error = 2.7 and 12.3 for use at 6.5 and 22.5 hr, respectively.

and Church, 1970) indicated sheep have a relatively high rejection threshold for ethanol (8.3 ml/100 ml). The fact that lambs were naive to browsing tarbush might account for the lack of consumption even after 22.5 hr. In general, no clear treatment effects were evident. The within-treatment variability may have been due to our inability to apply the spraying technique in a consistent manner.

Experiment 2. Differences ($P < 0.01$) among treatments were noted for percentage use (Table 2) after 23.5 hr of exposure. Ethanol- and denatured ethanol-immersed tarbush were used more than acetone-rinsed and control tarbush, while acetone-immersed tarbush was used to a greater extent than control bundles. At 6.5 hr after exposure, tarbush use was not different ($P > 0.05$) among treatments and was more variable than at 23.5 hr after exposure. Short-term use was generally low except for one bundle each from the ethanol and denatured ethanol treatments. Bundles composed of the same plants should have eliminated any confounding effects due to differences in plant genetics. Kainulainen et al. (1992) indicated that the terpene composition of conifers is under genetic control. No block effect was observed ($P > 0.05$) at 23.5 hr, suggesting that bundles from the two subsets of plants were not differentially used.

The consistent treatment response across bundles at 23.5 hr is in contrast to observations in experiment 1. Data would suggest that if tarbush use is improved in response to removal of surface secondary compounds, the immersion method may more completely (or uniformly) remove surface compounds. These results are substantiated by previous findings that the epicuticular wax (chloroform extraction of surface compounds) concentration of individual tarbush plants was related to the degree of use by livestock (Estell et al., 1992). Furthermore, these results imply a role for leaf surface secondary chemicals that are soluble in organic solvents as deterrents to use of tarbush by ruminants.

TABLE 2. PERCENTAGE USE BY RAM LAMBS OF TARBUSH IMMERSED IN ORGANIC SOLVENTS

Treatment[a]	Use (%)[b]	
	6.5 hr	23.5 hr
Control[c]	6	8[d] a
Acetone[c]	8	45 b
Ethanol[c]	40	88 c
DnEthanol[c]	40	85 c

[a] Treatment refers to the solvent used as immersion treatment (DnEthanol = denatured ethanol).
[b] Percent use based on ocular estimation after 6.5 and 23.5 hr of exposure.
[c] Least square means, $N = 2$; pooled standard error = 15.6 and 6.6 for use at 6.5 and 23.5 hr, respectively.
[d] Means in a column followed by different letters differ ($P < 0.01$).

Information obtained regarding relationships of secondary chemistry and herbivory could ultimately be exploited to modify consumption of shrubs by browsing herbivores.

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PURIFICATION AND PRELIMINARY CHARACTERIZATION OF A FROG-DERIVED PROTEINACEOUS CHEMOATTRACTANT ELICITING PREY ATTACK BY CHECKERED GARTER SNAKES (*Thamnophis marcianus*)

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Abstract—A potent proteinaceous chemoattractant, eliciting prey attack by checkered garter snakes (*Thamnophis marcianus*) was isolated from aqueous washes of the common frog *Rana temporaria* and purified by preparative continuous-elution electrophoresis. The biological activity of the frog crude extract or of the purified chemoattractive protein, measured by a snake bioassay, was unaffected by freezing, lyophilization, or dialysis but was lost after proteolytic digestion. The purified chemoattractant is glycosylated, has an apparent molecular mass of 24 kDa, estimated by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE), and a pI of 4.8. It gave one spot in two-dimensional electrophoresis. The bioassay showed that this protein is highly attractive to snakes. The lowest concentration yielding positive responses in the snake bioassay was approximately 25 μg/ml. These results suggest that a water-soluble Mr 24 kDa glycoprotein molecule produced by the common frog may be a vomeronasal stimulus used by checkered garter snakes for prey recognition.

Key Words—Chemoreception, chemical cues, frog extract, vomeronasal organ, garter snake, *Thamnophis marcianus*, *Rana temporaria*.

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INTRODUCTION

Among reptiles, the snakes probably exhibit the most specialized feeding habits. For example, the diet of natricine water snakes (genera *Natrix* or *Nerodia*) specifically consists of fish or amphibians. By comparison, other natricine snakes such as garter snakes (genus *Thamnophis*) show a wider variation in their diet (frogs, salamanders, fish, earthworms, slugs, or rodents) even if some species specialize among these prey types (Drummond, 1983). These prey preferences appear to be largely innate since they are not restricted to adult animals and have also been demonstrated in ingestively naive newborn snakes. The feeding responses are heritable but, in particular for neonates, may be altered in various ways by ontogeny or previous feeding experience (Burghardt, 1993).

Although vision is used for the detection of prey (Drummond, 1985; Teather, 1991), the specificity of the predatory behavior mostly depends on chemical stimuli (reviews by Burghardt, 1970, 1980, 1990). It is now well established that the tongue–vomeronasal system plays a major role in the perception of these stimuli. Tongue-flicking is crucial for the transfer of substances from the environment to the vomeronasal organ (VNO) (Halpern and Frumin, 1979; Kubie and Halpern, 1979; Halpern, 1987; Graves, 1993), although a contact of the lips seems to be sufficient to transfer a stimulus into the VNO (Halpern and Kubie, 1980). Tongue-flicking itself is an easily observable and quantifiable behavior and has been frequently used for the study of chemical prey preferences in squamate reptiles (Cooper and Burghardt, 1990). In contrast with the main olfactory system, the natural substances stimulating the vomeronasal organ and inducing behavioral responses seem to be nonvolatile in nature and of relatively high molecular weight (Sheffield et al., 1968; Halpern et al., 1984; Burghardt et al., 1988; Jiang et al., 1990).

To date, however, the sole chemoattractants involved in prey recognition that have been purified and characterized were obtained from extracts of earthworm (*Lumbricus terrestris*), which is one of the major components of the natural diet of some species of garter snakes (*Thamnophis sirtalis*). It has been known for many years that garter snakes readily respond by tongue-flicking and attack to cotton swabs coated with warm-water washes of earthworms (reviewed in Burghardt, 1970). Recently, several snake-attractive proteins have been isolated from warm-water washes of earthworm (EWW) (Wang et al., 1988, 1993) or from electric shock-induced earthworm secretion (ESS) (Jiang et al., 1990; Wang et al., 1993). From this kind of material, three major proteins, eliciting a prey attack behavior by the garter snakes, have been largely characterized: a low-molecular-mass protein of 3 kDa, a sulfhydryl group containing protein with a molecular mass of 20 kDa from EWW, and another 20-kDa molecule from ESS. Moreover there is evidence that binding sites for the 20-kDa protein from ESS are present on the vomeronasal sensory epithelium, suggesting the

existence of specific receptors for the chemoattractant. Finally, electrophysiological studies have recorded increased neuronal firing rates in the accessory olfactory bulb when the purified chemoattractants were applied to the vomeronasal epithelium (Jiang et al., 1990; Wang et al., 1993; Inouchi et al., 1993). Altogether, the data obtained in this model suggest that proteins present on the surface of potential prey may be one of the main stimuli responsible for prey recognition by garter snakes.

Garter snakes as well as other natricine snakes are able to discriminate among different kinds of prey available in their biotope. This could imply the existence of various prey-derived chemical attractants, which could be detected and recognized by snakes and be responsible for their specific feeding habits. The aim of the present study was the isolation and the characterization of a chemoattractant obtained from frog aqueous washes and eliciting prey attack behavior by checkered garter snakes (*Thamnophis marcianus*). This species is known to feed on a wide variety of prey in which frogs most probably represent a significant part of the natural diet (Wright and Wright, 1970; Conant, 1975).

METHODS AND MATERIALS

Animals

Common frogs (*Rana temporaria*), captured in a swamp area (Hollain, Belgium) were at least 2 years old. Their size, estimated by measuring snout–vent length, ranged from 4.1 to 5.5 cm and weight was 15–23 g. All animals were housed in 100 × 30 × 40-cm glass terraria containing a large water dish. The floor of the terrarium was covered with damp moss. The cages were submitted to a natural photoperiod and to temperatures ranging from 20–22°C during daytime to 14–15°C during the night. No source of artificial light was used. The food, consisting of larvae and adult insects—crickets (*Gryllus bimaculatus*), flies or maggots (*Calliphora sp.*) and meal-worms (*Tenebrio molitor*)—was given *ad libitum* every two days.

Checkered garter snakes (*Thamnophis marcianus*) were obtained from a local animal supplier. All individuals, four females and two males, were at least 1 year old with a size ranging from 43 to 61 cm (snout–vent length). They were allowed one month of acclimation before starting the experiments. Animals were submitted to a natural photoperiod and housed in individual 30 × 30 × 20-cm glass terraria maintained at 24–25°C during the day and at 17–18°C during the night. The floor of the terrarium was covered with towel paper and each cage contained a water dish and a hiding place. To restrict visual stimuli from adjacent cages, the lateral sides of the terraria were covered with sheets of black paper. All snakes were fed with raw fish (*Salmo trutta* or *Osmerus eperlanus*) biweekly, but preliminary observations have shown that this species willingly feeds upon

different frog species of the genus *Rana*. From November to March, the snakes were left in hibernation at a constant temperature of 12°C.

Snake Bioassays

In this study, the bioactivity of the fractions obtained during the successive purification steps was checked by a snake bioassay using the checkered garter snakes as test animal. This species was selected on the basis of its reliable behavior during preliminary testing, i.e., positive response (open mouth attack) to fragments of frog skin or to lures covered with concentrated frog extract.

All behavioral tests were conducted in late afternoon. Each animal was tested in one or two trials per day, and the time interval between two successive tests was never less than 20 min. At the time of presentation, each snake had fasted for four days. During each test the behavior of the snakes was monitored by a video camera (T.V. Bosch Low Level) set above the terrarium and recorded on video tapes (JVC HRS 210). The chemoattractivity of the samples was tested by using lures (macaroni, 5 mm diam., 30 mm length) provided on moist filter paper in a glass dish (90 mm diam.) and coated with 150 μl test sample or control solution. In preliminary experiments we compared different kinds of lures such as cotton swabs, sticks of agar-agar or artificial worm bits, but in our experimental conditions the macaroni gave more clear-cut and reproducible behaviors than any other lures.

Just before a trial, the water dish was removed from the terrarium and the dish containing the samples to be tested was placed in front of the snakes' shelter. The recording was started immediately before the withdrawal of the shelter from the cage. The actual beginning of a trial corresponded to the first tongue flick given on a sample and the duration of one test was limited to a maximum of 2 min. In our bioassays we adopted the all-or-none procedure described by Wang et al. (1988) yielding positive/negative results for each animal under test. A response was considered positive when the lure was attacked by the snake during the 120 sec of the trial. In any other case, the test was considered negative. Each sample was submitted twice to the bioassay with an interval of at least one week between the two presentations of the duplicate. The experimental snakes submitted to the test, as well as the order of presentation of the samples—test sample or control(s)—were chosen at random. To confirm negative results, food was presented to each snake at the end of the trial. If food was ignored, the results of the tests for the corresponding animals were not taken into account. This was generally the case when snakes were in preecdysis. Snakes apparently in the process of skin shedding (ocular scale opalescent) were not included into the tests until the day following the actual shedding.

In a last step, successive dilutions (in water containing 0.1% 2-mercapto-

ethanol) of the major protein (see Preparative Electrophoresis) ranging from 100 to 6.25 μg/ml were used to determine the lowest concentration yielding a positive snake bioassay. In these assays, the behavioral responses were also analyzed by the tongue-flick/attack score (TFAS) method (Cooper and Burghardt, 1990). After overall analysis of variance (ANOVA), individual score values were statistically compared by the Mann-Whitney U test.

Isolation and Purification of Chemoattractants

The main steps of purification and analysis of the chemoattractants obtained from aqueous washes of frogs are summarized in the flow diagram of Figure 1. The samples that have been submitted to the snake bioassay are numbered 1–7.

Preparation of Aqueous Washes of Frogs. Frog wash (FW) was obtained by placing living frogs for 1 hr in distilled water at 20°C in a ratio of 1 g frog/ml of water (3–4 cm depth in the container). The same operation was

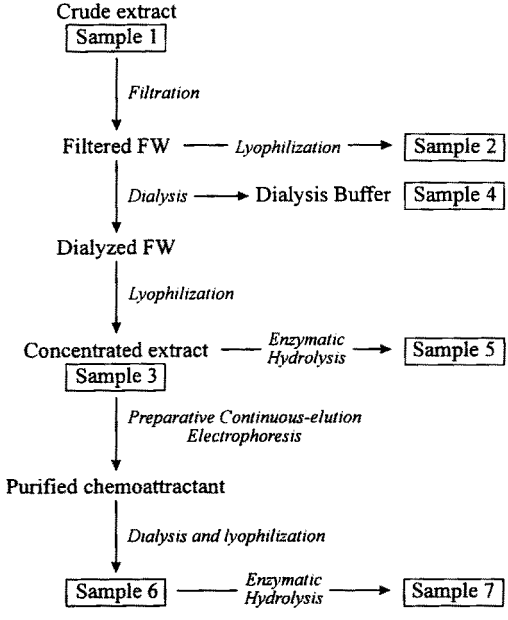

FIG. 1. Flow diagram of the main steps of isolation and purification of the snake chemoattractive material extracted from frog aqueous wash (FW). The numbered samples (see Methods and Materials and Results sections) have all been submitted to the snake bioassay.

repeated twice with the same wash at a 1-hr interval. The resulting solution was called the crude extract (sample 1). After each wash, the extract was temporarily stored at 4°C. Any preparation soiled with feces was discarded. After filtration, the wash solution was concentrated by lyophilization and dissolution in either 1 ml distilled water or 1 ml 0.1% 2-mercaptoethanol in water, and submitted to the bioassay (sample 2). In the next step, the filtered FW was dialyzed (molecular weight cutoff 12,000 Da) twice against 2 liters of 1 mM Tris HCl buffer, pH 7.5, for 3 hr at 4°C. The dialyzed solution was lyophilized, and the resulting material was used either for enzymatic hydrolysis, SDS-PAGE, or preparative continuous-elution electrophoresis. A fraction of this material was dissolved in either 1 ml distilled water or in 1 ml 0.1% 2-mercaptoethanol and submitted to the snake bioassay (sample 3). The material present in the dialysis buffer was also concentrated by lyophilization and tested on the snakes (sample 4).

Preparative Continuous-Elution Electrophoresis. Preparative electrophoresis was performed on the continuous-elution SDS-PAGE apparatus model 491 Prep Cell from Bio-Rad. Six hundred microliters of concentrated dialyzed extract (sample 3; 0.5 mg protein/ml) in sample buffer containing 10% 2-mercaptoethanol (see SDS-PAGE) were loaded onto a cylindrical polyacrylamide gel (28 mm ID; 5 cm length) of 14% T/2.64% C. Electrophoresis was carried out in running buffer (see SDS-PAGE) at 40 mA constant current for 7 hr at 20°C. The protein bands were continuously eluted with elution buffer (25 mM Tris, 192 mM glycine, pH 8.4) at a flow rate of 60 ml/hr. Elution of proteins was monitored by UV absorbance at 280 nm and fractions of 1.5 ml were collected. The proteinaceous content of the fractions was analyzed by SDS-PAGE, and the fractions corresponding to the major protein band (chemoattractant of 24 kDa) were pooled. After dialysis in 10 mM Tris HCl, pH 7.2, lyophilization, and dissolution in water (100 μg/ml, with or without 0.1% 2-mercaptoethanol), this concentrated major protein was submitted to the snake bioassay (sample 6).

Protein Analysis by SDS-PAGE. SDS-PAGE was done on 14% polyacrylamide gels according to Laemmli (1970). Protein samples were dissolved in sample buffer (125 mM Tris HCl, pH 6.8, 4% SDS, 20% glycerol) containing reducing conditions or not (nonreducing conditions) 10% 2-mercaptoethanol and heated at 80°C for 4 min before loading on Minigels (10 × 10 cm, 0.5 cm thick). Electrophoresis was performed in running buffer (25 mM Tris, 192 mM glycine, pH 8.4, 0.1% SDS) at 20 mA/gel (constant current) for 90 min at room temperature. The gels were stained by the silver method of Deutscher (1990) or by the periodic acid–Schiff reaction of Leach et al. (1980) to stain glycoproteins.

Two-Dimensional Polyacrylamide Gel Electrophoresis. The two-dimensional PAGE analysis was performed following the procedure described by

O'Farrell (1975). The pH range of Ampholines in the first dimension separation was 3.5–9.5.

Determination of Isoionic Point (pI). An Ampholine PAG plate (Pharmacia-LKB, pH 3.5–9.5) was used to determine the pI of the chemoattractant. The anolyte was 0.5 M acetic acid, pH 2.6; the catholyte was 1 M NaOH, pH 13. The electrofocusing was performed at 500 V (20 mA) for 90 min at 4°C.

Total Protein Determination. The total protein concentration was determined by the Coomassie blue G-250 colorimetric procedure of Bradford (1976) using bovine serum albumin as a protein standard.

Pronase Digestion. After dialysis and lyophilization, the frog extract was dissolved in 0.5 ml of 0.15 M ammonium bicarbonate pH 7.5, 1 mM $CaCl_2$ (0.5 mg/ml) and incubated with pronase (0.33 mg/ml) for 1 hr at room temperature (Wang et al., 1988). After incubation, the resulting sample 5 was tested in the bioassay. Similarly, the purified 24-kDa chemoattractive protein (see Results) was treated with pronase and tested in the bioassay (sample 7). Pronase in buffer or buffer alone served as control.

Amino Acid Microsequence Analysis. The protein, either dissolved in 10% acetonitrile (v/v) or electroblotted onto a PVDF membrane (Bio-Rad) after SDS-PAGE, was applied to a Beckman LF 3400 protein sequencer equipped with an on-line system Gold 126 Microgradient HPLC and a model 168 diode array detector (Beckman Instruments, Inc.), and sequenced using standard Beckman sequencer procedure 4 on 1–10 pmol of protein.

The phenylthiohydantoin (PTH) derivatives were quantitatively identified by reversed-phase HPLC on ODS ultrosphere 5-μm spherical 80 Å pore micro (2.0 × 250.0 mm; Beckman Instruments, Inc.).

RESULTS

Snakes' Predatory Behavior in Captivity

In preliminary experiments, we have analyzed the predatory behavior displayed by checkered garter snakes using as baits fragments of frog skin (*Rana temporaria*) or lures (macaroni) rubbed on frog dorsal skin. The behavior of the snakes typically consisted of a sequence of four successive steps. A latency period immediately followed the removal of the snake's shelter from the cage. This latency was highly variable among individuals (10–240 sec) and was accompanied by infrequent tongue-flicking (0–5 during the latency). Afterwards the snake began exploratory behavior of the terrarium for a duration ranging from a few seconds to 2–3 min and was characterized by an increased tongue-flicking rate (4–15/min). The first tongue-flick given on the frog skin immediately induced an elevated tongue-flicking rate (10–75/min), which was generally followed by attack and ingestion of the bait. The time elapsed between the first

tongue-flick on the prey and the actual attack was extremely variable among individuals (ranging from 2 to 75 sec) but remained nearly identical for each animal in the successive trials. No striking differences were observed in the reactions of the individual snakes whether fed with raw fish or with bioactive substances from frog extracts during the tests.

During the bioassays we have recorded the different parameters that are generally used in the scoring methods applied to the chemical discrimination of prey by snakes (Cooper and Burghardt, 1990); i.e., the number of tongue flicks directed toward or away from the sample, the latency before the first tongue flick given on the sample and the latency before attack. After several tests we have observed that the behavior of the snakes was remarkably constant and that they always responded to samples containing chemoattractive substances by displaying an easily observable open-mouth attack reaction. Moreover, the TFAS results for every sample (see below) have been submitted to statistical analysis, but failed to provide additional information about a putative response variation among effective fractions by comparison with the bioassay used in this study. Consequently, in the analysis of observations, we have only taken into account an actual attack of the lure (positive result) as opposed to any other behavior (negative result). The results obtained from the different snake bioassays (samples and controls) are summarized in Table 1. A positive or negative response means that, in at least one of the two trials, all snakes ($N = 6$) of the experimental group attacked or ignored a sample, respectively.

Bioactivity of Crude Extract

The crude extract (sample 1), submitted to the snake bioassay, showed no chemoattractivity. In the next step, the crude extract was filtered and concentrated (50-fold) using lyophilization, to a protein concentration (see below) of about 300 μg/ml (sample 2). In contrast to the crude extract, this concentrated extract was highly attractive for garter snakes. These observations suggest that our method of preparation of aqueous frog wash gave a chemoattractant concentration below the threshold needed to obtain a positive response. The concentrated frog extract retained its biological activity after freezing at $-25°C$ for one month.

Previous observations (Wang et al., 1988) have shown that bioactive material extracted from earthworm washes forms aggregates during isolation in the absence of reducing agents, leading to a decrease of its bioactivity. Accordingly, we have tested the biological activity of the successive samples obtained during purification in the presence and in the absence of reducing agents (2-mercaptoethanol). In contrast to what has been previously reported, the absence of 2-mercaptoethanol in the concentrated crude extract did not apparently influence the positive response of the snakes in the bioassay (Table 1). The attractivity

TABLE 1. BIOACTIVITY OF CHEMOATTRACTANT MATERIAL DERIVED FROM FROG AQUEOUS WASH

Samples[a]	*Thamnophis marcianus* ($N = 6$)[b]
Sample 1	−
Control water[c]	−
Sample 2[d]	+
Sample 2 + mercaptoethanol	+
Control water (+mercaptoethanol)[e]	−
Sample 3[f]	+
Sample 3 + mercaptoethanol	+
Sample 4	−
Sample 4 + mercaptoethanol	−
Buffer + (mercaptoethanol)[e]	−
Sample 5	−
Buffer + (pronase)[g]	−
Sample 6[h]	+
Sample 6 + mercaptoethanol	+
Sample 7	−

[a] The sample numbers refer to the different steps of purification of the chemoattractant as indicated in Figure 1. See also Methods and Materials for the preparation of the extracts and for the purification of the successive samples. Aliquots of 150 μl of each sample were used for each trial with the snakes.

[b] A positive (+) or negative (−) response indicates that, for a given sample, all the snakes of the experimental group attacked or ignored the lure respectively.

[c] Control water were included in the tests, in parallel with each sample, and gave negative responses.

[d] The protein concentration was about 300 μg/ml. The same sample was tested after freezing at −25°C for one month and gave also positive results.

[e] The control, tested in the presence or in absence of 0.1% mercatoethanol, gave identical results.

[f] The protein concentration was 210 μg/ml.

[g] The buffer alone or the buffer containing pronase (0.33 mg/ml) gave identical results.

[h] Positive responses were recorded for protein concentrations ≥25 μg/ml.

of the concentrated extract was not altered by dialysis (sample 3) but disappeared after proteolytic digestion (sample 5). After concentration by lyophilization, the dialysis buffer displayed no biological activity (sample 4). After addition of 0.1% 2-mercaptoethanol to these samples, all snakes in the bioassay exhibited the same behavior as in the previous trials without reducing agent. These observations suggested that the chemoattractant is of a proteinaceous nature, with an apparent molecular weight greater than 12 kDa (molecular weight cutoff of the dialysis membrane). At first sight, it seems that reducing conditions are not essential to maintain the chemoattractivity of the extract.

The total protein concentrations, determined by the Bradford's procedure (1976) were, respectively, 300 μg/ml for the concentrated crude extract (sample

2), 210 μg/ml for the concentrated crude extract after dialysis (sample 3), and about 6 μg/ml for the crude extract (sample 1).

Purification and Characterization of Chemoattractants

The proteins present in the concentrated crude extract were analyzed by SDS-PAGE in reducing and nonreducing conditions. In the presence of 2-mercaptoethanol the electrophoresis gel (Figure 2, lane B) showed three major bands corresponding to apparent molecular weights of 66, 31, and 24 kDa, respectively, and a set of minor bands. In nonreducing conditions the migration pattern of the crude extract was different and gave four major bands at 55, 22 (doublet), 18 (doublet) and 16 kDa, respectively (Figure 2, lane D) along with numerous minor protein bands. These results suggest the formation of oligomers by cross-linking of sulfhydryl groups in nonreducing conditions. Protein analysis by SDS-PAGE has been duplicated for different FWs from the same animal and for FWs obtained from different frogs (data not shown). In all cases, the electrophoresis gels showed similar patterns of protein bands with similar relative densities after staining. Finally, electrophoresis gels stained by the periodic acid–Schiff procedure indicated that the proteins bands at 66, 31, and 24 kDa were glycosylated (data not shown).

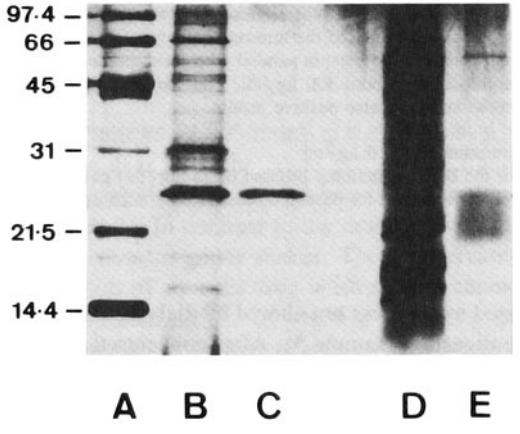

FIG. 2. SDS-PAGE of crude extract and purified chemoattractant. The loaded samples were: crude extract (sample 3, 300 μg protein/ml) with 2-mercaptoethanol (lane B), purified chemoattractant (sample 6, 100 μg/ml) with 2-mercaptoethanol (lane C), crude extract in water (lane D), and purified chemoattractant in water (lane E). Lane A shows, from top to bottom, the mobilities and the molecular weights of marker proteins: phosphorylase *b* (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa).

In the next step, the proteins of the concentrated crude extract reduced with 2-mercaptoethanol were separated by preparative continuous-elution electrophoresis. The material present in each collected fraction was analyzed by SDS-PAGE in reducing conditions. In fractions 66–90, a single protein band corresponding to an apparent molecular weight of 24 kDa was observed (Figure 3). The isolated material (fractions 70–79; sample 6), after dialysis in 10 mM Tris HCl, pH 7.2, and concentration by lyophilization, gave one single sharp band in SDS-PAGE in presence of reducing agents (Figure 2, lane C). In contrast, a single broad band was observed in nonreducing conditions (Figure 2, lane E). The homogeneity of this isolated protein was further analyzed by two-dimensional PAGE where only one single protein spot was observed (Figure 4). The

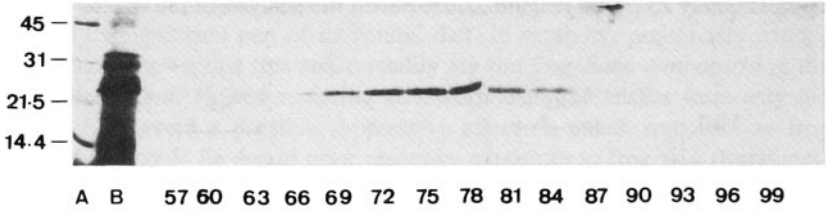

FIG. 3. SDS-PAGE of fractions obtained from preparative continuous-elution electrophoresis. The dialyzed and concentrated crude extract (sample 3) was loaded on a cylindrical polyacrymamide gel and the protein bands continuously electroeluted (see Methods and Materials section). The proteinaceous contents of each fraction (from 57 to 99) were analyzed by SDS-PAGE in reducing conditions. Lane A: molecular markers (see Figure 2). Lane B: crude extract (sample 3) in 0.1% 2-mercaptoethanol

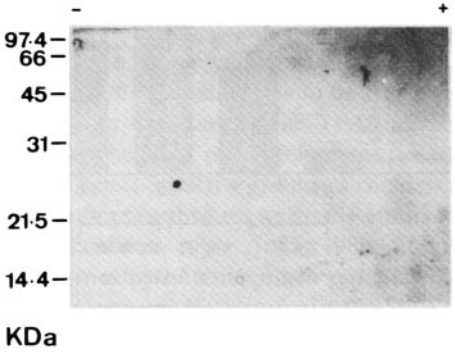

FIG. 4. Analysis of the purified chemoattractant by two-dimensional PAGE. In the first dimension the chemoattractant was separated on isoelectrofocusing gels (1000 V for 4 hr) in an Ampholine pH range of 3.5–9.5. The second dimension was SDS-polyacrylamide (14%) gel electrophoresis (20 mA/gel). The gels were stained by the silver method.

same diagram was obtained whatever the amount of material used (data not shown). This protein has an isoionic point of about 4.8 as determined on a pH 3–10 range PAG plate with known pI protein standards (data not shown). Amino acid microsequence analysis of the chemoattractant either dissolved in 10% acetonitrile (v/v) or electroblotted onto PVDF membrane after SDS-PAGE (100 pmol) failed to give any *N*-terminal sequence; this problem is currently under investigation.

This purified protein, in the presence and absence of reducing agents (0.1% 2-mercaptoethanol), appeared highly active on the snakes and triggered the predatory behavior of all tested animals (sample 6, Table 1) whereas its chemoattractive properties were lost after proteolytic digestion (sample 7). The lowest possible concentration yielding positive responses in the snake bioassay was approximately 25 μg/ml (Figure 5). However, the analysis of the snakes' behav-

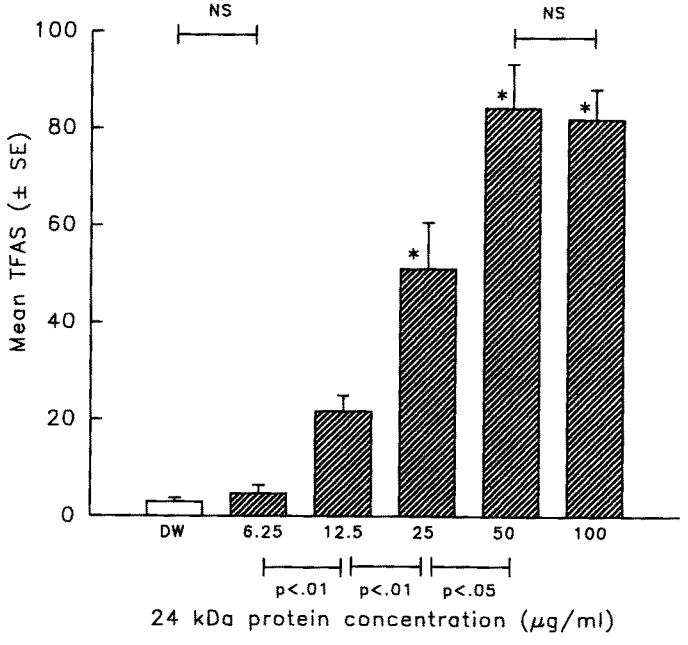

FIG. 5. Effect of concentration on the chemoattractive activity of the purified Mr 24 kDa protein. The bioactivity was estimated by the tongue-flick/attack score (TFAS ± SE). The asterisks indicate which concentrations of the test sample (150 μl) induced an open-mouth attack on the lure (DW: distilled water). After overall analysis of variance (ANOVA) ($P < 0.001$), individual comparisons were performed by the Mann-Whitney U test. NS: no significance.

ior during the tests by the TFAS method (see Methods and Materials section) revealed that bioactivity remained observable at 12.5 μg/ml; at this concentration, the frequency of tongue-flicking was consistently high but was never followed by an attack on the lure. At lower concentrations, the bioactivity of the purified chemoattractant was abolished and not significantly different from water controls.

DISCUSSION

Thamnophis marcianus, the species that we examined in our study, eat a wide variety of prey including amphibians (Wright and Wright, 1970; Conant, 1975). Owing to its geographical distribution, frogs of the genus *Rana* are most probably a significant part of its natural diet. In captivity, preliminary experiments have shown that this snake readily ate the frog *Rana temporaria* at the first presentation. However, during all experiments, the snakes were only fed with fish to avoid a possible suppressive effect on attack responses to frog extracts that could be due to prior repetitive exposures to frog skin (Burghardt, 1992). Our results also show that the feeding behavior observed during the biological assays with frog extracts is quite similar to the predatory behavior already described for other snake species in captivity and for various kinds of prey (Drummond, 1979; Burghardt, 1980; Mushinsky and Lotz, 1980; Teather, 1991). Altogether these observations suggest that in our experimental conditions, chemical stimuli alone may trigger the predatory behavior, thus allowing the characterization of a frog chemoattractant in artificial conditions. This was also suggested by the previous work of Kahmann (1932) showing that the grass snake (*Natrix natrix*) is able to follow tracks laid by rubbing frog skin on the substrate of a test arena.

In squamate reptiles, the tongue vomeronasal system plays a major role in the perception of chemical stimuli and seems to be responsible for the specific feeding habits of snakes. In this context, the TFAS method (see above) has been used to investigate chemical prey preferences in snakes (Cooper and Burghardt, 1990) and to detect attractive chemical components during the successive steps of isolation from earthworm washes (Reformato et al., 1983; Halpern et al., 1984; Kirschenbaum et al., 1985, 1986; Burghardt et al., 1988). However, in this work we adopted a simplified procedure based on the observation of clear-cut attacks triggered by the samples (positive or negative results), as already described by Wang et al. (1988). In the study of such chemoattractants eliciting a prey attack behavior, we also believe that attacks of the lure are better indicators of the "interest" of the snake than any scoring method combining tongue flicks with other variables.

In our experiments, we have not demonstrated that a functional vomero-

nasal organ was needed to detect the chemoattractants extracted from FW (i.e., by suturing the vomeronasal ducts or by peripheral deafferentation). Nevertheless, there is enough evidence (Halpern and Frumin, 1979; Kubie and Halpern, 1979; Burghardt, 1980; Halpern, 1987) that tongue-flicking is indicative of the use of the vomeronasal organ, especially when the substances to be tested are nonvolatile and of high molecular weight. Moreover, in our tests we have consistently observed that the behavioral responses were preceded by several tongue-flicks along with a physical contact between the tongue tips and the samples.

Among vertebrates, the vomeronasal organ is involved in many behaviors such as prey recognition or courtship (see Introduction). However, the chemical nature of the vomeronasal stimuli eliciting these various behavioral or physiological responses remains to be uncovered. To date only a few compounds displaying a biological activity mediated by the vomeronasal organ have been isolated and identified. The first is the protein aphrodisin, isolated from female hamster vaginal secretions (Singer et al., 1986; Henzel et al., 1988; Macrides and Singer, 1991). Three other chemoattractive proteins (Mr 3 kDa and Mr 20 kDa or Mr 20 kDa) have been isolated from aqueous earthworm wash (Wang et al., 1988, 1993) or from electric shock-induced earthworm secretions, respectively (Jiang et al., 1990; Wang et al., 1993). Moreover, Burghardt et al. (1988) have isolated from the fish (*Pimephales promelas*) vomeronasal stimulants containing both high- (159 kDa and 27 kDa) and low-molecular-weight (1.35 to 1.80 kDa) components. In the present work we have purified from frog aqueous wash another chemoattractive compound eliciting prey attack behavior by a natricine snake.

The chemoattractants of FW were extracted from frogs by water washes at normal environmental temperature (1 hr in distilled water at 20°C). This is in contrast with previous works where snake-attractive proteins were extracted from earthworm by warm-water washes (60°C) (Burghardt, 1966; Halpern et al., 1984) or from earthworm secretions obtained after electric shocks (Jiang et al., 1990). The data obtained from the first bioassays showed that, with our method of FW preparation, the concentration of the chemoattractant in the crude extract was too low to obtain a positive response. This was not a major drawback since the attractiveness of the extract was recovered after a simple concentration by lyophilization. These observations further suggest that the bioactive components, regardless of their histological origin, are present on the surface of the frog skin and can be released in the environment. This is also confirmed by the fact that lures rubbed on frog skin produced a positive response. Therefore, it is reasonable to assume that, in natural conditions, the concentration of the chemoattractant on the skin surface is sufficient to trigger the predatory behavior of the snakes. Another advantage of the mild extraction method that we applied was that the same frogs could be used for successive extract preparations. This

implies that the chemoattractive substances are regularly renewed on the skin surface.

The data obtained from the experiments described here suggest that the major active component in FW is a protein with an apparent molecular weight of 24 kDa as estimated by SDS-PAGE. The homogeneity of the purified chemoattractive component was demonstrated by the presence of a single protein band after SDS-PAGE in reducing conditions and of a single spot after two-dimensional electrophoresis in identical conditions. At the present time, amino acid microsequence analysis failed to provide an *N*-terminal amino group. The pI of the component was 4.8. The effectiveness of the FW chemoattractant was not altered by lyophilization or freezing but disappeared after proteolytic digestion. This confirms that the chemoattractant is protein in nature and suggests that the bioactivity of the attractant is associated with an intact protein and is not due to a nonproteinaceous ligand or a contaminant linked to the purified protein. The chemoattractant seems to be glycosylated, as indicated by the Schiff's reaction, but further analysis is needed to determine the nature of the carbohydrate moiety. Finally, the comparison of the SDS-PAGE data in reducing and nonreducing conditions shows that the latter induce the formation of oligomers and a broadening of the Mr 24-kDa band, probably by oxidation of protein sulfhydryl residues. In both types of conditions, the extracts exhibited chemoattractivity. However, the biological activity as well as the analytical behavior of the fully oxidized chemoattractant remain to be investigated according to Wang et al. (1988). It is worth noting that the reducing agent alone exhibited no chemoattractivity and did not modify the behavior of the snakes in the bioassays. As for warm-water washes of earthworms (Kirschenbaum et al., 1985; Wang et al., 1988) some other components of the frog washes might also be snake chemoattractive proteins. These proteins have been partially characterized but, due to the small amounts of material recovered after preparative electrophoresis, it has not been possible to test their putative chemoattractivity in the bioassay. Additional data, and particularly the primary structure, are needed to compare the chemoattractive protein isolated from the frog wash to the snake attractive proteins isolated from earthworm and to aphrodisin, and to reveal their possible similarities. Like aphrodisin, the chemoattractive proteins extracted from frog, earthworm, or fish have molecular weights in the range of 20 kDa and could be related to the α_{2u}-globulin superfamily of extracellular proteins (Macrides and Singer, 1991).

Successive dilutions of the purified chemoattractant have shown that the lowest concentration giving positive responses in the snake bioassay (attack of the lure) was approximately 25 μg/ml. The minimal concentration needed to trigger the feeding behavior is probably lower, since bioactivity (high tongue-flicks rates) remained observable at 12.5 μg/ml; at this concentration tongue-flicking was never followed by an attack of the lure. This further suggests that

the same chemoattractant not only plays a key role for prey recognition but could also, at lower concentrations, be involved in prey trailing as already demonstrated for *Thamnophis sirtalis* and *T. radix* (Kubie and Halpern, 1978). For earthworm chemoattractant (20-kDa protein from ESS), Jiang et al. (1990) have determined that a concentration of 3 μg/ml of the purified protein was effective to give a positive bioassay. The protein extracted from FW, at this concentration, displayed no attractivity in the bioassay. However, our observations seem to correlate well with electrophysiological studies (Inouchi et al., 1993) showing that the 20-kDa protein from earthworm is an effective stimulus for the accessory olfactory bulb neurons at concentrations ranging from 53 to 533 μg/ml (25 μg/ml and over for the 24-kDa protein from FW). Any additional comparison with our data seems difficult since the experimental models are too different.

Finally, preliminary observations (data not shown) indicate that other frog-eating water snakes (*Natrix natrix* or grass snakes; *Natrix maura* or viperine snakes) (Steward, 1971) respond positively to the concentrated crude extract as well as to the purified chemoattractant (24-kDa protein; 200 μg/ml). However, these results were not included in this paper since the behavior displayed by these two species during testing was more erratic as compared to checkered garter snakes, at least in our experimental conditions. Additional data are thus needed before a valid comparison can be made with the observations reported here for *Thamnophis marcianus*. In particular, it remains to be demonstrated for these two species of *Natrix* that a functional vomeronasal system is required to detect the purified chemoattractant. Nevertheless it seemed to us important to point out these preliminary observations because they suggest that a protein isolated from one frog species possesses chemoattractive properties not only for *Thamnophis marcianus* but also for two other snake species of different geographic distribution. We believe that this kind of comparative approach, using purified chemoattractants, will become a valuable method both for a detailed study of snakes' prey preferences and for the characterization and comparison of chemoattractive substances isolated from other frog species.

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A NEW BIOASSAY FOR TESTING PLANT EXTRACTS AND PURE COMPOUNDS USING RED FLOUR BEETLE *Tribolium castaneum* HERBST

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Abstract—We designed a new bioassay to test plant extract activity against stored product pests. Plant compounds were added to feed disks composed of wheat flour and yeast and fed to the red flour beetle (*Tribolium castaneum*). By measuring insect mass, disk mass, and insect mortality over time it was possible to calculate a phagodepression index, an antifeedant index, the amount of treatment chemical ingested by the beetles, the mortality rate, and the efficiency of conversion of ingested food. The assay was performed for 60 hr to allow for possible habituation effects and to discriminate between phagodeterrency and physiological stress caused by treatments. α- and β-Pinene, eugenol, kaurenic acid, sparteine, essential oils of *Minthostachis mollis* and *Melaleuca quinquenervia*, and extracts of *Sapindus saponaria* were tested. Using this assay we detected the presence of both phagodepressant and phagostimulant compounds in *S. saponaria* extracts, and we quantified the pronounced effects of sparteine on *T. castaneum*.

Key Words—Bioassay, red flour beetle, *Tribolium castaneum*, Coleoptera, Tenebrionidae, ECI, *Minthostachis mollis*, *Melaleuca quinquenervia*, *Sapindus saponaria*, α-pinene, β-pinene, eugenol, kaurenic acid, sparteine, phagodepression, phagostimulation.

INTRODUCTION

Plants produce a staggering diversity of toxic or deterrent substances (Bernays and Chapman 1987; Klocke, 1989) thought to inhibit herbivory and attack by

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microorganisms (Feeny, 1976; Rhoades and Cates, 1976; Coley et al., 1985; Green and Hedin, 1986; Rosenthal and Berenbaum, 1991). Various bioassays have been devised to test the activities of these plant secondary compounds (Alkofahi et al., 1989). The use of insects in various stages of development in these assays (Lewis and Van Emden, 1986) has allowed the identification of over 2000 plant species with antiinsect properties (Ahmed et al., 1984). However, few natural products have reached the status of commercial pesticides (Jermy, 1984; 1990) in spite of the need to reduce the use of synthetic insecticides. This situation has given rise to new advances in insect bioassay techniques (Lewis and Van Emden, 1986; Pickett et al., 1989; Kubo, 1991; Escoubas et al., 1992; Escoubas et al., 1993). Following this current of thought and to exploit the hitherto unexplored antiinsect potential of tropical plants in Venezuela, we initiated a screening program using stored products insects. Among these, *Tribolium castaneum* (Herbst) stands out as an agricultural pest of primary importance in the tropics (Kahn and Mannan, 1991) and elsewhere (Zettler, 1991) with a growing resistance to synthetic insecticides (Subramanyan et al., 1989; Collins, 1990; Zettler and Cuperus, 1990; Zettler, 1991). This beetle has been found susceptible to a number of plant-derived chemicals (Sighamony et al., 1984; Jilani et al., 1988; Mostafa, 1988; Qureshi et al., 1988; Liu et al., 1991; Sharma et al., 1992; Shukla et al., 1992). Therefore, it appeared feasible to use *T. castaneum* for testing compounds of ecochemical and economical importance.

Grain pests are often bioassayed by treating kernels or flour with organic solvent solutions of the test compound. Such assays provide information regarding repellency, contact toxicity, oviposition perturbation, and long-range effects. However, these tests rarely quantify phagostimulation of depression, the amount of material consumed, or physiological stress other than death itself.

We thought that the concept of the classical leaf disk bioassay (Lewis and Van Emden, 1986), normally applied to larval Lepidoptera, could be adapted to *T. castaneum* in order to quantify feeding, as well as parameters indicative of physiological perturbation such as the efficiency by which insects transform ingested food into body mass and metabolic energy, usually expressed by ECI (efficiency of conversion of ingested food) (Waldbaum, 1968). This would allow a greater sensitivity and a higher quantitative resolution in antifeedant bioassays, facilitating chemical screening as well as our understanding of the ecological function of plant semiochemicals. In this paper we report the successful modification of the leaf-disk bioassay concept. We selected a variety of plant compounds for testing according to their physical characteristics and chemical groups in order to cover most situations usually encountered in screening bioassays. Compounds were chosen for their reported or potential antiinsect activity, and plants were selected based on the ecological and ethnobotanical status that we could derive from field observations.

METHODS AND MATERIALS

Insect Rearing. A colony of *T. castaneum* was started in September 1989 with 150 insects collected locally from infested corn and wheat. The colony was maintained on whole wheat flour according to Smith (1966).

Disks of Artificial Diet (DAD). White wheat flour (100 g), water (60 ml), commercial margarine (7 g), and powdered baker's yeast (10 g) were thoroughly mixed and passed several times through a mechanical pasta roller until a thin dough leaf 1 mm thick was obtained. This round leaf of approx. 20 cm diam. was placed on a flat plastic surface on which nylon strings of calibrated diameter (0.3 mm) had been laid along parallel lines approx. 5 cm apart. The dough leaf was flattened further with the aid of a glass roller until the nylon strings appeared across the dough. Disks 0.8 cm diameter (0.5 cm^2) were cut with a metal borer, placed on a 20 × 20-cm glass plate, warmed to 40°C in a ventilated oven for 2 hr, and allowed to equilibrate with ambient relative humidity (70 ± 5%). The disks averaged 8 ± 1% moisture, 0.3 mm thickness, and 15–20 mg. Their weight remained constant (±0.1 mg) if stored under 70 ± 5% relative humidity.

Insect Preconditioning. Newly emerged adults of *T. castaneum* reared on whole wheat flour were exposed to disks of artificial diet (DAD) for 48 hr prior to the assay. (Adults thrived for several weeks on disks without detectable negative effects.) Insects were then starved for 48 hr to drive the beetles to feed actively on flour disks, then used for bioassay and discarded.

Assessment of Insect Aggregation on DADs. Eight 2-day-old, starved beetles and four untreated DADs were placed in a 10-cm-diam. Petri dish under the same ambient conditions of the bioassay (see below). The number of beetles either feeding or standing on the disks was counted every hour for 8 hr without disturbing the insects. Five replicates were made of this experiment.

One-Way Disk Bioassay (DAD-TC Assay). DADs placed on a glass surface were treated with 20 μl (10 μl on each side) of organic solvent solution (methanol, acetone, chloroform, or hexane depending on compound solubility) containing the test compound/extract so as to obtain 10, 100, 250, and 500 μg of treatment compound per square centimeter of DAD. This became 0.31 ± 0.016 to 15.17 ± 0.08 μg of compound/mg of DAD, that is 310 ppm to 1.5%. In turn, if insects accepted treated DADs as much as the controls, the ingested treatment chemical during the 60 hr of exposure was in the range 0.14–5.8 μg/insect, roughly a 1:40 range. The uncertainty stems from the weight variance of the DADs. Occasionally, some compounds such as α- or β-pinene or eugenol were added to DADs in larger quantity to force the appearance of otherwise weak responses by *T. castaneum*. However, this procedure sometimes introduced modifications in the mechanical properties of DADs that could possibly influence the acceptability of the diet by the insects. Nevertheless, this was not the case with the tested compounds, as Table 1 indicates. The control DADs

TABLE 1. RESULTS OF DAD-TC ASSAY APPLIED TO *T. castaneum*[a]

Compound or plant	Dose (g/cm)	Actual dose (g/cm)	Dry DAD mass consumed per insect (mg)	*P(ds)i*	Mortality (%)	Ich (g/insect)	ECI	ECI relative to control	AI (choice ass.)
Control			0.47 (0.06)a		0		51.5 (3.1)a	1.00a	
α-Pinene	10	0.30 (0.02)	0.43 (0.4)a,b	0.96 (0.08)a,b	0	0.15 (0.03)	59.2 (2.8)b	1.17 (0.06)b	53 (9.8)a
	100	2.7 (0.2)	0.51 (0.7)a	1.1 (0.2)b,c	0	1.5 (0.3)	47.4 (3.4)a	0.93 (0.06)a	54 (9.3)a
	500	15.6 (3.3)	0.52 (0.4)a	1.57 (0.08)c	0	8 (2.3)	45 (4.5)a	0.92 (0.08)a	45 (13)a
	1000	33.4 (4.5)	0.35 (0.4)b	0.78 (0.08)a	0	12.3 (0.7)	63.2 (4.4)b	1.21 (0.06)b	52 (7)a
β-Pinene	10	0.31 (0.03)	0.44 (0.05)a	0.75 (0.08)a	0	0.15 (0.02)	66.2 (2.4)b	1.31 (0.05)b	61 (9.4)a
	100	2.86 (0.15)	0.57 (0.08)b	0.91 (0.05)a,b	2	1.8 (0.3)	53.6 (6.2)a	1.05 (0.13)a	34 (8.1)b
	500	14.9 (0.5)	0.41 (0.06)a	0.71 (0.1)b	0	6.6 (1.1)	73.3 (6.9)b	1.45 (0.13)b	54 (6.3)a
	2500	73.9 (6.2)	0.35 (0.04)a	0.61 (0.07)b	0	28.3 (3.0)	69.1 (5.9)b	1.36 (0.12)b	62 (5.8)a
Eugenol	10	0.30 (0.03)	0.61 (0.06)b	1.23 (0.11)a	0	0.20 (0.04)	40.8 (3.6)b	0.81 (0.07)c	33 (7.9)a
	100	3.13 (0.15)	0.54 (0.08)a,b	1.10 (0.16)a,b	0	1.9 (0.3)	43.3 (3.9)b	0.85 (0.08)c	44 (8.2)a
	500	14.6 (1.4)	0.37 (0.04)c	0.75 (0.09)c	0	4.9 (1.7)	61.2 (4.2)c	1.20 (0.08)b	18 (2.6)b
	2500	77.2 (4.1)	0.44 (0.09)a	0.89 (0.17)b,c	0	33.7 (1.2)	53.7 (3.9)a,c	1.06 (0.08)ab	4 (2.3)c
Kaurenic acid	10	0.32 (0.04)	0.47 (0.07)a	0.86 (0.13)a	0	0.17 (0.04)	55.4 (7.3)a	1.09 (0.14)ab	54 (9.7)a
	100	3.16 (0.4)	0.45 (0.04)a	0.84 (0.07)a	0	1.56 (0.35)	58.9 (2.9)a	1.16 (0.06)b	37 (16)a
	250	7.1 (0.5)	0.45 (0.05)a	0.83 (0.08)a	0	3.47 (0.54)	58.8 (3.4)a	1.15 (0.08)b	10 (3.8)b
	500	14.7 (0.4)	0.49 (0.03)a	0.91 (0.05)a	0	8.04 (0.73)	52.4 (2.2)a	1.03 (0.04)ab	4 (2.9)b

Sparteine	10	0.32 (0.04)	0.06 (0.04)d	0.29 (0.16)a	2	0.02 (0.01)	1.1 (1.4)c	0.002 (0.0)d	49 (14)a
	100	3.1 (0.3)	0.08 (0.04)d	0.37 (0.2)a	2	0.26 (0.15)	0 (0)c	0 (0)d	13 (14)b
	500	15.3 (1.4)	0.02 (0.01)d	0.11 (0.06)b	2	0.41 (0.22)	0 (0)	0 (0)	3 (4)b
	1000	29.3 (1.4)	0.018 (0.008)d	0.09 (0.04)b	2	0.59 (0.28)	0 (0)	0 (0)	3 (4)b
Minthostachis mollis	10	0.31 (0.02)	0.61 (0.06)b	0.89 (0.09)a	0	0.21 (0.03)	48.7 (3.5)a,b	0.96 (0.07)a	40 (11)a
essential oil	100	3.22 (0.2)	0.60 (0.08)b	0.88 (0.1)a	0	2.1 (0.4)	44.9 (4.3)a	0.88 (0.08)a	34 (5)a
	250	7.9 (0.9)	0.58 (0.07)b	0.85 (0.1)a	0	4.9 (0.6)	48.9 (3.0)a,b	0.96 (0.06)a	30 (7)a
	500	14.8 (1.8)	0.55 (0.06)b	0.80 (0.09)a	0	8.7 (0.5)	52.9 (4.2)a,b	1.04 (0.04)a	35 (16)a
Melaleuca quinquenervia	10	0.31 (0.03)	0.55 (0.04)b	0.88 (0.06)a	0	0.17 (0.02)	52.5 (3.2)a	1.03 (0.07)a	30 (18)a
essential oil	100	3.15 (0.04)	0.52 (0.06)a,b	0.84 (0.08)a,b	2	1.6 (0.15)	53.1 (5.0)a	1.05 (0.11)a	9 (6)b
	250	9.11 (0.88)	0.64 (0.05)b	1.02 (0.08)a	0	5.5 (0.2)	45.9 (2.7)a	0.90 (0.06)a	7 (3)b
	500	16.3 (1.2)	0.42 (0.09)b	0.68 (0.14)b	0	6.8 (1.1)	50.0 (3.8)a	0.98 (0.08)a	22 (7)a
Sapindus saponaria									
Hexane extract (H1)	1000	28.6 (1.2)	0.42 (0.06)ab	0.9 (0.12)a	0	13.0 (1.9)	45.6 (3.1)a	0.90 (0.06)a	1.2 (0.4)a
Chloroform extract (C1)	1000	28.3 (2.5)	0.34 (0.05)b	0.56 (0.09)b	0	9.7 (1.7)	61.6 (7.0)b	1.21 (0.15)b	11 (4.5)b
Chloroform extract (C2)	1000	27.9 (2.2)	0.007 (0.004)c	0.016 (0.008)o	0	0.22 (0.11)	0 (0)c	0 (0)c	0 (0)a

[a] *P(ds)i* = phagodepression/stimulation index. Ich = amount of treatment chemical ingested by the insect. ECI = efficiency of conversion of ingested food. AI = antifeedant index. For the calculation of these and other parameters see text. Parameters relative to controls are in reference to the particular controls of each set of assays per compound. Standard deviations appear in parentheses. Numbers followed by different letters are statistically differentiated. Comparison of means was performed using Tuckey's test at the $\alpha = 0.05$ level of significance.

received 20 μl of clean solvent. DADs were then placed in a ventilated oven at 40°C for 20 min to evaporate the solvent and then were immediately used for the assays. One treated DAD of known weight was placed on the center of a paper-towel-lined Petri dish 10 cm in diam., containing 10 preconditioned and starved 4- to 5-day-old unsexed *T. castaneum* adults of known weight. Only one disk was exposed to the beetles in each replicate because only about 20–25% of the DAD was eaten in any test period. This was enough to prevent competition or feeding inhibition by excessive population density/available food ratio. A larger number of disks also may have resulted in an unmeasurably small variation in weight of each DAD.

The Petri dish was closed and placed in the dark at 28 ± 1°C and 70 ± 5% relative humidity. Readings were conducted after 60 hr of exposure to DADs to give ample opportunity for habituation or desensitization of the insects to the test material (Schoonhoven, 1969; Munakata, 1977; Jermy et al., 1982). After the feeding period, insects and the remainder of the DAD were weighed separately and discarded. Five replicates and a control were performed for each dose of chemical. All the material separated from the DAD became insect feed, as no crumbs remained on the Petri dish. For this one-way assay, the following parameters were obtained:

1. Amount of ingested food: ΔF. Correction for the 8% moisture content was introduced. Comparison with the separate control experiment gave the phagodepression/stimulation index $P(ds)i$ (Alkofahi et al., 1989):

$$P(ds)i = \frac{\Delta F(T)}{\Delta F(C)}$$

where T = treatment; C = control.

2. Death rate (if any).

3. Variation in insect weight, W_g. The amount of ingested treatment chemical I_{ch} was estimated by calculating the amount of compound added to the initial DAD as follows:

$$\text{Dose} = \frac{\text{added compound } (\mu\text{g/cm}^2) \times 0.5 \text{ (cm}^2\text{/DAD)}}{\text{weight of DAD (mg/DAD)}}$$

$$= \mu\text{g of compound/mg DAD}$$

and

$$I_{ch} = \text{Dose} \times \Delta F$$

If dead insects were observed, then I_{ch} could be used to determine LD_{50} in assays employing the various concentrations of the test chemical indicated above.

4. Efficiency of conversion of ingested food (ECI) with the following considerations. Adults of *T. castaneum* lose weight steadily when starved (10% in

60 hr > 70% rh). The weight gain parameter required for the calculation of ECI according to the formula below (Waldbauer, 1968) was estimated by adding the weight loss (W_s) of the starved insect (metabolic cost) and the weight gain (W_g) during the 60-hr period of exposure to the artificial diet.

Consequently:

$$ECI(T) = \frac{W_g + W_s}{\Delta F(T)} \times 100$$

A similar formula was used for the control DAD. When weight loss of experimental beetles was superior to that of the starved insects, then $ECI(T) = 0$ for that test.

5. Antifeedant index (AI). A two-way bioassay was also conducted in parallel using one treated and one control DAD per cage. This assay provided the concentration-dependent antifeeding index (Alkofahi et al., 1989):

$$AI = \frac{\%T}{\%T + \%C} \times 100$$

where T and C represent the consumption of treated and control disks, respectively.

Insects and DADs were weighed at the start and end of each experiment as in the one-way assay (above). In all experiments the means of each value were compared for statistical significance using Tukey's test at $\alpha = 0.05$ (Zar, 1984).

Chemicals. The following criteria was applied in selecting the test chemicals: α- and β-pinene and eugenol represent volatile liquids of the monoterpene and phenolic series, while sparteine is an oily alkaloid. All these are only sparingly soluble in water. Kaurenic acid is a diterpenic crystalline solid and partially soluble in water. Essential oils of *M. mollis* and *M. quinquenervia* are multicomponent mixtures of relatively volatile mono- and sesquiterpenes, while extracts of *S. saponaria* are complex mixtures of compounds belonging to various unknown chemical groups. Eugenol was obtained from Firmenich Chemical Company; α- and β-pinene were purchased from Aldrich Chemical Co. Sparteine and kaurenic acid were isolated from *Lupinus* sp. and *Baccharis trinervis*, respectively. Essential oils of *Minthostachis mollis* and *Melaleuca quinquenervia* were obtained by steam distillation of the aerial parts.

Plant Extraction. Sapindus saponaria seeds were collected in the vicinity of Maracay, north-central Venezuela at approx. 450 m altitude and were extracted as follows: 100 g of dried, mashed whole seeds was boiled in two 1500-ml portions of petroleum ether (40–60°C) for 2 hr. Evaporation of solvents gave 25% w/w of a yellowish oil (H1 extract). The seed mull was divided in two equal sized portions. The first was suspended in chloroform (1500 ml) and

refluxed for 2 hr. The hot suspension was then filtered through glasswool and solvents were evaporated to yield ca. 900 mg of brown material identified as C1 extract. The second seed mull portion was added to a warm water–methanol 3:1 mixture (2.5 liters) and refluxed for 2 hr. Filtration of the hot suspension through cheesecloth, evaporation to approx. one third of the initial volume by rotary evaporation under vacuum, followed by extraction of the resulting solution with chloroform (3 × 250 ml), drying over anhydrous sodium sulfate, and evaporation of solvents gave a crude light brown oil material (850 mg) that was identified as C2 extract.

The crude extracts of *S. saponaria* were incorporated into DAD at a relatively high concentration: 1000 $\mu g/cm^2$ equivalent to 28 ± 0.2 $\mu g/mg$ of DAD. Assuming that the active compound(s) was 10% of this crude extract, it would be equivalent to 100 $\mu g/cm^2$ of DAD, a concentration at which strongly active compounds, e.g., sparteine might show a measurable effect on *T. castaneum* behavior and fitness. Indeed *S. saponaria* extracts showed a strong effect at this concentration, as Table 1 shows. Although higher concentrations would be desirable to test, the upper limit was set by the amount of resinous material that was present in the extracts. These introduced additional, misleading feeding deterrency due to stickiness and were thus avoided.

RESULTS AND DISCUSSION

Weight Changes of T. castaneum. In order to derive ECI and other parameters, it was necessary to assess the age-dependent weight progress of this beetle. Figure 1 plots weights of newly emerged unsexed adults, either starved or

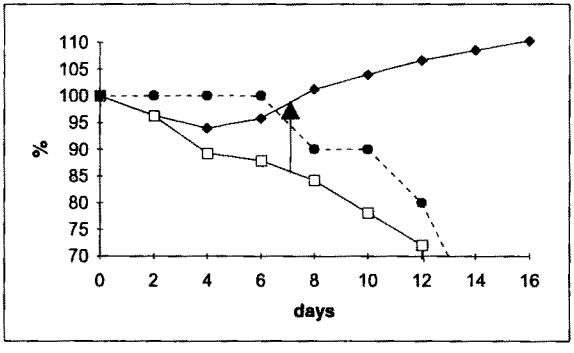

FIG. 1. Time-dependent weight changes as a percent of initial weight recorded for newly emerged *T. castaneum* adults: □, starved; ◆, exposed to DADs at day 0; ●, mortality percentage of starved beetles. For the meaning of arrow see text.

exposed to DAD. The moisture content of the beetles was assumed to remain constant under the conditions of the experiment, and feeding by cannibalism was not observed (Park et al., 1965; Sokoloff, 1974; Hastings and Constantino, 1987; Stevens, 1989). While the starved insects sustained a 28% weight loss in 12 days, DAD-fed beetles gained 17% from day 4 to day 10. The time- and diet-dependent progress of body weight of *T. castaneum* shown in Figure 1 was found large enough to provide a reasonable basis for the application of ECI in this test. The weight difference between starved and DAD-fed beetles after 60 hr starting on day 4 amounts to 8.5% of the insect body weight. This is far larger than the estimated weight of the gut contents at any given time of the feeding period (<1%). In the calculation of ECI, the weight loss due to metabolic and locomotion consumption was taken as the baseline to estimate the actual conversion of ingested material (arrow in Figure 1) and not the weight of the insect at time zero because the insect will convert the feed mass into metabolic energy plus body mass. According to the results of Figure 1, in principle the longer the starvation the greater the weight difference (length of arrow) and the more accurate the ECI. However, interference by the death of the insects started by day 8 (see dotted line). The cross point of the death line and the zero weight change line at day 4 was taken as a balance point. Therefore, the condition of the beetles for the DAD-TC assay was optimal between days 4 and 7.

Aggregation of Beetles during Feeding. Stored product beetle pests frequently aggregate during feeding. The results of the two-way DAD assay could be distorted if the aggregation behavior was significant in the *T. castaneum*-DAD binomium, because disk preferences may result not only from the treatment itself but also from a random first choice followed by accretion of other individuals. This was tested by exposing eight adult beetles to four untreated DADs and counting insect distribution after some time. The results of Figure 2 show first that aggregation is a reciprocal function of time. After the first hour there was no discrimination as to the rate of occupancy, and as much as 33% of the disks hosted between seven and eight insects. However, the beetles showed a tendency to disperse after a few hours and progressively settled to feed individually or with little gathering since after 8 hr 67% of the disks were occupied by one or two insects only. Therefore, insect agglomeration was likely not to interfere with two way DAD assays lasting over 60 hr.

Results of DAD-TC Assay. For each chemical tested, the parameters that could be extracted with confidence were those indicated in Table 1. The combined *AI* and $P(ds)i$ values were deemed more informative than either one taken individually. *AI* indicated the deterrency to feeding where an alternative food source was accessible to the beetles, as is found, for example, by herbivorous insects in species-diverse plant communities, whereas $P(ds)i$ showed the acceptability of DAD in the absence of other sources, as is the case with storage

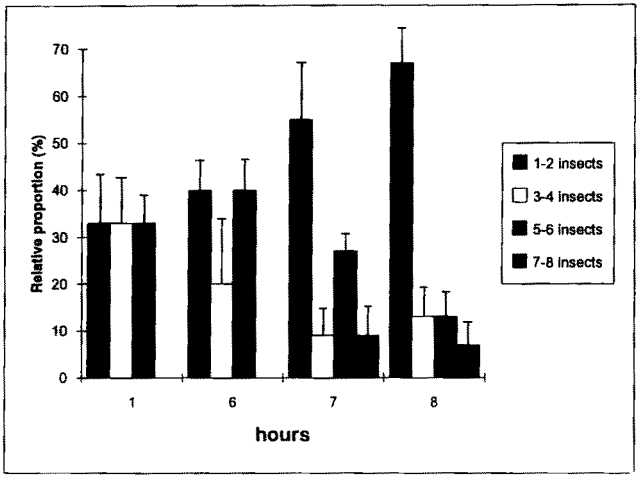

FIG. 2. Time dependent distribution of *T. castaneum* adults on untreated DADs represented as percent of occupied disks by groups of 1–2, 3–4, 5–6, and 7–8 insects.

insects in a large grain bin. Divergence in *AI* and *P(ds)i* indexes, e.g., low *AI* but nearly normal *P(ds)i*, as occurs in eugenol and kaurenic acid (Table 1), was interpreted as the effect of moderately feeding deterrent compounds that are nonetheless tolerated if food became scarce. The combined *P(ds)i* and *ECI* values also helped in discriminating compound induced feeding deterrency and lack of insect activity caused by physiological stress. That is the case of the C1 extract of *Sapindus saponaria* where the reduced *P(ds)i* value is not likely to be the result of insect stress because the *ECI* is still high. However, the low *P(ds)i* values brought about by sparteine on *T. castaneum* may have resulted from insect discomfort and stress stemming from the toxicity of this alkaloid.

The specific results of each compound tested are shown in Table I with the following comments:

For α- and β-pinene, extensive studies exist on their attractant (Byers et al., 1985; Chenier et al., 1989; Schroeder, 1987; Schroeder and Lindelow, 1989; Shore et al., 1990; Fuhrer et al., 1991; Nordenhem and Eidmann, 1991), repellent (Kostal, 1992), and toxic effects (Gijzen et al., 1993) or those of their autooxidation products (Sharaby, 1987; Hunt et al., 1989) on various insect species. This is probably the result of, or the reason for, their ubiquitous presence in many tree resins and exudates. These compounds also appear associated with multiple defensive roles in some arthropods (Hamilton et al., 1985; Everaerts et al., 1990; Roisin et al., 1990; Lindstrom, 1990). However, we detected no statistically significant behavioral or physiological effect of α- and β-pinene

on *T. castaneum*. Other insects such as *Choristoneura occidentalis* (Clancy et al., 1992) are also not affected by pinenes. Notably, *T. castaneum* adults tolerated as much as 28.3 μg of ingested β-pinene without any reduction in ECI.

Eugenol and methyl eugenol are potent attractants to various Coleoptera (Ladd et al., 1983; Yaro et al., 1987; Klein and Edwards, 1989; McGovern and Ladd, 1990; Lance and Elliot, 1991; Allsopp, 1992) and Diptera (Stark and Vargas, 1992). Our results indicate that at moderate to high concentration, eugenol elicits a modest antifeedant response in *T. castaneum*. We observed also limited phagodepression, although beetles tolerated well a large amount of ingested eugenol (33 μg) in the gut with no detectable physiological effect [*ECI* rel = 1.06 (0.08)].

Kaurenic acid, whose molecular structure is closely related to the gibberelins, has been isolated from a number of plants, notably *Baccharis* (Jakupovic et al., 1990) and *Espeletia* (Usubillaga and Capra, 1988). It also has been extracted from trichome exudates of *Polymnia sonchifolia* (Kakuta et al., 1992), and therefore its role as a defensive chemical is conceivable. The DAD-TC assay (Table 1) showed that kaurenic acid is possibly a contact feeding deterrent when alternative food sources are available (as *AI* indicates), but does not fend off feeding if it is found in the only source of food [as *P(ds)i* shows]. *Baccharis trinervis*, our source of kaurenic acid, grows in the Mérida area interspersed with other medium-sized vegetation of considerable diversity. Hence in this habitat there is ample opportunity for herbivores to find alternative foods. The moderate antifeedant effect of kaurenic acid may imbue *B. trinervis* with an adequate defense in this habitat. The results on kaurenic acid attest to the reproducibility of the DAD-TC assay.

Sparteine, an oily alkaloid, usually found in several species of the *Lupinus* genus (Kinghorn et al., 1980) has been linked to disturbances in larval development and survivorship of *Spodoptera eridania* (Johnson and Bentley, 1988). In our assays *T. castaneum* proved to be exceedingly sensitive to sparteine (Table 1) in terms of feeding deterrency and perturbation of the *ECI*. The lowest concentration here tested (0.3 μg/mg of DAD) induced a significant phagodepression [*P(ds)i* = 0.29 (0.16)], probably impairing the beetles' acceptance of the control disks in the two-way assay, since only 0.06 mg/insect of control DAD was consumed instead of the usual 0.5 mg/insect. A lack of preference between control and baited DADs [*AI* = 49 (14)] accompanied by reduced discriminatory behavior (large SD) also could be recorded. Most remarkable was that only 20 ng of ingested sparteine elicited a drastic reduction in *ECI*, which dropped to practically zero. Therefore, in addition to being a potent phagodepressant, sparteine strongly disturbed the insect's digestion–metabolism. The body weight of sparteine-fed insects decreased at a faster rate than the starved controls, possibly from the combined effect of starvation and intoxica-

tion. Insects died soon after the assays ended, in spite of the negligible mortality during the first 60 hr of exposure (2%).

Minthostachis mollis is a strongly aromatic, mint-scented plant of montane neotropics. The plant is unapparent in the field and rarely shows signs of herbivory. It is used by some Peruvian farmers to preserve stored potatoes against insect attack and prevent the appearance of sprouts. In addition, extracts of the aerial parts are mutagenic to human AHH1 lymphoblast cells (Carvajal and Thilly, 1988). Droplets of essential oil are visible just under the leaf surfaces (Usubillaga, personal communication). Hence, extracts of *M. mollis* were expected to show activity against insects. However, the essential oil brought about only a moderate value of *AI* on *T. castaneum*. Baited DADs in one-way assays became significantly less acceptable, but effects were not concentration dependent. This plant may possibly prove more active against other insects, such as those found in stored potatoes in Peru.

Melaleuca quinquenervia, a plant native to Australia with a remarkable capacity for invasion in tropical habitats (Myers, 1983) is a source of 1,8-cineole-rich essential oil (Aboutabl et al., 1991) with potential for insect repellency. In our DAD-TC assay it showed a distinct decrease in *AI* at moderate concentration, but there was no appreciable reduction on DAD biomass consumption. The negative correlation of AI concentration that is abruptly interrupted at 500 mg/cm^2 may be explained as the result of oversaturation of *T. castaneum*'s sensory discrimination of the active component.

Sapindus saponaria was studied because local peasants use the seeds for protection against human lice, also *S. saponaria* seeds are colonized by the aposematic unpalatable Hemipteran *Jadera haematoloma* (Carroll, 1988; Ribeiro, 1989). In addition, other *Sapindus* species have been shown to possess bioactive components (Kimata et al., 1983; Kumar and Thakur, 1988), although none of the compounds so far discovered in *S. saponaria* (Wahab and Selim, 1985) exhibited strong antiinsect activity in past reports, but the DAD-TC assay proved just the opposite.

We separated *S. saponaria* seed components in three solvent extracts of differing polarity as a first approximation to its bioassay-oriented chemical purification. All extracts showed depleted antifeedant indexes (AI). However, the most powerful activity was concentrated in the extract of water followed by chloroform. The baited DADs (one-way assay) were rejected almost entirely with no habituation to the treated feed, and as a result insects lost weight rapidly along a curve similar to the starved beetles, dying accordingly. By feeding so poorly, *ECI* could not be estimated and was assumed to be null.

Paradoxically, the DAD-TC two way assay also revealed that the extracts H1 and C2, but not C1, contain, in addition to the indicated phagodepressant, a strong phagostimulant for *T. castaneum*. Figure 3 depicts the observed consumption for controls (black bars, two-way assay) and treated DADs (white

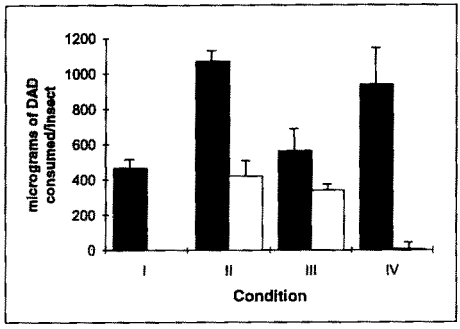

FIG. 3. Phagostimulation in *T. castaneum* by *Sapindus saponaria* extracts in DAD-TC assays. Black bars: amount of control of DAD consumed (μg/insect). White bars: amount of treated DAD consumed. Conditions: I, control without any treatment; II, DADs in the presence of H1 extract; III, DADs in the presence of C1 extract; IV, DADs in the presence of C2 extract. All extracts were added at 1 mg/cm^2 of disk. Error bars represent standard deviation of five replicates; 10 insects and two disks per replicate.

bars, one-way assay). The expected consumption of untreated DAD was 0.466 (0.06) mg/insect in 60 hr, (condition I). The C1 extract did not elicit any statistical variation of this quantity (condition III). By stark contrast, when *T. castaneum* was exposed to DADs treated with either H1 or C2 extracts, the insects moved on to the control disk and devoured 1.07 (0.07) and 0.9 (0.2) mg/insect of it, respectively. This is twice the expected amount and far superior to any control so far observed in our laboratory. Therefore, in addition to a potent phagodepressant, *S. saponaria* also contains a powerful feeding stimulant for *T. castaneum* concentrated in the H1 and C2 fractions. The DAD-TC assay not only proved useful in revealing which extract contained the antifeedant activity, but it was also able to demonstrate unambiguously the existence of another phagostimulant component without further testing and purification of the sample.

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EFFECT OF CULTIVATION ON ALLELOPATHIC INTERFERENCE SUCCESS OF THE WEED, *Pluchea lanceolata*

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Abstract—*Pluchea lanceolata*, a perennial noxious weed, is rapidly spreading into cultivated fields in semiarid regions of India. The objective of the present study was to evaluate the effect of cultivation on the interference success of *Pluchea lanceolata* by comparing chemical characteristics of the weed and its associated topsoil and subsoil in cultivated and uncultivated habitats. Weed plants from both cultivated and uncultivated habitats were analyzed for four biotic characteristics. Leaves were analyzed for nine chemical characteristics. Soils (topsoil and subsoil) were analyzed for 13 chemical characteristics. Nutrient concentrations of the weed and its associated soils, both in cultivated and uncultivated habitats, showed that plant response with reference to nutrient uptake was inversely related to that of soils. Thus, the weed does not create nutrient stress. With cultivation, leaf area and Cu and Na contents increased, while leaf ash, leaf weight, and Mg and Ca decreased. In the topsoil and subsoil, however, concentrations of total carbonates, total phenolics, and Ca increased with cultivation, while organic carbon, phosphate, and K decreased. High phenolic content of the cultivated fields could be explained due to leaching of water-soluble compounds from the plant parts either through natural leaching or through various agricultural practices such as ploughing and irrigation. We concluded that water-soluble phenolics, leached from the weed into the soil, increased with cultivation.

Key Words—Allelopathy, cultivation, discriminant function analysis, interference, *Pluchea lanceolata*, weed.

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INTRODUCTION

Pluchea lanceolata (DC) C.B. Clarke (Asteraceae), a hoary pubescent undershrub, is a common weed of cultivated fields and also occurs in uncultivated areas in and around Delhi and in sandy and saline tracts of northwest India (Anonymous, 1969; Bhandari, 1979). Our earlier research established that this weed achieved its interference success by inhibiting growth and establishment of certain plant species through water-soluble phenolics synthesized and released into its associated soil (Inderjit and Dakshini, 1990, 1991b, 1992a,b). *Pluchea lanceolata* grew more in cultivated than in uncultivated lands (Dakshini and Sabina, 1981). Furthermore, with irrigation, it competes successfully with crop plants. However, interference with crop plants was less severe in drier conditions. *P. lanceolata* has the potential to modify its life cycle in response to ecological stress by acting as a competitor, a reactor, or a resistor (Dakshini and Sabina, 1981). Such versatility has not only helped *P. lanceolata* to survive, but probably has encouraged its spread throughout the semiarid tracts of northwest India. To understand the causes of its success in cultivated habitats, a comparative study was made between weed-infested cultivated and uncultivated habitats. The objective of the study was to evaluate the effect of cultivation on the phenolic content of the soil as contributed by *P. lanceolata* and thus the allelopathic interference success of this weed.

METHODS AND MATERIALS

Study area. Two sites, Alwar (latitude 27°34′ N, longitude 76°38′ E) and Dharuherra (latitude 28°12′ N, longitude 76°40′ E), infested with the weed *Pluchea lanceolata* were selected for the present study. At each site, cultivated and uncultivated habitats were marked. Weed plants and associated soils (topsoil and subsoil) of the cultivated and uncultivated habitats were sampled in three different seasons, i.e., February, July, and October from 1990 through 1991 to maximize heterogeneity among sites. Analyses were made in triplicate and repeated twice to obtain ecologically and statistically relevant results.

Uncultivated habitats were defined as areas having no record of cultivation or irrigation and no biotic disturbance. Cultivated habitats were defined as being cultivated during the year, irrigated, and frequently ploughed before sowing of crop seeds. In such habitats, damage to the weed and its propagules is frequent. The climate of this region is semiarid with a marked dry and hot summer (May through June, temperature 30–45°C) and a dry and cold winter (mid-November to February, temperature 4–15°C). Precipitation is moderately low (60–120 cm annually) and the wettest months are July and August. The soils of this region are of the sandy loam textural class. The major crops cultivated in this region

are mustard (*Brassica juncea*), wheat (*Triticum aestivum*), and chick pea (*Cicer arietinum*).

Chemical Analysis. Plants were randomly sampled at each sampling site, Alwar (AW) and Dharuherra (DH), in three different seasons. Plant samples were collected, washed, and dried at 45°C. Leaves of the plants were separated and stored in paper bags for later chemical analysis. The weed was analyzed for four biotic characteristics: density (Daubenmire, 1959), height, leaf area (Delta-T leaf area meter), and leaf weight (average of ten leaves), and for nine chemical characteristics: leaf ash (muffle furnace at 550°C for 3 hr), total phenolics (Swain and Hillis, 1959), and nutrients such as PO_4, Cu, Zn, Na, K, Mg, and Ca (Grimshaw et al., 1989).

Soil samples from surface (identified as topsoil) and at the depth of 30 cm (identified as subsoil) were collected (Brady, 1990). Soil samples were air-dried, sieved (2-mm sieve), and stored in paper bags. Soils were analyzed for pH, electrical conductivity (EC), organic carbon (OC), soluble chloride, total carbonate (Piper, 1966), total phenolics (Swain and Hillis, 1959), and nutrients such as PO_4, Zn, Na, K, Mg, and Ca (Grimshaw et al., 1989).

Statistical Analysis. The data on the various parameters were analyzed statistically by comparing the group mean for each variable of cultivated and uncultivated habitats using ANOVA (COMPARING GROUP MEAN, range Tukey), $P < 0.05$ (SPSSPC, 1986). Since ANOVA can not bring out the relative contribution of each variable to the overall distinction between the two groups (namely cultivated and uncultivated), the data of all the variables were subjected to discriminant function analysis (DFA, DISCRIMINANT Procedure, Method Direct). Group membership was the grouping variable and the various parameters the predictor variables. The two groups referred to the cultivated and uncultivated habitats.

RESULTS

Both cultivated and uncultivated habitats at sites AW and DH had wide and overlapping ranges for all the 13 factors analyzed for plant, topsoil, and subsoil. Plants of cultivated habitats of DH had significantly higher Cu content ($0.0037 \pm 0.0009\%$) as compared to uncultivated habitats ($0.0026 \pm 0.0007\%$). Although the values of several other factors analyzed were distinct for plants from cultivated and uncultivated habitats, the differences were not statistically significant.

Topsoils and subsoils of uncultivated habitats in AW, as compared to cultivated habitats, had significantly higher ($P < 0.05$) values for OC and K, respectively. Other factors analyzed displayed some amount of differences, but these were nonsignificant.

Phosphate of both the topsoil and subsoil of uncultivated habitats of DH was significantly higher ($P < 0.001$) than that of cultivated habitats, while Ca of cultivated habitats of the topsoil and subsoil was significantly higher ($P < 0.05$) than that of uncultivated soils. Other values were not significantly different. TP of both the topsoil and subsoil of cultivated habitats of AW and DH was not significantly higher than that of soils of uncultivated habitats.

DFA classified all the cases correctly at the level of plants, topsoil, and subsoil (Figure 1). An all-group histogram from DFA showed that the cultivated and uncultivated habitats were distinct in both AW and DH sites (Figure 1). On the basis of DFA, the (cultivated and uncultivated) groups in DH were best discriminated by Ca, Na, and Cu in plants (Table 1); by PO_4, total carbonate, Zn, OC, Cl, and Mg in topsoil (Table 2); and by Mg, Ca, EC, total carbonate, Cl, pH, K, TP, and Na in subsoil (Table 2). In AW, the best discrimination between the two groups was brought out by leaf ash, Na, Zn, K, leaf area, Ca, Cu, and TP in plants (Table 1); by Zn, K, Mg, OC, and Na in topsoil (Table 2); and by Zn, K, Na, Mg, Ca, PO_4 and OC in the subsoil (Table 2).

DISCUSSION

A comparative evaluation of the data showed that the concentration of phenolics in the weed-associated soils increased with cultivation. Such an addition was through natural leaching of water-soluble phenolics from the weed *P. lanceolata*, or cultivation of the plant into the soils followed by irrigation and is supported by our earlier research (Inderjit and Dakshini, 1990, 1992b) and also widely reported in nature (Bhowmik and Doll, 1984; Heisey, 1990; Inderjit and Dakshini, 1991; Rice, 1984). A comparative evaluation of the nutrient concentrations in the weed and its associated soils, both from cultivated and uncultivated habitats, brought out that plant response for nutrient uptake was inversely related to that of the soil status. Thus, while the concentrations of particular cations (Mg and Ca) or anions (PO_4) were higher in the soils (particularly subsoils), these were lower in the plant and vice versa. On the basis of topsoil, Zn, Mg, and OC were common discriminants of two habitats; on the basis of subsoil, K, Mg, Na, and PO_4 were the main discriminants. Soils of cultivated habitats had higher TP content, which could influence nutrient accumulation and availability (Rice, 1984). In view of earlier reports (Chichester and Hauser, 1991; Ewel et al., 1991; Sharma and Dakshini, 1991), and with the data presented, it is not difficult to visualize the role of *P. lanceolata* in bringing about alteration of soil characteristics with cultivation. In soils, the concentration of total carbonate, TP, and Ca increased, whereas OC, PO_4, and K concentrations decreased, with cultivation. This could be due to breaking of soil crumbs and modification of soil structure through cultivation rather than

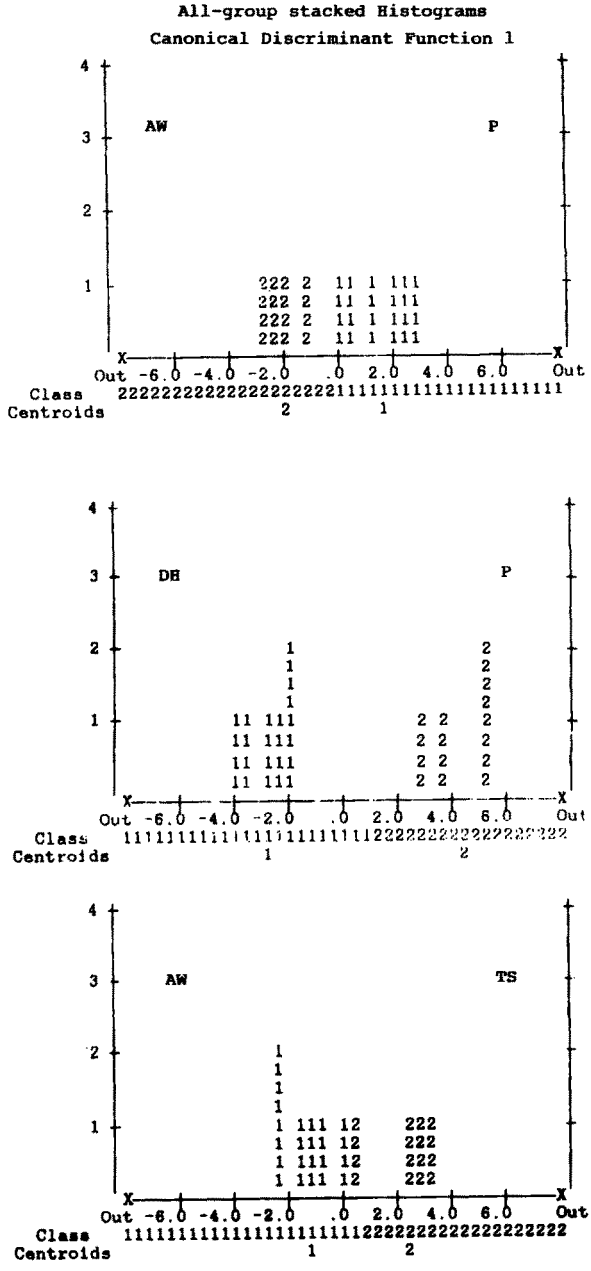
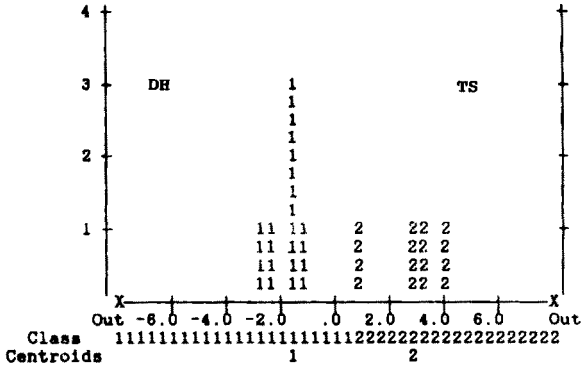
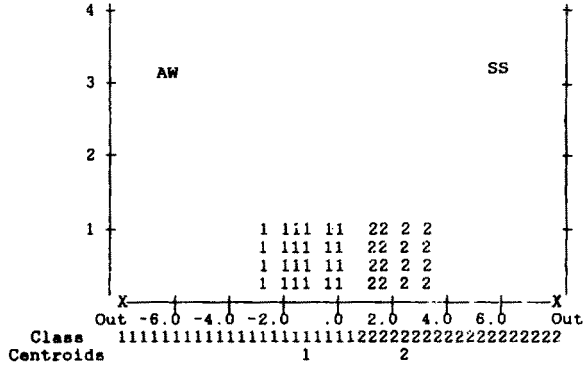


FIG. 1. All-group histograms from discriminant function analysis on the basis of 13 variables of plants (P), topsoil (TS), and subsoil (SS) of cultivated (class 1) and uncultivated (class 2) habitats of two sampling sites, Alwar (AW) and Dharuherra (DH).





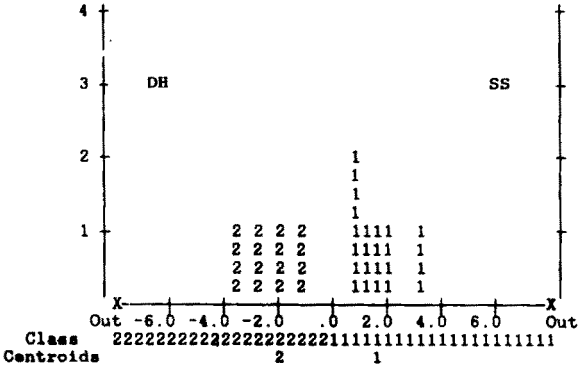


FIG. 1. Continued

TABLE 1. POOLED WITHIN-GROUP CORRELATIONS BETWEEN DISCRIMINATING VARIABLES AND CANONICAL DISCRIMINATING FUNCTION (DF) ON BASIS OF PLANT CHARACTERISTICS OF TWO SAMPLING LOCATIONS, ALWAR AND DHARUHERRA

Discriminating variable	DF correlations	
	Alwar	Dharuherra
Leaf ash	−0.30226[a]	0.07229
Na	0.22007[a]	0.21205
Zn	0.18894[a]	0.03805
K	−0.17671[a]	−0.04077
Leaf area	0.15811[a]	−0.02561
Ca	−0.14491[a]	0.46698[a]
Cu	0.13607[a]	−0.19071[a]
TP	0.13159[a]	0.10860
Mg	0.11065	0.08922
PO_4	−0.04676	−0.02241
Leaf weight	−0.02805	0.05144

[a]Significant correlations within each discriminating function.

effect of the plant, as the accumulation pattern of the nutrients in plants was inverse to that of soil. Since the weed was present in both cultivated and uncultivated habitats, TP was not a significant discriminating variable but TP concentration in the weed-associated soils of cultivated habitats (AW: topsoil, 12.31 mg/100 g; subsoil, 7.33 mg/100 g, and DH: topsoil, 8.0 mg/100 g; subsoil, 9.47 mg/100 g) was rather high as compared to that of uncultivated habitats (AW: topsoil, 8.16 mg/100 g; subsoil, 4.25 mg/100 g, and DH: topsoils, 6.9 mg/100 g; subsoil, 5.20 mg/100 g). This increase in TP could be a natural consequence of leaching of water-soluble phenolics from the weed or due to the microbial degradation of chemicals in the soil (Blum and Shafer, 1988; Blum et al., 1987; Martin and Haider, 1976; Rice, 1984; Tanrisever et al., 1987). Weed plants of uncultivated habitats were shorter and clumped as compared to cultivated habitats (Dakshini and Sabina, 1981). Furthermore, the nutrient concentrations of weed plants from uncultivated habitats were lower and those of soils were higher as compared to those from cultivated habitats. This would suggest that in general, in uncultivated habitats the uptake of nutrients by the weed was poor and growth conditions were not as favorable as in cultivated habitats. Higher OC content in weed-associated soils of uncultivated habitats was probably due to slow decomposition. This could be due to the relatively more compact nature of soils of uncultivated habitats, and with the poor exchange of gases and thus poor microbial activity, resulting in increased OC (except in subsoil of DH) and PO_4 (Table 2). Further, physical, chemical, and microbial

TABLE 2. POOLED WITHIN-GROUP CORRELATIONS BETWEEN DISCRIMINATING VARIABLES AND CANONICAL DISCRIMINATING FUNCTION (DF) ON BASIS OF TOPSOIL (TS) AND SUBSOIL (SS) OF TWO SAMPLING LOCATIONS, ALWAR AND DHARUHERRA

Discriminating variable[a]	DF correlations			
	Alwar		Dharuherra	
	TS	SS	TS	SS
Zn	0.58077[b]	0.34981[b]	0.29430[b]	0.04682
K	0.56098[b]	−0.34104[b]	0.10822	−0.14053[b]
Mg	0.46525[b]	−0.29700[b]	0.18239[b]	−0.21636[b]
OC	0.40828[b]	0.18717[b]	0.24622[b]	0.00081
Na	0.24045[b]	−0.30645[b]	−0.15541	0.09302[b]
EC	0.17814	−0.03495	0.00397	0.18245[b]
PO_4	0.14933	0.19792[b]	0.40346[b]	0.07709
Ca	0.14704	0.25478[b]	−0.12862	0.19434[b]
TP	−0.11605	−0.13538	−0.03703	0.13937[b]
Cl	−0.09145	0.00732	0.20414[b]	0.15276[b]
HCO_3	0.08049	−0.07455	−0.10776	−0.04052
pH	0.04462	0.04937	−0.04929	0.14720[b]
TCO_3	−0.00586	−0.12265	−0.31961[b]	0.17039[b]

[a]OC, organic carbon; EC, electrical conductivity; TP, total phenolics; TCO_3, total carbonate.
[b]Significant correlations within each discriminating function.

processes should also be involved in loss of OC residues from the cultivated habitats (Bristow et al., 1986; Parr and Papendick, 1978; Stott et al., 1986; Stroo et al., 1989). However, as compared to weight loss by microbial degradation, physical and chemical processes often seem minor (Douglas and Rickman, 1992), and this may explain the direct relationship of OC and PO_4 in cultivated and uncultivated habitats.

Our results establish that the phenolic content of the weed-associated soils increases with cultivation, and thus its interference potential increases. Further, since *P. lanceolata* is perennial, it maintains continuous availability and periodic replenishment of phenolics in the soils, and if not controlled may bring about permanent damage to the cultivated land with time, especially in dry and semiarid regions, as in such regions the decay and degradation of allelochemicals is slow and delayed (Del Moral and Muller, 1970).

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DIRECT AND MEDIATED EFFECTS ON *Bactrocera oleae* (GMELIN) (DIPTERA; TEPHRITIDAE) OF NATURAL POLYPHENOLS AND SOME OF RELATED SYNTHETIC COMPOUNDS: STRUCTURE–ACTIVITY RELATIONSHIPS

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Abstract—Among the main polyphenols occurring in olive oil vegetation waters (VW), catechol showed the most deterrent action on the oviposition of *Bactrocera oleae* (Gmelin); 4-methylcatechol was less active, whereas hydroxytyrosol and tyrosol were inactive. In contrast, synthetic *o*-quinone was found to be stimulant at 7.5×10^{-2} M. Two other synthetic derivatives of catechol, diacetylcatechol and guaiacol, were also deterrent, suggesting these compounds undergo a biochemical transformation into catechol by means of the bacterial symbionts of *B. oleae*. VW and their phenolic extracts showed deterrence only when highly concentrated, while natural olive juice was strongly deterrent. Experiments carried out to evaluate the effect of olive juice and catechol on the fecundity of *B. oleae* showed that they strongly reduce this function. Moreover, the possible utilization of VW and their bioactive polyphenols in protection of olives against *Batrocera oleae* is discussed.

Key Words—*Olea europea*, *Bactrocera oleae* Gmelin, Diptera, Tephoitidae, *Pseudomonas syringae* subsp. *savastanoi*, olive oil vegetation waters, polyphenols, ovipositional deterrence, fecundity.

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INTRODUCTION

Olive oil mill waste waters, known also as vegetation waters (VW), cause disposal problems because they contain powerful pollutants; they cannot, therefore, be spread on fertile land or discarded in sewage plants. They contain many inorganic and organic compounds among which polyphenols, whose content ranges from 0.6 to 1.6% (Pacifico, 1989), are recognized to be responsible for their phytotoxicity (Capasso et al., 1992a). On the other hand, polyphenols could be used as recycling products, as suggested by many researchers (De Caro and Galamini Ligori, 1959; Fiume and Vita, 1977; Girolami et al., 1981; Camurati and Fedeli, 1982; Bartolini et al., 1993; Capasso et al., 1993).

The ecological problems raised by the disposal of these waste waters and the opportunity to find methods for their detoxification and recycling have prompted the authors of this paper to analyze their organic extracts and the resulting exhausted solution. This research is aimed at identifying the compounds that are responsible for the reported antimicrobial (Rodriguez et al., 1988), insect ovipositional deterrent, phytotoxic, and plant growth regulating activity of these waste waters, in order to find natural products for protection of the olive plant against its parasites. In a recent paper, Capasso et al. (1992a) described the isolation and the spectroscopic characterization of VW main polyphenols. In addition they reported on the phytotoxicity of these compounds and the corresponding acetyl derivatives assayed in vitro. In the present paper we describe the effects of the same polyphenols and related compounds on the oviposition of the olive fly *Bactrocera oleae* (Gmelin). The possible interference of bacterial symbionts of *B. oleae* has been also evaluated and the bioactivity has been correlated with the structure of the compounds considered. The effects of olive juice (OJ), VW, and their phenolic extracts (PE) are also described. Moreover, we report the results of a study on the influence of different substrates, including olive juice and catechol, on the fecundity of *B. oleae*. Finally, the possible use of bioactive products, VW, and their PE for the protection of olive plants against the olive fly is discussed.

METHODS AND MATERIALS

General. The analytical and preparative TLC were carried out on silica gel plates (20 × 20 cm, Merck, kieselgel 50 F_{254}, 0.25 and 0.5 mm, respectively), with C_6H_6–EtOAc (85:15) (eluent A) or C_6H_6—EtOAc—MeOH (60:30:10) (eluent B) or C_6H_6-EtOAc (70:30) (eluent C). The bands were detected by exposure to Vis or UV selected at 254 nm, while the spots were visualized by exposure to Vis or UV selected to 254 nm and by spraying the plates first with 10% H_2SO_4 in MeOH and then with 7% phosphomolybdic acid in MeOH, followed by heating for 5 min at 105°C. IR spectra were recorded in $CHCl_3$ or

neat. UV spectra were obtained in MeOH. ^{1}H NMR spectra were recorded in $CDCl_3$ or neat. UV spectra were obtained in MeOH. ^{1}H NMR spectra were recorded in $CDCl_3$ at 270 MHz. EI mass spectra were recorded at 70 eV. Catechol **(1)**, 4-methylcatechol **(6)**, hydroxytyrosol **(8)**, 1,4-tyrosol (known as tyrosol, **11**) and the corresponding acetyl derivatives **5, 7, 10,** and **15** were obtained according to the procedures previously described (Capasso et al., 992b). Guaiacol **(2)** and veratrole **(3)** were purchased from Fluka (Buchs, witzerland); 1,2-tyrosol **(12)** and 1,3-tyrosol **(13)** were from Aldrich (Steineim, Germany). Fresh VW were supplied by mills from Avellino province Italy). PE extracts were obtained as previously described (Capasso et al., 1992a).

o-Quinone (4). This compound was prepared by oxidation of catechol according to the procedure employed by Adler et al. (1962) to prepare **4** from guaiacol. To catechol (200 mg) dissolved in ultrapure H_2O (4.5 ml) was added a solution of $NaIO_4$ (1.4 g) in ultrapure H_2O (10 ml), at 0°C, under continuous stirring. The reaction mixture was left for a few seconds, then was extracted with CH_2Cl_2 (3 × 15 ml). The red combined extracts were directly chromatographed by preparative TLC (eluent A) in the dark, producing a red pure product (4.5 mg): UV, 250 nm (ϵ = 500); IR ($CHCl_3$), 1673 (CO—CO group), 1603 (C=C group) cm^{-1}.

Diacetyl-1,2-hydroxytyrosol (4-Hydroxyethyl-1,2-diacetoxybenzene, 9). To a sample of hydroxytyrosol (**8,** 15 mg) was added Ac_2O (20 μl) followed by 96% H_2SO_4 (195 μl) at 0°C and under constant stirring. Afterwards, a mixture (150 μl) composed of ice (6.5 g) and $NaHCO_3$ (1.20 g) was added and the resulting neutral solution was extracted with $CHCl_3$ (3 × 2 ml). The combined extracts were dried and evaporated under reduced pressure. The crude product (11.2 mg) was purified by preparative TLC (eluent B), to an uncrystallizable oily residue (3 mg), identified as **9:** UV, 239 nm (ϵ = 390), 265 (470), 271 (440); IR (neat), 3560 and 3450 (alcoholic OH), 1765 (C=O, phenolic ester), 1502 and 1370 (Me of acetyl groups), 1220 (C—O, ester) cm^{-1}; ^{1}H NMR, δ 7.15 (1H, *d*, $J_{5,6}$ = 8.0 Hz, H-5), 7.10 (1H, *d*, $J_{5,6}$ = 8 Hz, H-6), 7.06 (1H, *br s*, H-3), 3.86 (2H, *t*, $J_{1',2'}$ = 7.3 Hz, H_2-2'), 2.86 (2H, *t*, $J_{1',2'}$ = 7.3 Hz, H_2-1'), 2.30 (3H, *s*, Ac) and 2.29 (3H, *s*, Ac); EI-MS, *m/z*, rel. int.: 238 $[M]^+$ (4), 196 $[M-CH_2CO]^+$ (20) 194 $[M-CH_2CHOH]^+$ (3), 178 $[M-AcOH]^+$ (2), 154 $[M-2CH_2CO]^+$ (95), 136 $[M-CH_2CO-AcOH]^+$ (14), 123 $[M-2CH_2CO-CH_2OH]^+$ (100), 94 $[M-2CH_2CO-CH_2OH-HCO]^+$ (5), 77 $[C_6H_5]^+$ (17), 65 $[C_5H_5]^+$ (3), 43 $[Ac]^+$ (90).

Monoacetyl-1,4-tyrosol (4-Hydroxyethylacetoxybenzene, 14). A sample of 1,4-tyrosol (**11** 50 mg), was converted into **14,** using the same method to obtain **9** from **8.** The crude product (47 mg) yielded from the work-up, was purified by TLC (eluent C) to an uncrystallizable oily residue (25 mg), identified as 4-hydroxyethylacetoxybenzene **(14):** UV, 240 nm (ϵ = 390), 264 nm (ϵ = 340), 270 nm (ϵ = 305); IR (neat), 3556 and 3390 (alcoholic OH), 1756 (C=O,

phenolic ester), 1509 and 1370 (Me of acetyl group), 1220 (C—O, ester) cm^{-1}; ^1H NMR, δ 7.23 (2H, *d*, $J_{2,3} = J_{5,6} = 8.6$ Hz, H-2 and H-6), 7.02 (2H, *d*, $J_{2,3} = J_{5,6} = 8.6$ Hz, H-3 and H-5), 3.86 (2H, *t*, $J_{1',2'} = 7.3$ Hz, H_2-2'), 2.86 (2H, *t*, $J_{1',2'} = 7.3$ Hz, H_2-1'), 2.29 (3H, *s*, Ac); EI-MS *m/z*, 180 $[M]^+$ (15), 138 $[M-CH_2CO]^+$ (100), 136 $[M-C_2H_4O]^+$ (30), 120 $[M-AcOH]^+$ (24), 108 $[M-CH_2CO-CH_2O]^+$ (45), 107 $[M-CH_2CO-CH_3O]^+$ (70), 91 $[C_7H_7]^+$ (20), 77 $[C_7H_5]^+$ (44).

*Diacetyl-1,3-tyrosol (2-Acetoxyethylacetoxybenzene, **16**).* 1,2-Tyrosol (**12**, 240 mg) was acetylated, using the usual methods previously described (Capasso et al., 1992b): UV, 239 nm ($\epsilon = 290$), 261 nm ($\epsilon = 320$), 268 nm ($\epsilon = 260$); IR ($CHCl_3$), 1762 (C=O, phenolic ester), 1740 (C=O alcoholic ester), 1491 and 1368 (Me of acetyl groups), 1213 (C—O, ester) cm^{-1}; ^1H NMR, δ 7.29 (1H, *dd*, $J_{3,4} = 7.7$ Hz, $J_{3,5} = 1.90$ Hz, H-3), 7.24 (1H, *td*, $J_{4,5} = J_{5,6} = 7.7$ Hz, $J_{3,5} = 1.9$ Hz, H-5), 7.04 (1H, *dd*, $J_{5,6} = 7.7$ Hz, $J_{4,6} = 1.9$ Hz, H-6), 4.24 (2H, *t*, $J_{1',2'} = 7.3$ Hz, H_2-2'), 2.87 (2H, *t*, $J_{1',2'} = 7.3$ Hz, H_2-1'), 2.34 (3H, *s*, Ac), 2.04 (3H, *s*, Ac); EI-MS, *m/z*: 180 $[M-CH_2CO]^+$ (25), 162 $[M-AcOH]^+$ (80), 138 $[M-2CH_2CO]^+$ (15), 120 $[M-CH_2CO-AcOH]^+$ (100), 107 $[M-CH_2CO-CH_2OAc]^+$ (70), 91 $[C_7H_7]^+$ (65), 77 $[C_6H_5]^+$ (20).

*Diacetyl-1,3-tyrosol (3-Acetoxyethylacetoxybenzene, **17**).* 1,3-Tyrosol (**13**, 204 mg) was acetylated, using the usual methods previously described (Capasso et al., 1992b): UV, 239 nm ($\epsilon = 290$), 261 nm ($\epsilon = 320$), 268 nm ($\epsilon = 260$); IR, 1762 (C=O, phenolic ester), 1740 (C=O, alcoholic ester), 1213 (C—O, ester) cm^{-1}; ^1H NMR, δ 7.30 (1H, *t*, $J_{4,5}, = J_{5,6} = 8$ Hz, H-5), 7.08 (1H, *br d*, $J_{5,6} = 8$ Hz, H-6), 6.96 (1H, *br d*, $J_{4,5} = 8$ Hz, H-4), 6.95 (1H, *br s*, H-2), 4.28 (2H, *t*,, $J_{1',2'} = 7.3$ Hz, H_2-2'), 2.94 (2H, *t* $J_{1',2'} = 7.3$ Hz, H_2-1'), 2.29 (3H, *s*, Ac), 2.04 (3H, *s*, Ac); EI—MS, *m/z*: 180 $[M-CH_2CO]^+$ (15), 162 $[M-AcOH]^+$ (50), 138 $[M-2CH_2CO]^+$, 120 $[M-AcOH-CH_2CO]^+$ (100), 107 $[M-CH_2CO-CH_2OAc]^+$ (30), 91 $[C_7H_7]^+$ (25), 77 $[C_6H_5]^+$ (20).

*Evaluation of VW, VW extracts, OJ, and phenols **1–17** on ovipositional behavior of* Bactrocera oleae *Gmelin. B. oleae* adults were obtained from infested olives collected in an olive grove near Pisciotta (Salerno, Italy). The adults were reared in tulle cages (12 × 6 × 7 cm height), under a light–dark cycle of 14–10 hr at 23 ± 1.5°C and relative humidity above 60%. Crepuscular light, which favors mating, was simulated by decreasing the luminous intensity in the last hour of the light cycle. The adults were supplied with water from small bottles overturned on the cage surface and were fed with a mixture of yeast autolysate, water, and saccharose (1:7:10), brushed on the tulle. Soon after emergence, females were caged with males and sound green olives and reared in controlled conditions as above described, for five weeks, during which the tests were carried out. Each week three days of test were performed with these females, alternating three days of tests and four days of permanence with olives.

Test cages were provided with Plexiglas walls and horizontal faces in tulle. Oviposition by test females was obtained into false olives in form of agar domes (12 mm diameter × 15 mm high), which were green colored and covered by parafilm. Two agar domes were placed, in each cage one treated with the test substance, the other treated only with the solvent (methanol). The substances to be tested, dissolved in methanol (40 μl), were spread on the dome surface. For each substance tested, four test cages were used, each with five females and two males on three consecutive days for a total of 12 replicates. Every day ovipositions were registered and agar domes replaced and treated with new samples.

Evaluation of OJ and catechol (1) for fecundity on Bactrocera oleae *Gmelin.* Five females and two males, just emerged, were introduced into the described Plexiglas–tulle cages. Five cages were used concurrently. In each of four cages the olives were introduced as follows: five sound olives in the first cage, five infested olives in the second cage, five sound olives spread with OJ in the third cage, five sound olives spread with 50 μl of catechol (3×10^{-2} M, methanol) in the fourth cage. In the fifth cage no olives were introduced. The cages were kept in the same conditions for two weeks, replacing the substrates daily. During the third and fourth weeks, the olives were replaced with false olives (domes) obtained from green-colored agar. Ovipositions in these domes were registered every day. Each test was replicated five times.

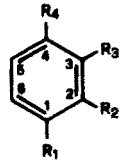
The *C/T* ratio between the number of eggs laid in control olives (*C*) and treated olives (*T*) was calculated.

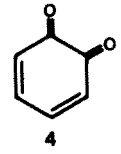
Evaluation of growth inhibition of Pseudomonas syringae *subsp.* savastanoi *by phenols 1–3, 5–7, 13, and 17.* A strain of *P. s.* subsp. *savastanoi* was maintained on nutrient agar (NA) (0.8% nutrient broth and 1.7% agar; BBL). All tests were carried out in 9-cm-diameter Petri plates containing 20 ml of NA and stored at 25°C. The different substances were added to the NA substrate at 50°C after filter sterilization. Each plate was inoculated with a suspension of 10^8 cells/ml and after two days the colonies were analyzed and compared with the control. Test concentration was 5×10^{-4} M.

RESULTS AND DISCUSSION

Among the polyphenols previously identified in our VW samples (Capasso et al., 1992a), catechol (Figure 1, **1**) showed the strongest deterrent activity on the oviposition by *B. oleae*; 4-methylcatechol (**6**) was less active; while hydroxytyrosol (**8**) and tyrosol (**11**) were inactive (Table 1).

Among the compounds closely related to catechol, guaiacol (**2**) and diacetylcatechol (**5**) were less deterrent than **1**; veratrole (**3**) was inactive; while *o*-quinone (**4**) stimulated the oviposition at 7.5×10^{-2} M (Table 1). It is worth





	R_1	R_2	R_3	R_4
1	OH	OH	H	H
2	OH	OCH_3	H	H
3	OCH_3	OCH_3	H	H
5	$OCOCH_3$	$OCOCH_3$	H	H
6	OH	OH	H	CH_3
7	$OCOCH_3$	$OCOCH_3$	H	CH_3
8	OH	OH	H	CH_2CH_2OH
9	$OCOCH_3$	$OCOCH_3$	H	CH_2CH_2OH
10	$OCOCH_3$	$OCOCH_3$	H	$CH_2CH_2OCOCH_3$
11	OH	H	H	CH_2CH_2OH
12	OH	CH_2CH_2OH	H	H
13	OH	H	CH_2CH_2OH	H
14	$OCOCH_3$	H	H	CH_2CH_2OH
15	$OCOCH_3$	H	H	$CH_2CH_2OCOCH_3$
16	$OCOCH_3$	$CH_2CH_2OCOCH_3$	H	H
17	$OCOCH_3$	H	$CH_2CH_2OCOCH_3$	H

FIG. 1. Structure of natural polyphenols (**1, 6, 8, 11**) and corresponding synthetic derivatives (**2–5, 7, 8–10, 12–17**).

noting that catechol is the most structurally simple molecule among the tested orthodiphenolic compounds. In fact, compared with **1**, 4-methylcatechol and guaiacol possess a methyl and methoxy group at C-4 and C-2, respectively, of the benzene ring, while diacetylcatechol and veratrole present both the hydroxyl groups transformed into an ester and ether, respectively. These differences cause a decreasing deterrent activity. In particular, in regard to **6** the methyl group at C-4 could reduce the affinity of the orthodiphenolic system with the site of action of **1**.

TABLE 1. EFFECTS ON OVIPOSITION (*C/T*) OF *Bactrocera oleae* OF OLIVE JUICE (OJ), VEGETATION WATERS (VW), THEIR PHENOLIC EXTRACT (PE) AND POLYPHENOLS, AND STRUCTURALLY RELATED COMPOUNDS[a]

Compound	Concentrations		
	7.5×10^{-2} M	3×10^{-2} M	1.5×10^{-2} M
Catechol (**1**)	1.96	1.57	1.43
Guaiacol (**2**)	NA	1.74	NA
Veratrole (**3**)	NA	NA	NA
o-Quinone (**4**)	0.62[b]	NA	NA
Diacetylcatechol (**5**)	1.34	1.25	NA
4-Methylcatechol (**6**)	1.39	1.36	NA
1,3-Tyrosol (**13**)	NA	1.41	NA
	×10	×5	×1
OJ			2.69
VW	1.84	1.32	NA
PE	1.28		NA

[a] *C/T* = ratio between the number of eggs laid in control olives (*C*) and treated olives (*T*); the values reported are only those statistically significant determined with Student's *t* method. NA = no activity.
[b] Value corresponding to a stimulant activity.

On the other hand, guaiacol and diacetylcatechol most likely show deterrence because of their enzymatic transformation into catechol by means of, respectively, a mixed oxidase and a hydrolase present in the bacterial symbionts of *B. oleae*, which could interfere in the olive exploration phase by the olive fly with its labellum. The biochemical activation of natural and synthetic compounds by organisms such as plants, insects, and bacteria has been often reported (Uchtil and Durbin, 1980; Capasso et al., 1992a). Howard et al. (1985) suggested that a possible role of bacterial symbionts of *Rhagoletis*, a fruit fly genus related to *Bactrocera*, which are housed in the esophageal diverticulum as they are in *B. oleae*, might be the metabolic transformation of exogeneous compounds. Petri (1909) identified the phytopathogenic gram-negative *Pseudomonas syringae* subp. *savastanoi* as the bacterial symbiont of *B. oleae*; later, Lüthy et al. (1983a,b) did not confirm this identification, but demonstrated that the bacterial symbionts of *B. oleae* were gram negative. In order to support the hypothesis of the mediate enzymatic transformation described above, we tested compounds **1–3**, **5–7**, **13**, and **17** for their ability to inhibit the growth of *P. s.* subsp. *savastanoi*, assuming this capacity to be a response to the metabolic activity of the bacterium. The results, summarized in Table 2, show that guaiacol

TABLE 2. GROWTH INHIBITION OF *Pseudomonas syringae* SUBSP. *savastanoi* BY SOME VW POLYPHENOLS AND RELATED COMPOUNDS AFTER 48 HR AT 25°C[a]

Compound (5×10^{-4} M)	Growth inhibition (%)
Catechol (**1**)	70 b
Guaiacol (**2**)	90 a
Veratrole (**3**)	0 c
Diacetylcatechol (**5**)	64 b
4-Methylcatechol (**6**)	100 a
Diacetyl-4-methylcatechol (**7**)	0 c
1,3-Tyrosol (**13**)	0 c
1,3-Diacetyltyrosol (**17**)	0 c
Control	0 c

[a]Each value is the average of four experiments (four replicates per experiment). Means in the same column followed by the same letter are, according to Duncan's test, not statistically different ($P = 0.01$).

and diacetylcatechol were more active than veratrole. The lack of deterrence of this last compound might be the effect of its weak tendency to be transformed into **1**.

The lack of deterrence of diacetyl-4-methylcatechol (**7**) is consistent with the response of the bioassy reported in Table 2, which suggests that *P. s.* subsp. *savastonoi* has no capacity to transform **7** into 4-methylcatechol (**6**).

Hydroxytyrosol (**8**), which Vita et al. (1977) showed to be deterrent and Girolami et al. (1981) less deterrent than catechol, was inactive in our experiments. The lack of activity of **8** could be justified by the presence of a hydroxyethyl group at C-4 of the ring of the *o*-diphenolic system, which could inhibit the interaction of **8** with the active site of **1**. The corresponding triacetyl (**10**) and diacetyl (**9**) derivatives were inactive too.

Tyrosol (**11**), another VW phenolic compound, and its diacetyl (**15**) and monoacetyl (**14**) derivatives were not deterrent. Among the isomers of tyrosol (**12** and **13**) and the corresponding acetyl derivatives (**16** and **17**), respectively, only **13** was active at 3×10^{-2} M, most probably because this compound is the only isomer having the hydroxyl groups spaced as in catechol, as observed by comparing the Dreiding models of catechol and 1,3-tyrosol (**13**).

In the light of our hypothesis on the mediation of bacterial symbionts, the lack of deterrence by **17** (Table 1) might be due to the weak capability of the symbionts to transform this compound in the corresponding **13**, as shown by the data of Table 2.

In order to achieve preliminary indications on the mechanism of action of catechol, *o*-quinone (**4**) was tested in the deterrence assay and showed to be a

stimulant (Table 1). Therefore **1**, which acts as deterrent, and **4**, which acts as stimulant, would interact with two independent sites.

Examination of the data of Table 1 points out the remarkable deterrence of the natural OJ as proof of a natural deterrence mechanism existing in the olives (Cirio, 1971). In addition, the activity of VW and PE, previously reported by Fiume and Vita (1977) and by Vita et al. (1977) has been confirmed. The lower activity showed by VW and PE with respect to OJ demonstrates that liposoluble compounds occurring in OJ, in addition to the tested natural hydrosoluble polyphenols of VW, might contribute to the overall deterrence of olive juice. The report by Girolami et al. (1981) is thus confirmed.

The results of the fecundity test are reported in Table 3. They show that newly emerged females of the olive fly, kept for two weeks in presence of olives spread with OJ or catechol, strongly reduced their fecundity compared to those that were reared in absence of olives or in presence of infested olives, suggesting for OJ and **1** a role in inducing depression of fecundity as well. In addition, by comparing the data concerning the effect to of OJ with that of **1**, it turns out that this compound could play a role much more important in depressing fecundity than in deterring. *B. oleae* females.

It is possible that an inhibiting effect similar to that shown by **1** on females might be exerted by the other phenolic compounds that showed a deterring action in the oviposition tests (**2**, **5**, **6**, and **13**).

In light of the results described above, the possibility of recycling VW for protecting olives from *B. oleae* might be taken into consideration. Fiume and Vita (1977) experimented with VW against *B. oleae* and concluded that their use is to be avoided, since they are easily oxidized by air and light and cause fruit and leaf drop. For the same reason the employment of the phenolic extract is to be excluded.

It is known that catechol **(1)** and 4-methylcatechol **(6)** are oxidized by air

TABLE 3. EFFECTS OF OLIVE JUICE (OJ) AND CATECHOL (**1**) ON FECUNDITY OF *Bactrocera oleae*[a]

	Mean Eggs (*N*) per female per day
Sound olives	13.0 a
Infested olives	7.8 b
Sound olives spread with **1**	7.5 b
Sound olives spread with OJ	7.0 b
No olives	6.3 c

[a]Means followed by the same letter are not statistically different ($P = 0.05$).

and light. In addition, our recent observations indicate that hydroxytyrosol **(8)** is responsible for olive drop (Bartolini et al., 1993). We therefore conclude that the VW need to be fractionated to isolate **1** from **6** and **8.** Successively, **1** should be acetylated to obtain **5,** which is much more stable and activated only by contact with *B. oleae*. The deterrent capacity of **5** and its activity against *P. s.* subsp. *savastanoi*, a pathogen of the olive plant (Iacobellis et al., 1985), allows us to propose this substance for possible use in an integrated pest management program. Further experiments are in progress to investigate whether these compounds have any influence on the physiological activities of olive plant.

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DETECTION OF HYPOGEOUS FUNGI BY TASMANIAN BETTONG (*Bettongia gaimardi:* MARSUPIALIA; MACROPODOIDEA)

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Abstract—The ability of Tasmanian bettongs (*Bettongia gaimardi*) to locate hypogeous fungi (their main diet) was tested in a controlled laboratory situation. Bettongs dug directly over buried fungi significantly more often than they did over buried glass marbles or over disturbed soil. This ability was not enhanced as they gained experience. Bettongs dug more often over buried filter paper onto which fungus extract was absorbed than over control papers, and showed no discrimination between the outer and inner layers of the fungi. They preferred the odor of whole fungi to individual volatile compounds. They showed no reaction to the odor of the steroid ergosterol.

Key Words—hypogeous fungus, olfactory, bettong, *Bettongia gaimardi*, rat-kangaroo, *Mesophellia*, Marsupialia.

INTRODUCTION

Many hypogeous fungi form symbiotic mycorrhizal associations with the rootlets of vascular plants forming underground fruiting bodies known as sporocarps or truffles (Trappe, 1962; Maser et al., 1988). Such fungi depend on animals to consume the fruiting bodies and disperse the spores (Kotter and Farentinos, 1984). As spores mature within a sporocarp, the odor of the fungus intensifies and with it the chance of detection by mycophagous mammals (Claus et al., 1981; Maser et al., 1988). This study was undertaken to determine whether the odor of underground fungi is utilized by *Bettongia gaimardi*, the Tasmanian rat-kangaroo or bettong, for fungus location, as has been suggested but not proven

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(Burbidge, 1983; Delroy et al., 1986). The bettong's diet consists almost exclusively of hypogeous fungi (Taylor, 1992). Confirmation of the bettong's ability to locate fungi by odor cues is a necessary precursor to investigations on the semiochemical activity of volatile and nonvolatile compounds present in the fungus. In this study we concentrate on truffles of the genus *Mesophellia*, a particularly favored food of the Tasmanian bettong (C. Johnson, personal communication).

METHODS AND MATERIALS

Fifteen bettongs, born in captivity and naive to hypogeous fungi at the initiation of testing, were used as experimental subjects. They were maintained in outdoor pens, with several individuals in each pen. At all times they were supplied with water, fruit, bread, and cereals, a standard captivity diet for this species. Although there was no departure from a 1 : 1 sex ratio in the group of subjects, no attempt was made to balance the sex ratio in each experimental replicate, following the application of a χ^2 heterogeneity test to the results of a preliminary study. Three bettongs were used in each replicate. Truffles were collected from around the bases of trees of the genus *Eucalyptus* in the southeastern part of Tasmania.

In the first part of the study we investigated whether bettongs use odor for truffle location. Plywood trays measuring 75 × 60 × 10 cm deep, partitioned into 20 cells, each 15 × 15 × 10 cm deep, were filled with dry sandy loam, which was regularly replaced to prevent its contamination by truffle odor. Each of the cells was subjected to one of three treatments. Five glass marbles and five small *Mesophellia* sp. truffles were distributed at random in holes, 2 cm deep, made in the center of each cell. Although it is recorded that *Mesophellia* sp. sometimes occurs at depths in excess of 40 cm (Claridge et al., 1992), our field observations indicated that they frequently occur very close to the surface. Ten cells were left untreated as controls, and all holes were filled in. The digging and filling of holes in the untreated cells ensured that disturbance of the soil was not a variable. Marbles represented a nonfood item of a similar size and shape to truffles, for the purpose of investigating whether bettongs show exploratory digging behavior towards buried nonfood items as has been shown in some other small mammals (Holling, 1958; Howard et al., 1968). The trays were placed in the outdoor enclosures.

Before the trials, the criterion for a "dig" was established as being any excavation that reached a depth greater than 2 cm. Data were recorded by direct observation and a trial ended when five cells experienced a dig. Two trials were performed each evening for five consecutive nights in each of four bettong enclosures. Data were analyzed by χ^2.

In the second part of the study several species of hypogeous fungi were analyzed chemically. A sample of air saturated with volatiles from a fresh fungus was manually injected into a Hewlett Packard 5980 gas chromatograph (GC) attached to a 5970 HP mass selector (MS). The samples were frozen onto an HP5 (polymethyl siloxane) column (film thickness 0.52 μm, ID 0.32 mm, length 25 m). Peaks present in each sample were identified by comparison of their spectra with reference spectra from the NIST mass spectral data base. GC-MS analyses were repeated on two or three samples of truffles.

Sporocarps of *Mesophellia* sp. were also extracted with dichloromethane and analyzed by thin-layer chromatography (TLC) and GC-MS to identify the less volatile truffle compounds. Elution times of the truffle fractions were compared with those of known plant oils and steroids. GC-MS analysis of extracts from several species of fungi involved injection of extracts into the GC-MS equipped with an HP1 column (film thickness 0.17 μm, ID 0.32 mm, length 25 m), using an autosampler. Sporocarp peridia and glebae of *Mesophellia* sp. were extracted separately and analyzed to determine whether the two parts of the truffle contained the same compounds.

In the third part of the study several fractions of *Mesophellia* sp. truffles were individually tested for bioactivity, using the testing procedures outlined above. The fractions were dissolved in vegetable oil and applied to pieces of filter paper that were buried in the cells. Fractions included extract and residue of whole truffles, extracts of peridia and glebae, and the individual compounds identified from *Mesophellia* sp. headspace analyses. The concentration of all compounds was 5×10^{-6} mol in 0.05 ml of vegetable oil solution. This concentration was arbitrarily low, but based on levels of fungal volatiles that have been reported to be stimulatory to insects (Bengtsson et al., 1991) and because natural concentrations of compounds in mixtures of fungal volatiles are reported as "low" (Hutchinson, 1971), but without quantification. Separate trials were run for each component and for the vegetable oil vehicle, using the same experimental testing procedures as before.

RESULTS

Heterogeneity χ^2 tests showed that the results from all trials were homogeneous ($\chi^2 = 4.3$, $P > 0.05$). Digging by bettongs was not random ($\chi^2 = 430.5$, $P \ll 0.005$) with significantly more digs in truffle-containing cells than in either marble-containing cells or untreated cells (Figure 1). There was no significant improvement in the accuracy of bettongs to locate truffles following first exposure (Figure 2, upper trace) or any reduction in the time taken by bettongs to complete a trial following experience (Figure 2, lower trace).

Classes of compounds present in truffle volatile mixtures were simple alco-

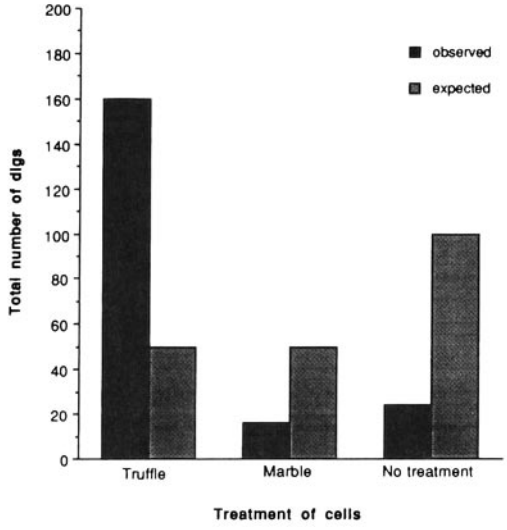

FIG. 1. Effect of buried truffles, buried glass marbles, or no treatment on foraging behavior by bettongs. The first five digs from 40 trials was recorded.

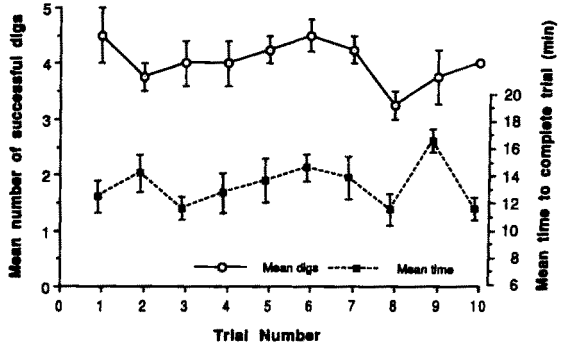

FIG. 2. Effect of experience on the accuracy of bettongs when locating buried truffles (upper trace), and effect of experience on the time taken for bettongs to complete a foraging trial, i.e., completion of five digs (lower trace) (mean ± 1 SE, for four replicates combined).

hols, aldehydes, ketones, alkenes, and esters. No single compound or group of compounds identified from the chromatographs was common to all species of hypogeous fungi tested. In all chromatographs, many minor components were present in concentrations too low for accurate mass spectral identification. The

GC-MS analyses of *Mesophellia* sp. volatiles revealed many compounds; most of the identifiable ones were esters. Both peridia and glebae of fresh *Mesophellia* sp. truffles contained the same volatile compounds. TLC and GC-MS analyses of truffle involatile extracts revealed that all extracts of both peridia and glebae consist of long-chain fatty acids (C_{16}–C_{24}) and the fungal steroid ergosterol.

χ^2 analysis of the data of bioactivity of truffle fractions revealed that bettongs significantly preferred cells that contained any truffle extract to cells that contained only soil ($\chi^2 = 12.8$, $P < 0.005$). Bettongs did not show a significant discrimination between extracts of peridia and glebae ($\chi^2 = 0.0$, $P > 0.05$). Bettongs were significantly attracted to the seven compounds (acetaldehyde, ethyl acetate, *n*-propyl acetate, isobutyl acetate, ethyl isobutanoate, ethyl butanoate, and ethyl propanoate) that dominate the *Mesophellia* sp. headspace chromatograph ($0.025 < P < 0.05$ for ethyl propanoate and $P < 0.005$ for the others). However, bettongs preferred the odor of the entire truffle to the odors of any of the individual compounds ($0.005 < P < 0.01$ for ethyl acetate and ethyl butanoate and $P < 0.005$ for the others). Bettongs did not respond to the separated components of truffle involatile compound extract (long-chain fatty acids and ergosterol) ($\chi^2 = 2.56$, $P > 0.05$ for both cases).

DISCUSSION

The observations reported here suggest that bettongs dig much more frequently in cells that contain truffles or pieces of filter paper onto which truffle volatile compounds have been applied than in cells that contain marbles or nothing other than soil. This suggests that bettongs respond to olfactory cues emanating from the fungi. We further suggest that bettongs do not need to learn the characteristics of truffle odor, since naive subjects were able to find hidden truffles as readily as experienced subjects. This conclusion is further supported by the observation that bettongs show no reduction in the time taken to locate truffles as they become more familiar with truffle odor. When truffles were located, they were eaten with great speed and apparent relish equally by experienced and inexperienced subjects. In this respect, bettongs differ from small rodents that have been reported to have to learn the characteristics of novel food items and exploit them with increasing efficiency (Holling, 1958). This is an interesting observation that requires further study.

Our observations reveal that while bettongs are significantly attracted to a range of volatile compounds found in truffles, they were more strongly attracted to the odor of whole truffles than to a single compound. This suggests that the cue that triggers digging behavior is chemically complex. Recent studies on European black truffles (*Tuber melanosporum*), however, have indicated that dimethyl sulfide is the compound that attracts not only dogs and pigs, but also

mycetophilous insects (Talou et al., 1990; Pacioni et al., 1991). In the experiments of Talou et al. (1990), one experienced pig and four dogs trained to truffle odor were used in a field-based series of location trials. Only dimethyl sulfide was detected on every occasion by both the pig and the dogs. It has previously been reported that pigs are attracted to a steroidal compound, 3α-hydroxy-5α-androst-16-ene, present in European truffle odor and also found as a sex pheromone in male pig saliva (Claus et al., 1981). Talou et al. (1990) found that, when presented alone, their test pig could not locate the buried source of this odor with the same accuracy and repeatability as it could locate either buried whole truffle odor or methyl disulfide alone. In our study with the Tasmanian bettong, we found no response to the odor of buried ergosterol, a steroid compound identified in *Mesophellia*, even though this compound has a strong odor to the human nose.

Tasmanian bettongs occur in dry forested areas characterized by poor quality soils, where the availability of plant and invertebrate food is generally low (Taylor, 1992). In such forests even epigeous fungi are uncommon. The detection of hypogeous fungi is therefore critically important for survival in this type of environment. A number of other species of rat-kangaroos also feed on hypogeous fungi (e.g., brush-tailed bettong, *B. penicillata*; rufous bettong, *Aepyprymnus rufescens*; long-nosed potoroo, *Potorous tridactylus*; and long-footed potoroo, *P. longipes*), but whether these species utilize odors in the same way as we have shown the Tasmanian bettong to do is not known. As all rat-kangaroos are endangered or vulnerable (Ride and Wilson, 1982), further research into this important aspect of bettong feeding ecology is clearly needed.

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ALARM PHEROMONE SYSTEM OF LEAF-FOOTED BUG *Leptoglossus zonatus* (Heteroptera: Coreidae)

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Abstract—The alarm pheromone system of *Leptoglossus zonatus* (Dallas) adults was shown to be composed of hexyl acetate, hexanol, hexanal, and hexanoic acid. Single components tested in the field elicited dispersive behavior of over 70% of adults. 2-(*E*)-Hexenal, found in the secretion of nymphs, but not in the exudates of adults, was also active against adults. In addition, first-instar nymphs responded to the four components of the alarm pheromone of adults as well as to 2-(*E*)-hexenal, a component of their own alarm pheromone system. Adults and nymphs possess different alarm pheromone systems, which are not specific to their own life stage. That hemipteran alarm pheromone systems are not species-specific was supported by the fact that both adult and nymph *L. zonatus* responded to butanoic acid, an alarm pheromone of Alydidae, which was not found in this Coreidae species.

Key Words—*Leptoglossus zonatus*, hexyl acetate, hexanol, hexanal, hexanoic acid, 2-(*E*)-hexenal, 4-oxo-hex-2-en-1-al, alarm pheromone, Heteroptera, Coreidae.

INTRODUCTION

In many parts of the world, leaf-footed bugs (Coreidae) are important agricultural pests. In California, for example, *Leptoglossus clypealis* and *L. occidentalis* are the most important bugs implicated in the pistachio epicarp lesion,

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causing considerable crop losses. *L. zonatus* (Dallas) is reported as a pest of corn in Brazil (Panizzi, 1989) and may feed on several other economically important plants, such as cotton, citrus, peach, sorghum, and watermelon (Allen, 1969; Solomon and Froeschner, 1981; Schaefer and Mitchell, 1983).

The conspicuous sexual activity of *L. zonatus* observed in corn fields in Londrina, Paraná, Brazil (latitude 23°11'S, longitude 51°11'W) prompted us to investigate its sexual behavior, with emphasis on the chemical cues involved in communication. However, as with other hemipterans (Leal and Kadozawa, 1992a; Aldrich et al., 1987), the presence of large amounts of defensive secretions in the extracts makes the task of isolating sex or aggregation pheromones a difficult challenge. Volatiles (either airborne or whole-body extract) collected from insects possessing sex, aggregation, and alarm pheromones would not elicit attraction. This fact is substantiated by the case of the bean bug, *Riptortus clavatus* (Alydidae), which possesses an aggregation pheromone (Leal et al., 1993) whose active chemicals were masked by an alarm pheromone (Leal and Kadozawa, 1992b). In order to overcome this obstacle, the alarm pheromone system was studied first, before the final goal of identifying the chemicals involved in the aggregation behavior of the insect could be achieved.

In this first report on the chemical ecology of *L. zonatus*, we describe the alarm pheromone system of adults, which was demonstrated in the field to elicit dispersive behavior of adults, even those engaged in copulatory behavior. Aggregated nymphs, although possessing a remarkably different secretion, also responded to the alarm pheromone of adults.

METHODS AND MATERIALS

Insects. Leptoglossus zonatus adults were collected in corn fields in the Londrina area (EMBRAPA/CNPSo Field Experiment Station) and kept in cages (0.5 × 0.5 × 0.5 m) in the laboratory. Corn ears were used as food and potted soybean plants were introduced in the cages to host the insects. Egg masses obtained were placed in an environmental chamber (25 ± 1°C, 60 ± 5% relative humidity, and 14 : 10 hr light–dark photoperiod). Early instar nymphs to be used in bioassays were kept in Petri dishes, and remaining nymphs were returned to the rearing cages.

Extraction. Defensive secretions were extracted from eight groups (four to five individuals per group) of laboratory-raised male and female insects (8, 11, or 14 days old) and from two groups of field-collected males and females (unknown age). The insects were immersed in hexane for 3 min, the extract was filtered while transferring to 5-ml ampules, and these were ice-cooled, sealed, and shipped to Japan, where the samples were chemically analyzed.

Chemical Analysis. GC analysis was performed in a Hewlett-Packard 5890

gas chromatograph either in splitless (injector 210°C) or cold on column injection mode. The column, either HP-1 (12 m or 25 m × 0.2 mm; 0.33 μm) or DB-Wax (30 m × 0.25 mm; 0.25 μm), was operated at 50°C (or 40°C) for 1 min, programmed at 4°C/min to 180°C, held at this temperature for 1 min, programmed again at 10°C/min to 210°C, and held at this temperature for 20 min [50(1)-180(1)/4-230(20)/10 or 40(1)-180(1)/4-230(20)/10]. Gas chromatography-mass spectrometry (GC-MS) analyses were done on a Hewlett-Packard 5891 mass selective detector, EI mode at 70 eV, splitless injection, and equipped with a DB-wax column (as in GC) operated at 50(1)-180(1)/4-230(20)/10.

Bioassay. Alarm pheromonal activity was investigated both in the lab and in a corn field at the EMBRAPA/CNPSo, Brazil. Samples were prepared by loading Pasteur pipets with strips of filter paper containing the candidate chemicals. One microliter of a 10 μg/μl solution was loaded on the filter paper and the solvent was evaporated for 30 sec; the control was prepared in a similar manner by loading the filter paper with solvent (hexane) only. Silicone bulbs (3 ml) were fixed on the pipets and the narrow tips were brought close (2–3 cm) to the test insects. For each test, air inside the control pipet was puffed three times on the body of the insects, then experiments were identically performed with pipets containing the samples. Recovery tests were done in the laboratory by puffing air in the same way out of sample pipets on small pieces of glass wool, containing 1 μg of an internal standard, hexyl isovalerate. The glass wool was washed with 100 μl of hexane, the extract concentrated, and the recovery rate was quantified by GC and GC-MS.

Statistical Analysis. Data comparing dispersive behavior of bugs elicited by sample and control were analyzed by a contingency table using JMP software (version 2) (Anonymous, 1989).

RESULTS AND DISCUSSION

GC and GC-MS analyses revealed that whole-body extracts of *L. zonatus* adults contained mainly hexanal, hexanol, hexyl acetate, and hexanoic acid. Except for hexanoic acid, these chemicals were previously found in the secretion of two other species, *L. oppositus* and *L. clypealis* (Aldrich and Yonke, 1975). The proportions of the four constituents varied from sample to sample, but hexyl acetate (27.1 ± 19.0 μg/bug) and hexanal (22.2 ± 20.1 μg/bug) were the major components. Only very small amounts of hexanoic acid were found in some samples (Figure 1), but it was the major component of others (average 9.3 ± 11.2 μg/bug), whereas hexanol (1.8 ± 1.3 μg/bug) was a minor component. These chemicals are secreted by the metathoracic scent gland, whose glandular cells are segregated from the reservoir wall into primary and secondary accessory glands. Variations in the proportions are due in part to compartmentalized bio-

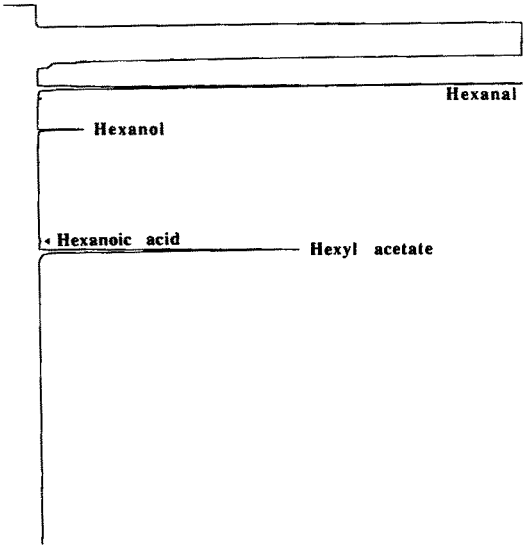

FIG. 1. Gas chromatogram of whole-body extract of *L. zonatus* adults obtained on a 12-m HP-1 capillary column operated at 40(1)-180(1)/4-230(20)/10. (Retention times: hexanal, 2.58 min; hexanol, 3.82 min; hexanoic acid, 7.24 min; hexyl acetate, 7.59 min).

synthesis. Hexyl acetate is secreted by the primary accessory glands into the reservoir, where esterase and dehydrogenase enzymes, apparently secreted by the secondary accessory glands, cleave the ester and oxidize the resulting alcohol (Aldrich, 1988). Nevertheless, it is not clear why insects of the same sex and nearly the same age possessed the four components in such different proportions.

In order to quantify the chemical stimuli during the bioassays, we investigated the amount of chemicals released during puffs from Pasteur pipets. Although the recovery rate changed from chemical to chemical, it was very low for all compounds (0.1–1%). Small recovery rates were also found by Todd and Baker (1993) in similar experiments. We found that for hexyl acetate, for example, out of 10 μg (ca. 0.4 individual-equivalent) loaded on a strip of filter paper, only 0.5% (50 ng on average) was effectively blown out of the pipet in one puff. The amount of alarm pheromone released by a bug in response to an environmental hazard is not accurately known, but it is certainly higher than the effective amount released in one puff under our experimental conditions (pipet loaded with 10 μg) and probably much less than the amount obtained by whole-body extraction.

Preliminary bioassays in the field showed that adults dispersed in response

to a mixture of hexyl acetate and hexanoic acid. Of 31 individuals tested, only one responded to puffs from the control, whereas 24 responded to puffs of the synthetic mixture. Over 50% of the bugs responded by flying away, and those engaged in copulatory behavior stopped mating and dispersed. Although only few nymphs of third, fourth and fifth instars were found in the field during the tests (January 19–21, 1993), their response was very clear: 0% to the control and 75–100% to the synthetic mixture.

The role of single components on the dispersive behavior of adults was further investigated in the field with synthetic chemicals. The test compounds were the metathoracic gland constituents: hexyl acetate, hexanoic acid, hexanal, and hexanol; 2-(*E*)-hexenal, a common alarm pheromone of stink bugs (Leal and Kadosawa, 1992a and references therein; Lockwood and Story, 1987; Kou et al., 1989), and butyric acid, a constituent of Alydidae species (Aldrich and Yonke, 1975; Leal and Panizzi, unpublished).

The responses to *L. zonatus* exudates (hexyl acetate, hexanoic acid, hexanal, and hexanol) were the strongest (>70%), whereas butyric acid gave the poorest response (<40%). 2-(*E*)-Hexenal also elicited a response in adults, even though this chemical was not found in the secretion of adults (Figure 2), but only in nymph exudates (see below).

It has been demonstrated by means of EAG measurements that a minimum chain length of six carbons and a terminal carbonyl group were required for optimal alarm pheromonal activity of the bedbug *Cimex lectularius*; however, a Δ^2-double bond was not essential (Levinson et al., 1974). Conversely, it can be speculated that 2-(*E*)-hexenal mimics hexanal, one of the alarm pheromone constituents of *L. zonatus* adults. Similarly, the response of *L. zonatus* elicited

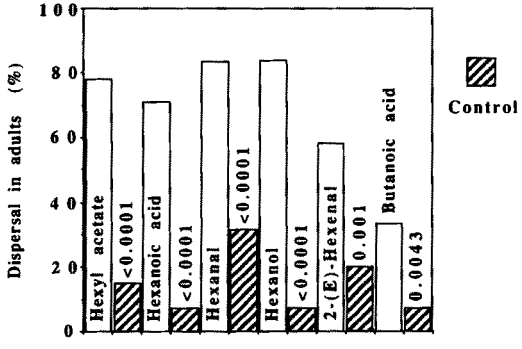

FIG. 2. Dispersal of *L. zonatus* adults ($N = 30$) in a corn field elicited by single components of the alarm pheromone system and related compounds. The results of Pearson chi-square tests ($P > \chi^2$) for each pair (sample × control) are given on the top of the control bars.

by butanoic acid may be explained by its similarity to hexanoic acid. Therefore, alarm pheromones of hemipterans are of little species specificity.

A prerequisite for the evolution of alarm pheromones is the evolution of group living (Nault and Phelan, 1984). Aggregations of *L. zonatus* adults are considerably more dispersed than those of first-instar nymphs. Nevertheless, the secretion of the former causes conspecific adults to disperse. As pointed out by Blum (1985), the evolution of chemical releasers of alarm behavior appears to have occurred in arthropods belonging to disparate taxa that, during at least one life stage, form groups in which the individuals are near enough to each other to rapidly communicate.

The alarm pheromone of adults and related compounds tested in the lab on first-instar nymphs gathered on Petri dish covers showed that dispersal due to the puffs of control was less pronounced in nymphs than in adults (Figure 3). 2-(*E*)-Hexenal elicited the greatest dispersal, only rivaled by hexanal. In fact, 2-(*E*)-hexenal was found in whole-body extracts of all nymph stages along with 4-oxo-hex-2-enal. These compounds were previously found in the secretions of *L. opposítus* and *L. clypealis* (Aldrich and Yonke, 1975).

The fact that, as a general rule, the exocrine chemistry of heteropteran nymphs is distinct from that of the adults (Aldrich and Yonke, 1975) suggests the existence of different alarm pheromone and defensive systems in adults and nymphs of true bugs. Our findings support this hypothesis in the case of the alarm pheromone systems of *L. zonatus* adults and nymphs. Similarly, nymphs of the bean bug, *R. clavatus*, responded to the alarm pheromone of adults, 2-(*E*)-hexenyl hexanoate (Leal and Kadozawa, 1992b), a chemical that was not found in the secretion of any nymphal stage (Leal, unpublished). Interestingly,

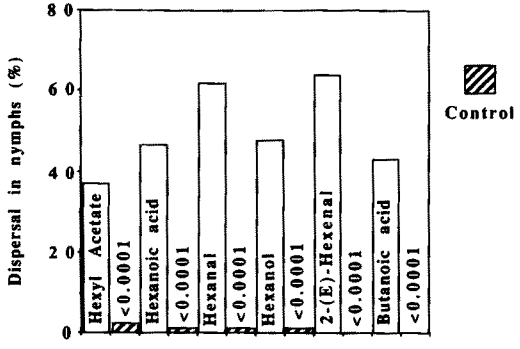

FIG. 3. Dispersal of first-instar nymphs of *L. zonatus* ($N = 44$) in a laboratory bioassay, elicited by the same chemicals tested against adult insects. The figures on the top of control bars are the $P > \chi^2$ according to the Pearson χ^2 tests applied to each pair (sample × control).

fifth-instar nymphs, found to be the most gregarious stage of the bean bug in the field (H. Higuchi, personal communication), displayed the strongest response to 2-(*E*)-hexenyl hexanoate (Leal and Kadozawa, unpublished).

Acknowledgments—Part of this work was carried out during a visiting professorship awarded to W.S.L. by Comissão de Aperfeiçoamento da Pesquisa e Ensino Superior (CAPES), Ministry of Education (Brazil). We are also indebted to Kohji Yamamura (National Institute of Agroenvironmental Sciences) for discussion on statistical applications as well as to Dr. Pierre Escoubas (Suntory Institute for Bioorganic Research) for a critical review of an earlier manuscript.

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Journal of Chemical Ecology, Vol. 20, No. 5, 1994

Book Review

Chemical Signals in Vertebrates VI. Richard L. Doty and Dietland Müller-Schwarze (eds.).
New York: Plenum Press, 1992. 637 pp.

This volume is the sixth in a series of updates on chemical signaling in vertebrates. It's the best yet, discussing current experimental findings and extending the area of investigation to new levels of excellence. The bibliographies are extensive, the 90 presentations are comprehensible, for the most part, and most of the papers are mercifully short. The volume deserves a place on the bookshelf of any investigator interested in chemocommunication.

The research and ideas reported in this volume were described at the Chemical Signals in Vertebrates VI symposium held at the University of Pennsylvania in the summer of 1991. The gathering of scientists was the largest of all of the symposia associated with the six volumes: 200 investigators from 22 countries participated. With a few exceptions, it's a virtual "Who's Who" and "What's What" in the area. Parenthetically, the organizing of the symposium and the publication of this volume was sponsored by several governmental agencies and private sources, reflecting the growing importance of chemocommunication in the understanding of behavior and the ability of diverse groups to pool resources for a worthy venture.

The 637-page volume is structured in seven major segments, reflecting vertical progression from the molecules of olfaction and taste to recent and provocative work with humans. Here they are: (1) anatomy and physiology, (2) chemistry and histology, (3) development of structure and function, (4) semiochemicals and the major histocompatibility complex (MHC), (5) signals and endocrine processes, (6) chemical repellents, and (7) behavior and social communication.

Within this structure are significant advances that anticipate trends for the future. One of the most provocative is the work on the MHC loci, with their relations to individual identification and mate selection. Who would have thought just a few years ago that the immune complex associated with recognition of "self" and "nonself" would also specify olfactory qualities of organisms that are used for individual recognition and differential genetic choice? The connection is almost too parsimonious to believe. This excellent work, originating with R.E. Brown, K. Yamazaki, G.K. Beachamp, and their associates late in the last decade, is expanded here. It now appears that the MHC class I molecules found

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in the urine and other bodily fluids are responsible for variations in odor signals. Amazingly, the molecules themselves do not convey odor differences; instead they seem to bind with bacterial volatiles in bodily fluids, transporting them to the urine where they are released into the environment. Thus, there are symbiotic relations between immunologic variation at the gene level, bacterial production of volatiles, and the transport of effective signals to release sites. The hypotheses surrounding these ideas are not free of problems, as with unaccountable differences between rats and mice, little understood effects of diet, and the presence of chemical identification cues not related to the MHC. Nevertheless, the hypotheses are new and daring. At least we can now begin to talk about "molecules of adaptive behavior."

At the behavioral level, ecological features that control communication systems assume more importance. Debora Cantoni and Laurent River show that three shrew species, *Crocidura russula*, *Sorex coronatus*, and *Neomys fodiens*, differ both in the frequency of social interactions during the breeding season and, in correlation, with the chemical complexity of their flank-marking gland secretions. Apparently, the evolution of complex social behaviors dictates an equally complex chemical communication system. In this same vein, Wolfgang and Roswitha Wiltschko are doing precise behavioral work with carrier pigeons, showing regional variations in homing orientation and in the effects of anosmia. Experiments like these are leading the way to the understanding of important relations between behavior, genes, environment, evolution, and physiology. Incidentally, this volume also shows an increasing awareness of how an understanding of "chemosensory ecology" can contribute to species and environmental conservation.

At the risk of slighting a great deal of superb anatomical, chemical (including hormonal), and behavioral work, I would like to highlight the recent work with humans described in this volume. Many of us believe that olfactory and gustatory processes are experimentally unmanageable at the human level and can not possibly contribute to the understanding of fundamental mechanisms, but even here, the work is becoming systematized and interpretable. Richard Frye, Richard Doty, and Paul Shaman, for example, demonstrate in careful experiments that there are olfactory differences in sensitivity for the right and left sides of the nose. A rhythm of airflow that switches back and forth between the two nostrils of the nose (test yourself) correlates with changes in physiology and olfactory sensitivity. Left airflow is related to parasympathetic dominance, greater right hemispheric integrated EEG activity, and decreased olfactory sensitivity. Conversely, right airflow is associated with sympathetic dominance, greater left hemisphere integrated EEG, and heightened olfactory sensitivity. Laterality seen at the periphery obviously extends into the deepest motor, perceptual, and cognitive regions of the brain. Also important among the human studies are the reports of odor-evoked brain potentials, odor effects on topo-

graphical EEG patterns, and variations in social preference associated with the MHC loci.

There seem to be changes from earlier volumes in reported attempts to identify specific chemosignals for regulating adaptive responses and in the view that the vomeronasal organ is a mediator of sexual pheromones. Fewer studies are aimed at isolating and identifying species-specific pheromones. The invertebrate model simply does not apply, pulling investigators toward a more detailed look at developmental imprinting of sensory information and the importance of learning in general. Most chemosignals in vertebrates are linked to the environment, their import depending in part on experiential events. Similarly, the vomeronasal organ may not be a specific pheromone receptor in higher vertebrates, opening up challenges about environmental control that many of us thought were closing. The question is still, what does this strange organ do?

The major deficiency of this volume is actually a deficiency of the area of vertebrate chemocommunication. Theoretical advances have not kept pace with empirical investigations. Exciting things are happening, but the big ideas are not there, and existing theory seems archaic. Evolutionary theory is changing rapidly, now including regulatory gene effects, punctuational events, allometric functions, and self-organizational processes. This "revolution of evolution" is not reflected in the area of chemocommunication. Similarly, "life-history" events so important in survival and reproduction, such as number, size, and sex ratio of offspring and age- and size-specific reproductive investments, are not taken into account. Sociobiology hardly has a place at all. One gets the feeling that theory may be reaching deeper into the genome, as with the MHC advances, but not upward toward integration. This may not be a continuing deficiency, as the data base is firming up, allowing for more integrating hypotheses involving genes, development, physiology, evolution, and ecology. Perhaps Volume VII of *Chemical Signals in Vertebrates* will add a new section on theory and chemicals. In any case, we can applaud this volume and look forward to the next.

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Journal of Chemical Ecology, Vol. 20, No. 6, 1994

ANNOUNCEMENT

We take much pleasure in our association with the *Journal of Chemical Ecology* and the International Society of Chemical Ecology over the past years. We also realize that editors are not forever, and we now announce our retirements as Editors of the journal. We do so in very good spirits because both the journal (20 years) and the Society (12 years) have helped define the new field of Chemical Ecology, thus ensuring its great potential.

Even more gratifying is the willingness of two highly qualified individuals to accept the responsibility as Editors: James L. Nation and David A. Jones, both of the University of Florida, Gainesville. We wish them many years of enjoyment.

The new Editors will quickly learn, as we have, that they will be indebted to the Editorial Board; to many additional reviewers; to the contributors who willingly accept the vicissitudes of peer review, patiently revise their manuscripts, and sometimes even thank the reviewers; and to the publisher. We wish to express special thanks to Eleanor Zaccaria, our secretary who held everything together during our stewardship.

To assure uninterrupted publication, **manuscripts submitted after June 30, 1994 should be addressed to the new Editors** (see the back inside cover for exact mailing address). We shall continue to process manuscripts scheduled for publication in 1994 and to prepare the author and key word indices for Volume 20 (1994).

R. M. Silverstein and J. B. Simeone

Journal of Chemical Ecology, Vol. 20, No. 6, 1994

Editor's Note

This issue of the Journal of Chemical Ecology contains papers from a symposium on *Chemical Ecology of Terpenoids* presented at the 19th Annual Meeting of the International Society of Chemical Ecology (ISCE) hosted by John C. Romeo at Clearwater Beach, Florida, July 31–August 4, 1993. An ISCE symposium especially directed toward terpenoids seemed appropriate as they are by far the largest and most diverse group of plant allelochemicals, and hence mediate numerous ecological interactions. The chemical significance of these compounds was recognized in an international symposium entitled *Ecological Chemistry and Biochemistry of Plant Terpenoids* held by the Phytochemical Society of Europe in Murcia, Spain, in September 1989. By comparison, the ISCE symposium focuses on higher plant terpenoids in terrestrial ecosystems and approaches these compounds in a more broadly based ecological perspective than the symposium held by the Phytochemical Society of Europe. Provocative issues mark all of the presentations in the ISCE symposium, with syntheses of previous ideas on the topics, generally accompanied by new data. Langenheim presents "Higher Plant Terpenoids: A Phytocentric Overview of Their Ecological Roles" in which she stresses the ecological importance of qualitative and quantitative variation of terpenoid mixtures and integrates the numerous roles of these terpenoids as contributing factors in determining some properties of terrestrial plant communities and ecosystems. Gershenzon discusses cost—a perennial thorny problem for all evolutionary and ecological considerations—specifically addressing "Metabolic Costs of Terpenoid Accumulation in Higher Plants," including very recent evidence that is accumulating to aid our understanding. He also relates the biochemical evidence to assumptions and predictions of three current chemical defense theories. Takabayashi et al. summarize some of their viewpoints in "Volatile Herbivore-Induced Terpenoids in Plant–Mite Interactions: Variation caused by Biotic and Abiotic Factors." Fischer et al. present a progress report, "In Search for Allelopathy in the Florida Scrub: The Role of Terpenoids," in which they address in this specific example the chemical mechanisms of terpenoid action as well as potential ecological effects at the community level. Finally, White discusses "Monoterpenes: Their Effects on Nutrient Cycling," focusing on the nitrogen cycle but making comparisons with the carbon cycle, thus projecting thinking toward impacts of terpenoids on ecosystem properties that have not been generally considered.

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Each of the authors acknowledges assistance with various parts of their presentations, including reviewers. However, here we would like to further express our appreciation for the helpful comments of the following reviewers: David Lincoln, Bess Ward, S. W. Whitman, U. Blum, and Jeff Weidenhamer.

Jean H. Langenheim
Symposium Chair and proceedings coeditor
R.M. Silverstein
John B. Simeone
Editors, Journal of Chemical Ecology

Journal of Chemical Ecology, Vol. 20, No. 6, 1994

HIGHER PLANT TERPENOIDS: A PHYTOCENTRIC OVERVIEW OF THEIR ECOLOGICAL ROLES

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(Received October 12, 1993; accepted February 2, 1994)

Abstract—Characteristics of higher plant terpenoids that result in mediation of numerous kinds of ecological interactions are discussed as a framework for this Symposium on Chemical Ecology of Terpenoids. However, the role of terpenoid mixtures, either constitutive or induced, their intraspecific qualitative and quantitative compositional variation, and their dosage-dependent effects are emphasized in subsequent discussions. It is suggested that little previous attention to these characteristics may have contributed to terpenoids having been misrepresented in some chemical defense theories. Selected phytocentric examples of terpenoid interactions are presented: (1) defense against generalist and specialist insect and mammalian herbivores, (2) defense against insect-vectored fungi and potentially pathogenic endophytic fungi, (3) attraction of entomophages and pollinators, (4) allelopathic effects that inhibit seed germination and soil bacteria, and (5) interaction with reactive troposphere gases. The results are integrated by discussing how these terpenoids may be contributing factors in determining some properties of terrestrial plant communities and ecosystems. A terrestrial phytocentric approach is necessitated due to the magnitude and scope of terpenoid interactions. This presentation has a more broadly based ecological perspective than the several excellent recent reviews of the ecological chemistry of terpenoids.

Key Words—Higher plant terpenoids, quantitative variation of mixtures, plant defense, plant pollination, allelopathy, tritrophic interactions.

INTRODUCTION

Increasing recognition of the ecological importance of terpenoids is reflected by two recent excellent reviews of the ecological chemistry of terpenoids (Harborne, 1991a; Gershenzon and Croteau, 1991). However, I shall view these compounds with a more broadly based ecological perspective than these two

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reviews and will, in addition, set the framework for the remainder of this International Society of Chemical Ecology symposium issue. Although all plants synthesize terpenoids necessary for growth and development, such as photosynthetic pigments, growth regulators and steroids, I shall stress how particular characteristics of a very large number of other terpenes produced by individual higher plants result in these compounds mediating interactions at different levels of ecological organization, i.e., within plant populations, terrestrial plant communities, and ecosystems. Some of these community and ecosystem relationships have been implicit in various studies, but generally the roles of terpenoids as contributing factors in determining some properties of terrestrial plant communities and ecosystems have not been made explicit and have not been presented in an integrated fashion. My terrestrial phytocentric perspective is obviously artificial, but some restriction is also obviously necessary for this presentation due to the magnitude and scope of terpenoid interactions. Even within a phytocentric framework, discussion will again necessarily be limited to selected examples.

ECOLOGICALLY RELEVANT CHARACTERISTICS OF HIGHER PLANT TERPENOIDS

First, in such an overview it seems helpful to think about the many ecologically relevant characteristics of higher plant terpenoids. Sixteen characteristics will be discussed sequentially as presented in a summary table (Table 1); however, a few will be emphasized (and elaborated upon in later sections) that have received little attention, showing how, as a result, terpenoids have perhaps been misrepresented in some chemical defense theories. These characteristics are also significant in numerous roles other than plant defense and will again receive some focus in later discussion of various interactions.

Chemical Characteristics (Table 1)

Biosynthesis. Terpenoids are the largest group of plant chemicals (15,000–20,000 currently fully characterized), with a common biosynthetic origin in mevalonate (Figure 1). Of the major biosynthetic reaction sequences involved in secondary metabolism, the terpenoid pathway is perhaps best suited to generate the incredible structural diversity and complexity characterizing these compounds (Gershenzon and Croteau, 1990; Gershenzon, 1994). The polymeric assembly of the basic five-carbon intermediates allows for formation of compounds with a different base number of carbon atoms. There is the potential for the terpenoid skeleton to be further modified, for example, with different oxygen functions and different conjugating moieties and cyclizations. Recent evidence shows the importance of further complexity contributed by stereoisomers (Harborne, 1991a). Additionally, terpenoids may contribute carbon skeletons to other

TABLE 1. ECOLOGICALLY RELEVANT CHARACTERISTICS OF HIGHER PLANT TERPENOIDS

A. Chemical characteristics
   1. Largest group of plant chemicals; have common biosynthetic origin.
   2. Terpenoid pathway perhaps best suited to generate great structural diversity and complexity of compounds, thus generating enormous potential for mediating ecological interactions.
   3. Although some of the most expensive compounds to synthesize per gram, some of them in the mixture may be recycled during senescence into primary metabolism, the remainder being emitted into the environment and in some cases may have community/ecosystem functions.
   4. Synthesized in specialized secretory structures protecting primary metabolic processes, but in areas of plant where most effective ecologically.
   5. Broadly lipophilic, but considerable water solubility demonstrated for some oxygenated monoterpenoids in addition to others that occur as glycosides.
   6. Some compounds volatile, in varying degrees, and others nonvolatile.

B. Characteristics relating to effects on other organisms
   *7. Occur in *mixtures* (constitutive or induced) either within five-carbon classes or among them.
   *8. Qualitative and quantitative variation in compositional profile of mixture (intraplant, intra- and interpopulation and interspecies) that is significant ecologically.
   *9. Individual constituents of mixture can have multiple ecological effects, thus having wider ecological impact.
   10. Although some compounds show apparently broad-spectrum effects, these effects are probably selective at specific dosages on different organisms within different communities.
   *11. Effects of individual components, combined components, or total quantity are concentration or dosage dependent.
   12. Variety of mechanisms producing toxic, deterrent or inhibitory effects.

C. Characteristics relating to their occurrence
   13. Terpenoids acting as allelochemicals are widely distributed throughout higher plants; some terpenoid classes characterize certain plant families whose occurrence may be characterized by specific environments.
   14. Plants with certain mixtures of terpenoid classes (e.g., essential oils and resins) are characteristic of certain climatic and vegetation types.
   15. Some resin mixtures fossilize, providing a paleobotanical record of chemical lineages and nature of some past forests.
   16. Occur in herbs, shrubs and trees that occupy all stages of succession, from early to late, hence have different degrees of apparency.

*Emphasized in later discussion.

kinds of allelochemicals, e.g., a large and diverse group of alkaloids. Thus, both the number and structural diversity of terpenoids provide enormous potential for mediating significant ecological interactions. Although terpenoids are the most expensive secondary compounds to synthesize per gram in leaves, some of them in the mixture in which they occur may be recycled into primary compounds during late stages of leaf development (Gershenzon, 1993). The remainder are ultimately emitted into the environment, either through volatilization, leaching, or decomposition of plant debris as it falls to the soil. Some of these

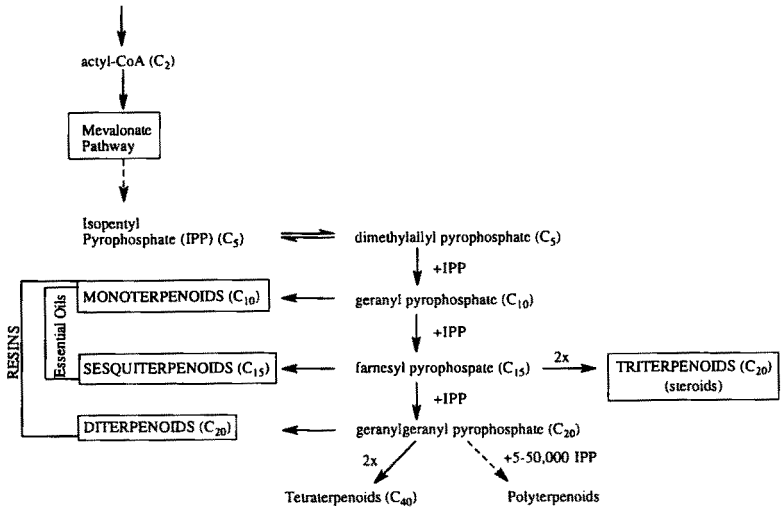

FIG. 1. Outline of terpenoid biosynthesis showing difference in components of resins and essential oils. Boxed classes of terpenoids are discussed in text.

compounds may thus occur in sufficient concentrations in plant litter to have an effect on community/ecosystem properties.

The accumulation in higher plants of terpenoids that subsequently can play numerous ecological roles is generally thought to be constrained by the metabolic cost of these compounds. Therefore, Gershenzon discusses the metabolic costs of the accumulation of higher plant terpenoids in this issue in the light of recent advances in terpenoid biochemistry, emphasizing the importance of not only considering costs in biosynthesis but those involved in storage and maintenance of these compounds. He also discusses how these results relate to several current chemical defense hypotheses, including those regarding the balance of carbon/nutrients (Bryant et al., 1983), resource availability (Coley et al., 1985) and growth differentiation (Herms and Mattson, 1992).

Amid a bewildering array of structures, the sequential combining of basic five-carbon units allows us to recognize the familiar categories of C_{10} (mono-), C_{15} (sesqui-), C_{20} (di-), C_{30} (tri-), C_{40} (tetra-) and $C > 40$ (poly-) terpenoids (Figure 1). Terpenoids characteristically occur in plants as mixtures of compounds *within* each five-carbon class as well as *among* different ones. The C_{10} and C_{15} compounds are often referred to as "lower terpenoids"; when they occur together, they are commonly called "essential oils." On the other hand, all compounds containing 20 carbons and above are often referred to as "higher terpenoids". Although the term "resin" is used variously in the literature, here "resin" will refer to a combination of nonvolatile terpenoids (either C_{20} diter-

penoids or C_{30} triterpenoids) and volatile terpenoids (C_{10} monoterpenoids and/or C_{15} sesquiterpenoids) (Langenheim, 1990). My examples of ecological interactions will center on mono- and sesquiterpenoids; di- and some triterpenoids will be discussed, although numerous outstanding examples will have to be omitted. Fortunately, both Harborne (1991a) and Gershenzon and Croteau (1991) provide numerous examples of interactions of these two classes of terpenoids. The tetra- and polyterpenoids will not be discussed, as there is little evidence yet for their ecological roles.

Terpenoids are synthesized in various cellular organelles, but are then stored in specialized secretory structures, thus protecting the plant's metabolic processes from their toxic effects. These structures are generally located in areas where they would most likely be effective in defense of various organs—e.g., trichomes on the surface of leaves, resin ducts and laticifers throughout tissues of trees, pockets near the epidermis of primary stems, in fruit, etc. (Gershenzon and Croteau, 1991).

Chemical Properties. Although terpenoids have generally been characterized as having broadly lipophilic properties (Harborne, 1991a,b), recent work has demonstrated that we need to consider this conclusion more carefully. For example, Fischer (1991) and Weidenhamer et al. (1993) have found that some oxygenated volatile monoterpenoids (previously thought to have only lipophilic properties) are water soluble at biologically active concentrations, the ecological implications of which are discussed by Fischer et al. (1994). These water-soluble monoterpenoids are, of course, in addition to those terpenoids that occur as glycosides. For example, the monoterpenoid iridoids and triterpenoids, such as cardenolides and saponins, have glucoside attachments.

Some terpenoids, such as mono- and sesquiterpenes, are volatile, whereas others, such as di- and triterpenes, generally are not. In fact, the monoterpenoids are noteworthy for their high degree of volatility, and how volatile compounds can significantly influence community/ecosystem properties will be discussed in some detail in later sections.

Characteristics Relating to Effects on Other Organisms (Table 1)

Throughout this discussion, I will emphasize the importance of terpenoid mixtures and their quantitative variation. For example, usually 30–40 terpenoids of at least 1% concentration compose the mixture in leaves of essential oil plants and in resin-producing trees. They may be either constitutive, induced, or both in a particular plant. I will emphasize the enormous diversity of terpenoids rather than diversity across other classes of secondary compounds. This is not to deny that compounds other than terpenoids may be significant in terpenoid-producing plants, and, in fact, other symposium contributors (such as Takabayashi et al., 1994 and Fischer et al., 1994) will refer occasionally to compounds occurring

with the terpenoids as being important in interactions they discuss. It has also been noted, however, that the diversity within the major group of secondary compounds in a plant is generally greater than across different kinds of compounds (Jones and Firn, 1991). I shall not focus only on the biological activity of individual or novel compounds occurring in the mixture but on characteristics of mixtures themselves.

Factors Influencing the Occurrence of Terpenoid Mixtures. A number of factors may contribute to the general occurrence of these terpenoid mixtures in plants, although there is still speculation regarding the relative importance of these factors. Genetic, biochemical, and ecological conditions are possible contributors. Genetically, mutations influencing the biochemical pathway could obviously influence the mixture. Furthermore, the composition of the accumulation of mixtures has often been shown to be under strong genetic control, which will be discussed in a later section. Biochemically, Croteau (1987) and Gershenzon (1993 and as illustrated in this issue) suggest that some mixtures may result simply from certain enzymes of terpenoid biosynthesis producing multiple products. However, because these multiproduct enzymes are likely to reduce the potential costs of producing mixtures of metabolites that may mediate ecological interactions, they become significant ecologically. From a strictly ecological perspective, complex mixtures may confound the capacity for herbivores to evolve resistance to all of the compounds and hence slow the rate of breakthrough of a plant defense (Pimentel and Bellotti, 1976; Schultz, 1983; Jones and Firn, 1991) or increase the potential for attracting benefactors, e.g., pollinators (Bergström, 1991). Other ecological considerations include: (1) the constituent compounds may act additively or synergistically, thus achieving an adequate concentration to produce an effect (McKey, 1979; Berenbaum, 1985; Espinosa-Garcia and Langenheim, 1991b); and (2) the ratio of volatile and nonvolatile compounds in a mixture (e.g., resins) determines the fluidity, viscosity, and polymerization rates, which in turn increase the capacity to engulf organisms, coat wounds, etc. (Langenheim et al., 1978; Synder, 1992). Schuck (1982) suggests that in resins one fraction, such as monoterpenes, may act toxically whereas another, such as diterpenes, may act as a physical barrier in defending against pathogenic fungi. However, Hinejima et al. (1992) have shown in bioassays that resin from *Pinus ponderosa* has broad antimicrobial activity, with monoterpenes generally being active against fungi as well as having some activity against gram-positive bacteria; diterpenes only displayed inhibition against gram-positive bacteria. Lerdau and Penuelas (1993) also suggest a toxic role for the diterpenoids and the importance of the solvent properties of the monoterpenes. Thus these differing reports emphasize how the different fractions of a mixture may serve different physical as well as biochemical roles.

Additionally, the variation of the constitutive compositional profile of the mixtures, i.e., the relative proportions (percentages) of the constituents com-

posing the mixtures is of considerable ecological consequence (Figure 2). The profile may vary qualitatively as well as quantitatively among organs and during organ development (Langenheim et al., 1978, 1986b; Crankshaw and Langenheim, 1981; Hall and Langenheim, 1986; R. Goralka, M. Schumaker, and J. H. Langenheim, unpublished ms.). Furthermore, there may be ontogenetic variation (i.e., between juvenile and adult plants) in leaf compositional profile in trees with the quantitative variation greatly increased in tropical rainforests compared to dry tropical forests (Langenheim and Stubblebine, 1983; Macedo and Langenheim, 1989b). Population variation of the compositional profile has commonly been noted; in fact, it is characteristic of terpenoids. The compositional profile or type may vary considerably in degree in different parts of a geographic range of species (Martin et al., 1974, 1976; Von Rudloff, 1975; Von Rudloff and Rehfeld, 1980; Zavarin and Snajberk, 1975; Lincoln and Langenheim, 1976; Cates et al., 1983; Langenheim, 1984; Sturgeon and Mitton, 1986; Hall and Langenheim, 1987). Examples of repetitive compositional types that occurred in populations of *Hymenaea* spp. over a wide geographic range are shown in Figure 3. The ecological significance of compositional variation will be discussed in detail in later sections.

Effect of Abiotic Factors on Terpenoid Concentrations. A large body of evidence exists for variation in total amounts of terpenoids due to abiotic factors of the environment. Generally, it is considered that increases in accumulation occur with increased light intensity, but decreases in accumulation occur with N, P, or K fertilization (Gershenzon and Croteau, 1991). It is hypothesized that the decreases result from an addition of resources that enhances photosynthate production allocated to growth. Synthesis of terpenes has been thought to be directly dependent on the amount of structural carbohydrate available as a substrate (Croteau, 1987). However, Lincoln and Couvet (1989) showed that the amount of essential oils in *Mentha piperita* were not influenced by CO_2 supply and concluded that allocation to allelochemicals is closely regulated and not sensitive to carbon supply as such. Furthermore, Fajer et al. (1992) demonstrated in a study of iridoid glycosides in *Plantago lanceolata* that excess carbohydrates were a "necessary but insufficient trigger" for increased terpenoid production. Particular interest has been focused on the role of nitrogen availability since Bryant et al. (1983) suggested that additions of N result in a reduction in the production of C-based secondary chemicals, such as terpenoids. However, in subsequent studies, there has been a lack of consistent patterns relating terpenoid production to the effect of nitrogen concentrations (Lerdau et al., 1994), with reports varying from inverse or negative (Mihaliak 1985; Mihaliak and Lincoln, 1989) to direct or positive (Bjorkman et al., 1991). Muzika et al. (1989) also point out considerable genetic variation within local populations of *Abies grandis* in responding to N treatment. They further conclude that the proportion of photosynthate allocated to terpenoid synthesis is

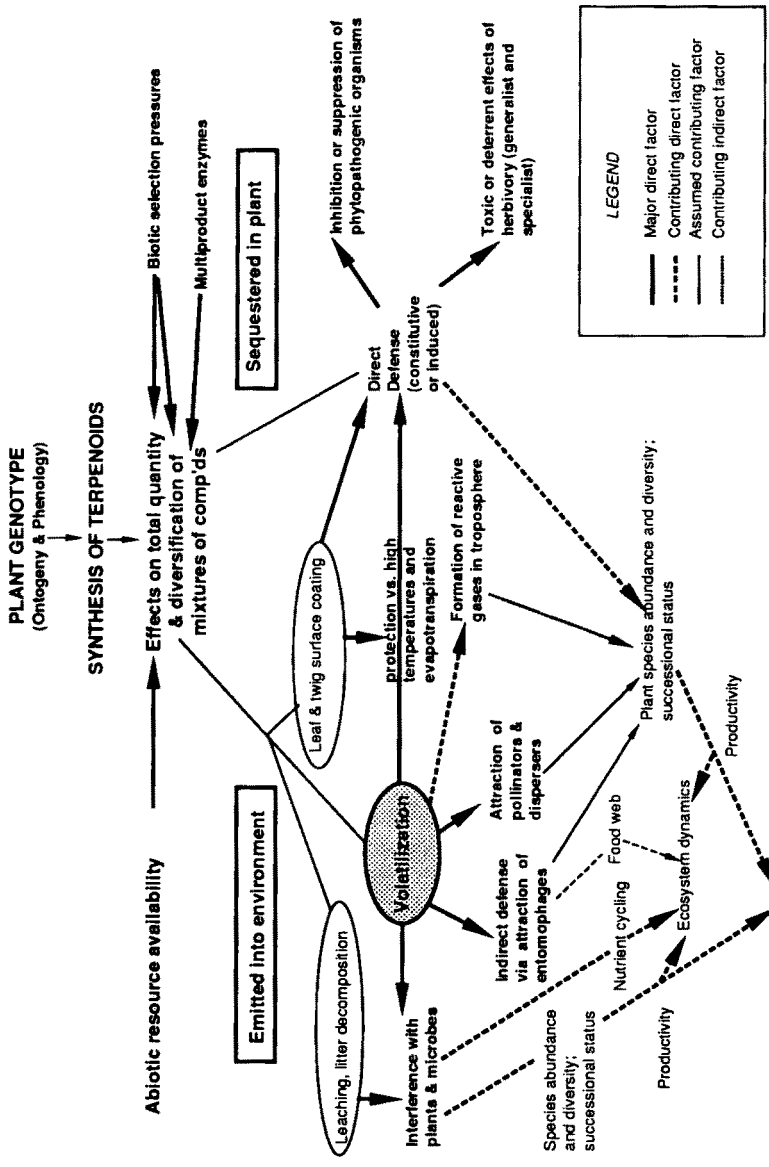

FIG. 2. Major phytocentric roles for terpenoids from higher plants in plant terrestrial communities and ecosystems.

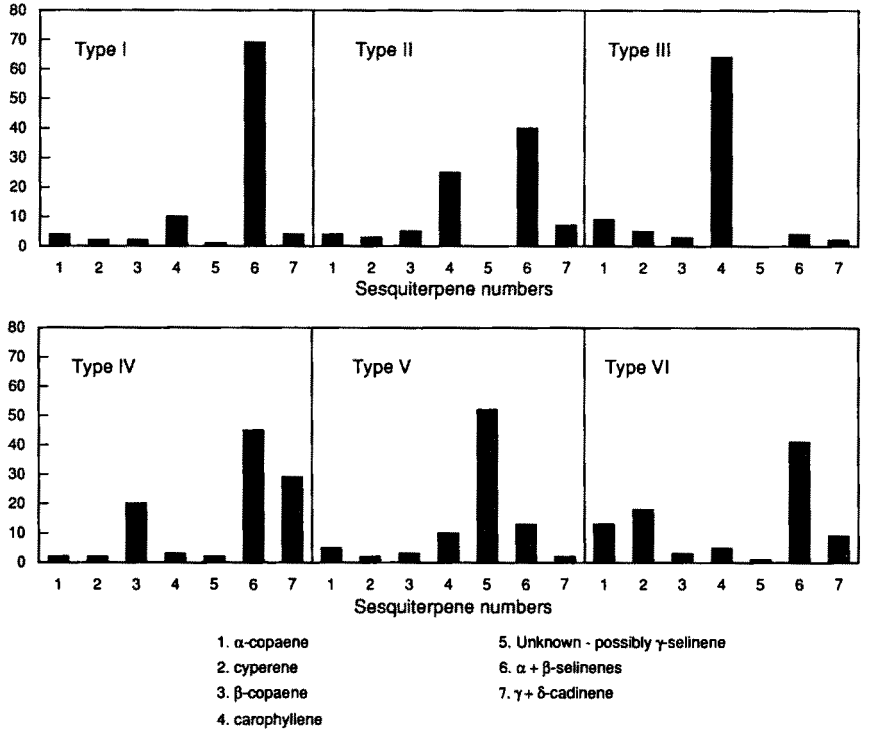

FIG. 3. Examples of variation in terpenoid compositional types (profiles) as shown among six of nine types found in populations of various species of *Hymenaea*.

controlled by the genotype, rather than strongly influenced by the environment. Contrary to this emphasis upon genetics, Reichardt et al. (1991) indicate that the carbon/nutrient balance hypothesis may correctly predict the effects of fertilization (N and P) on concentrations of "end products," but that it fails for many compounds because of dynamics associated with metabolite turnover (such as had been thought to occur in terpenoids).

As chemosystematists have discovered, the compositional profile of a mature organ of individual plants often remains little affected by abiotic factors (Von Rudloff, 1975; Langenheim, 1984; Zavarin et al., 1977, 1990). Kotzias et al. (1992) found that, although the total amount of monoterpenes of *Picea abies* decreased 42% during the summer months, the relative proportion of all terpenoids in the profile and as well as the ratio of the optical isomers of α-pinene remained the same throughout the year. However, conditions during the growing season, have been reported to modify the relative proportions of the components

of the mixture (Gershenzon and Croteau, 1991). Some of these reported seasonal changes have been confounded with changes associated with the development of the organ through the growing season (e.g., Hall and Langenheim, 1987; R. Goralka, M. Schumaker, and J.H. Langenheim, unpublished ms), whereas some changes have now been related to seasonal periods of herbivory (to be discussed later). Gambliel and Cates (1994) have shown that Rocky Mountain Douglas fir (*Pseudotsuga menzeisii*) has the ability to simultaneously change the relative concentrations of components in a biogenetic group (tricyclene, camphene, and bornyl acetate) during the growing season while maintaining the optical activity within a narrow range. They indicate that this represents "surprising flexibility of the plant and suggests strong genetic control at this level."

Multiple Effects of Individual Terpenoids. Harborne (1991a) has clearly shown that multiple ecological effects can be attributed to mono-, sesqui-, di-, and triterpenoid classes. Also, Picman (1986) has demonstrated that amazing kinds of biological activity of the sesquiterpene lactones. However, Table 2 demonstrates how numerous individual monoterpenoids commonly occurring in mixtures have been demonstrated in particular cases to have multiple allomonal

TABLE 2. MULTIPLE ECOLOGICAL INTERACTIONS OF COMMON MONOTERPENOIDS[a]

	Direct plant defense (vs. herbivores and pathogens)	Allelopathy	Formation of reactive gases in troposphere	Indirect plant defense (entomophages)	Plant pollination
Limonene[b]	a	e	f, h		k
α-Pinene[b]	a, c	e	g, h	j	k
β-Pinene[b]	a, c	e	f		k
Myrcene[b]	c	e	h		k
Δ-3-Carene	c		h		k
Camphor	a, b	e			k
Pulegone	a, b	e			
Cineole	d	e	h		k
Citronellol	b				k
Linolool			h	i	k
Ocimene			h	i	k

[a] References indicated with letters; in some cases citations are specific article whereas others are those cited in major reviews. The list of references is illustrative, not exhaustive. a, Harborne (1991a); b, Gershenzon and Croteau (1991); c, Raffa and Klepzig (1992); d, Reichardt et al. (1990a); e, Fischer (1991); f, Grosjean et al. (1992); g, Lopez et al. (1988); h, Winer et al. (1992); i, Dicke et al. (1990); j, Camors and Payne (1971); k, Knudsen et al. (1993); and references cited within each listed paper.

[b] Produced by pinene cyclase.

effects, i.e., direct plant defenses against herbivores, microbial diseases, and other plants, as well as attractants for enemies of insect herbivores or pollinators. Obviously, once these volatile compounds are emitted from the plant, any organism that encounters them may be either a potential user or be affected by some of them. Two common compounds, limonene and α-pinene, may be involved in most kinds of interactions, in fact, α-pinene in all and limonene in all except attraction of entomophages. Moreover, nine of the listed monoterpenoids have been reported in cases of both direct plant defense and pollination, and seven have allelopathic effects as toxic, deterrent or inhibitory compounds (e.g., β-pinene, myrcene, camphor, and cineole); three are reported as attractants to both pollinators and to predators and parasitoids of herbivores (e.g., linolool and ocimene). Interestingly, Gershenzon (1993) has noted that reduced compounds are more expensive to produce per gram than oxygenated compounds. However, some of these (e.g., limonene, α- and β-pinene, camphene, myrcene) that are very common in certain plants may be produced by a single cyclase that may reduce the cost of their synthesis (Gershenzon, 1993). A diverse role for terpenoids also may decrease "opportunity costs" as defensive compounds, i.e. from diversion of resources in production of new plant tissue.

The multiple effects of a single compound bring up two questions: (1) what are the possible detrimental effects of a generalist defense, and (2) are these effects truly broad spectrum? In the first case, Krishick (1991) has argued that a single plant defensive compound that has generalized effects on insect herbivores and plant pathogens could be a possible detriment to the plant, in that this defense could not only deter herbivores and inhibit plant pathogens but also inhibit insect pathogens. Thus, a specialized herbivore could potentially gain protection if it could tolerate higher concentrations or dosages of chemicals found constitutively in some individual plants or induced by the plant. With regard to the second question, Berenbaum (1988) has suggested that most plant allelochemicals have broad-spectrum activity, i.e., attacking metabolic processes common to most organisms. However, I suggest that although multiple effects may appear to be broad spectrum, they may well be selective by having different effects on different organisms within any particular community (e.g., the following comparison of caryophyllene and caryophyllene oxide). In other words, the inhibitory or stimulatory effects may not be truly general but rather quite specific in terms of dosage levels on different populations of interacting organisms in different communities. Thus, this dosage factor can create a degree of specificity in actual effect.

Dosage-Dependent Effects. A survey of recent recorded ecological effectivity of terpenoids shows them universally to be concentration or dosage dependent (Harborne, 1991a), either as individual compounds, combined components or with respect to their total quantity. These results are contrary to the predictions of both the apparency and resource availability models of plant

defense against insect herbivory (Feeny, 1976; Rhoades and Cates, 1976; Coley et al., 1985). In both models or theories, terpenoids were considered to occur in concentrations too low to produce dosage effects. Although terpenoids in leaves often are in the range of 1–2% dry wt/g, they can attain higher levels in leaves (e.g., 15–20% dry wt) as well as in other organs (Gershenzon and Croteau, 1991), and deterrent and inhibitory effects have been demonstrated at even the low concentrations (to be discussed later). Furthermore, the fact that the plant has the potential to quantitatively vary constitutive and induced components of a mixture in numerous ways to achieve dosage effects that enable it to meet a diversity of rapidly evolving enemies was not considered in these theories. Thus, the terminology in which terpenoids are only considered "qualitative" in their effects, whereas polyphenolics are considered "quantitative," obviously can lead to confusion when quantitative effects of terpenoids are discussed.

Mechanisms of Toxic, Deterrent, and Inhibitory Effects. As might be expected from the diversity of structures, terpenoids exhibit a variety of toxic, deterrent, and inhibitory effects (Harborne, 1991a; Gershenzon and Croteau, 1991). Toxicity results from such effects as inhibition of ATP formation, alkylating of nucleophiles, disrupting molting hormonal activity, complexation with protein or binding to free sterols in gastrointestinal system of herbivores, disturbing the nervous system, etc. The exact mechanism of common insect feeding and ovipositional deterrence often is not known, although evidence is accumulating regarding the interaction of some terpenoids with insect sensory receptors (Gershenzon and Croteau, 1991). The basis for odor and bitter taste deterrent effects in mammals is often related to known behavioral responses of humans (Harborne, 1991b); however, Bryant et al. (1991) suggest that terpenoid toxicity may be closely associated with feeding deterrence.

Characteristics Relating to Their Occurrence (Table 1)

Some Terpenoid Classes Characterize Certain Plant Families. Although the five-carbon classes of terpenoids generally are widespread among higher plants, some groups characterize or at least are particularly well known in certain plant families and/or genera within them. Gershenzon and Croteau (1991) have even suggested that the abundance of higher plant defensive terpenoids may be a "simple consequence" of the fact that some very successful phylogenetic groups, e.g., conifers, composites, mints, and euphorbs happen to employ terpenoids as protective agents.

Among the lower terpenoids, both the mono- and sesquiterpenoids are common among higher plants and we know more about their distribution and ecological interactions than most of the other terpenoids, possibly in part because they are relatively easy to analyze and quantify. In fact, the monoterpenoids are

emphasized by other participants in this symposium issue. The sesquiterpenoids are the largest group of terpenoids (6500), with more than half occurring as sesquiterpene lactones that are characteristic of all but one tribe of the family Compositae.

Among the higher terpenoids, the C_{20} diterpenoids are another large and structurally diverse group widespread among plant families. However, among the 20 main structural types, some occur predominantly in certain plant families, e.g., the tiglanes and daphanes in the closely related Euphorbiaceae and Thymelaeaceae, the clerodanes in the Compositae and Labiatae, and resin acids with labdane, kaurane, abietane, and pimarane skeletons in leguminous (Caesalpinioideae) and coniferous (particularly Pinaceae) trees. Although the triterpenoids also are generally widespread among higher plant families, and thus through a variety of environments, two groups of compounds tend to be more restricted. The cucurbitacins characteristically occur in the Cucurbitaceae, the members of which are most abundant in the tropics, and the quassinoids also occur predominantly among tropical and subtropical trees in the Simaroubaceae.

Characteristic Distribution of Essential Oil and Resin-Producing Plants. Plants with mixtures of different classes of terpenoids, such as essential oils and resins (Figure 1), are noteworthy for their characteristic occurrence in certain climatic and vegetation types. Forty-nine percent of the essential oil-producing genera (frequently in the families Labiatae and Compositae) occur in regions with a Mediterranean-type climate and associated vegetation (Ross and Sombrero, 1991). Di Castri (1981) describes this climate, characterized by mild, wet winters and long, hot dry summers, as being transitional between temperate and tropical dry ones. Ross and Sombrero (1991) point out that occurrence of plants producing essential oils is also generally high in the tropics.

Resins produced by trees occur predominantly among the conifers and tropical angiosperm members of the Dipterocarpaceae, Burseraceae, and Leguminosae (Langenheim, 1969, 1990). In fact, the greatest number of tree taxa producing copious amounts of resin occur in the moist tropics. Although conifers occur primarily in temperate-zone vegetation types, their most copious production of resin also occurs in the subtropical and tropical portion of their distribution (Howes, 1949; Langenheim, unpublished ms.). These resins add another interesting dimension in that some readily fossilize (known as amber), providing a long paleobotanical record of their tropical existence that further supports the role of both abiotic and biotic conditions characteristic of these regions in producing large quantities of resins (Langenheim, 1969, 1990). Amber also provides information regarding chemical changes in the resin among genetic lineages as well as important evidence for the nature of past forest communities in which resin-producing trees occurred (Poinar, 1992). Despite phylogenetic constraints on the occurrence of essential oils and resins in certain plant families, some relation to the environment is also evident, i.e., within these taxa the occurrence

of ecologically effective chemistry may have been influenced by both abiotic and biotic components of the environment.

Relation of Terpenoid Occurrence and Apparency of Plants. Terpenoids occur in herbs, shrubs, and trees that occupy all stages of succession from early to late. For example, many terpenoid-producing trees occur in both early and late stages of succession and many terpenoid-producing shrubs involved in allelopathic interactions occur in mature communities. Therefore, terpenoids should not be described as characteristic of plants occurring predominantly in early stages of succession, i.e., categorized as unapparent plants by Feeny (1976) and as unpredictable plants by Rhoades and Cates (1976). In these apparency theories, however, the authors did indicate that chemicals, such as terpenoids, in apparent trees, would occur in unapparent tissue, such as young leaves. In leaves, terpenoids generally occur in highest quantity in young, developing leaves (Gershenzon and Croteau, 1991), although there are exceptions (Langenheim et al., 1986b; Kepner et al., 1974; R. Goralka, M. Schumaker, and J.H. Langenheim, unpublished ms). Likewise, even though the total quantity of terpenoids may decrease during leaf development, attention by these theorists again has not been given to the quantitative changes in constituents of the mixture, i.e., to their relative proportions that may determine the ecological effectivity of the mixture (to be discussed in some detail later in this paper).

Terpenoids also can be present in enormous quantity in trunks of terpenoid-producing trees, which are obviously apparent or predictable tissue. Furthermore, some coniferous trees occupy early stages of succession and others occupy late stages. An excellent example of the contrasting defensive "strategies" of two such trees is provided by Raffa and Berryman (1987). They document a comparison of the effect of terpenoids in defense against the mountain pine beetle in the early successional lodgepole pine (*Pinus contorta*) vs. defense against the fir engraver in the late successional grand fir (*Abies grandis*). They show that in the seral lodgepole pine, which occurs generally in even-aged stands, there is low terpenoid diversity and rapid quantitative decline after maturity. However, the late successional grand fir, occurring usually in mixed stands, has a high terpenoid diversity and only a gradual decline in quantitative responses with age. These differences in terpenoid variation are clearly related to the position of the different species in their communities, and the success of older trees of grand fir in a mature community particularly runs counter to predictions of the mentioned chemical defense theories. Resource availability theory (Bryant et al., 1983; Coley et al., 1985) predicts that late successional plants with long-lived leaves make large investments in antiherbivore defense. This is clearly true for many conifers, boreal deciduous trees, and other terpenoid-producing trees, such as tropical legumes, dipterocarps, etc. (Langenheim, 1990). However, the resource availability theory also predicts that these plants would be

expected to use "immobile" rather than "mobile" defenses as they characterized terpenoids.

I draw attention again to the summary of ecologically relevant characteristics that have been discussed (Table 1); those that will be emphasized with more detailed discussion in later sections are marked with an asterisk.

PHYTOCENTRIC ROLE OF TERPENOID MIXTURES IN DIRECT PLANT DEFENSE

I shall focus on phytocentric examples of the role of mixtures of higher plant terpenoids and their qualitative and quantitative variation in individual plant and population interactions within a terrestrial plant community/ecosystem framework (Figure 2). Differences in the kind of lines and arrows (Figure 2) indicate whether the effect is considered to be a major direct effect (including tritrophic level interactions) for individual plants, whether there is a lesser contributing effect or whether the effect is only assumed, with little current evidence to support it. I shall refer to plant species abundance and diversity, their resultant patterning and structure as well as their successional status as community properties, and those involved in the functional system relating the community to the environment, i.e., productivity, food chains, and nutrient cycling as ecosystem properties. This reference to the contributions of terpenoids to community and ecosystem properties will generally be in ecological rather than evolutionary time. I further shall view the ecological roles of the terpenoids from the perspective of whether they are sequestered in the plant or emitted into the environment.

The most detailed evidence for ecological effects of terpenoids is available for bitrophic level plant defense; both Harborne (1991a) and Gershenzon and Croteau (1991) have admirably enumerated many studies, particularly regarding herbivores, through 1989. A defensive role also has persistently been presented as the *raison d'être* of secondary compounds, including terpenoids, since the field of chemical ecology developed essentially around the concept of coevolution (Feeny, 1992). I shall not only emphasize the role of the terpenoid mixture and/or the variation in its composition, but some other pertinent research since 1990 as well. Emphasizing the chemical diversity produced by terpenoid mixtures is not to ignore the potential defensive role of mixtures that include different classes of compounds (Berenbaum, 1985), and thus what Kubo and Hanke (1985) call a "multifaceted defense," and what has led Jones and Lawton (1991) to formalize into a "diverse defense hypothesis." Rather, in this presentation my aim is to recognize and analyze the great defensive potential existing in mixtures of terpenoids (both within and among classes of them) at different concentration dosages.

Comparative Effects on Fungi and Insects of Two Common Terpenoids in Mixtures

Relatively small changes in chemical structure of co-occurring compounds may have large effects on insect feeding responses. Bowers and Puttick (1988) point out that despite the two iridoid glycosides, aucubin and catapol, being very closely related biosynthetically and having similar structures, they have different effects on the lepidopteran, *Spodoptera eridania*. In this respect, it is interesting to contrast the effects of two relatively common sesquiterpenoids that occur in varying concentrations in mixtures in some plants. Comparison of the differential effects of caryophyllene and caryophyllene oxide as part of mixtures of terpenoids is instructive in illustrating how these compounds defend different plants against different insect herbivores and fungi within different communities (Table 3).

Differential Effects of Caryophyllene and Caryophyllene Oxide in Hymenaea. Studies of the effects of variation in leaf resins in the tropical leguminous tree *Hymenaea* showed different effects of caryophyllene and caryophyllene oxide on leaf-spotting fungi and on lepidopteran insects. In culture experiments, where resin compositional types were dominated by caryophyllene in various *Hymenaea* species through a wide range of tropical communities (Arrhenius and Langenheim, 1983), there was no effect on the potentially pathogenic leaf-spotting genus *Pestalotia* (*Pestalotiopsis*) *subcuticularis*. On the other hand, if caryophyllene oxide in the leaf resin reached sufficiently high, naturally occurring concentration levels, it had a significant fungistatic effect on *Pestalotia*. The importance of the individual compound in this case was supported by experiments using only caryophyllene or caryophyllene oxide. We, as well as others (Hubbell et al., 1983), have found caryophyllene oxide to be an effective fungistatic agent against a wide range of fungi. Thus, caryophyllene oxide is a compound that may function in general resistance, i.e., affects a broad spectrum of fungi that occur on plants, but importantly, at specific dosages for different fungi (S.P. Arrhenius, unpublished data). It is not known if this compound could also affect insect pathogens, which would support the previously discussed conceptual scenario presented by Krischik (1991).

In contrast to the lack of effects of caryophyllene on leaf-spotting fungi in *Hymenaea*, Langenheim et al. (1980) found that caryophyllene at sufficiently high naturally occurring dosage levels within the resin mixture had highly significant effects on both generalist and specialist insects. In a bioassay study with the generalist *Spodoptera exigua*, caryophyllene had a highly significant effect on mortality, whereas high selinenes similarly affected larval growth and time to pupation. Under field conditions. high concentrations of caryophyllene in several populations of *Hymenaea stigonocarpa* were correlated with low levels of lepidopteran herbivory (adapted *Stenoma ferrocanella*), whereas in other pop-

TABLE 3. COMPARISON OF EFFECTS OF CARYOPHYLLENE AND CARYOPHYLLENE OXIDE IN REPELLING, ATTRACTING, OR BEING NEUTRAL ON DIFFERENT ORGANISMS IN DIFFERENT ECOSYSTEMS

Plant origin	Caryophyllene	Caryophyllene oxide
Hymenaea spp. (various tropical ecosystems)	No effect on potentially pathogenic leaf fungus *Pestalotia* in culture	Strong fungistatic effect on *Pestalotia subcuticularis*
H. courbaril	Significant effects on mortality of *Spodoptera exigua* in bioassays	Not analyzed
H. stigonocarpa (tropical savanna)	Significant effects in field analysis on *Stenoma ferrocannella*	Insignificant effects
H. courbaril + other tropical plants (subdeciduous forest)	No effect on survival of *Atta cephalotes*; 50% inhibition symbiotic fungus	Strong deterrent effects on *A. cephalotes*; 100% inhibition symbiotic fungus
	No difference in response to varying concentrations in captive colonies of *Atta cephalotes* and *Acromyrex octospinosus*	*Atta cephalotes* and *Acromyrex octospinosus* differ significantly in response to varying concentrations
Gossypium hirsutum (agroecosystem)	*Campoletis* did not respond	Parasitic wasp *Campoletis sonorensis* responds, but weakly so compared to mixture with other terpenoids
	Attracts predator *Chrysopa carnea*	Does not attract *Chrysopa*
	Collops less attracted than to oxide	Attracts predator *Collops vittatus*
	Directly affects growth of *Heliothis virescens* and acts synergistically with low concentrations of gossypol.	Significant effects on *Heliothis* in lower concentration than caryophyllene; also acts synergistically with higher concentrations of gossypol.

ulations it was correlated with high concentrations of γ-muurolene (Langenheim and Hall, 1983; Langenheim, 1984). In both cases low levels of herbivory were also correlated with a high amount of variability of the other compounds in the resin mixture. The possible effectiveness of caryophyllene oxide against some lepidopterans, however, cannot be excluded, since many lepidopterans detoxify such lipophilic compounds through a mixed-function oxidase system, and we

did not analyze the effects of the oxide in either our early laboratory experiments or field studies with *Hymenaea*.

Differential Effects of Caryophyllene and Caryophyllene Oxide on Attine Ants. Leaf-cutting ants and their symbiotic fungi provide another set of contrasting effects of caryophyllene and caryophyllene oxide. These ants can do extensive damage in short periods to undefended tropical trees. Hubbell et al. (1983) reported that the antifungal caryophyllene oxide defended *Hymenaea courbaril* against the fungus-growing *Atta* ant. They found that caryophyllene was 10–20× less deterrent to the ant than caryophyllene oxide. Howard et al. (1988) further demonstrated that caryophyllene from *Hymenaea* and other tropical trees had no detectable effect on the survival of adult *Atta cephalotes* ants, although it decreased the growth of their mutualistic fungus by 50%. On the other hand, caryophyllene oxide produced both strong deterrent effects on the ant and completely inhibited fungal growth. Later, Howard et al. (1989) reported that captive colonies of *Atta cephalotes* and *Acromyrmex octospinosa* differed significantly in their response to caryophyllene oxide (*Acromyrmex* was more sensitive to low concentrations and less to high concentrations of caryophyllene oxide than was *Atta*) but did not differ with regard to caryophyllene. However, both ant genera responded to a minimum concentration of caryophyllene oxide and deterrent effects occurred at 3–4× lower concentration than previously reported (Hubbell et al., 1983), but caryophyllene was only effective at a concentration 10× that of caryophyllene oxide. The authors attributed these differences in response to a possible difference in ecological roles of the two genera within the tropical forest community. *Atta* and *Acromyrmex* are the two most advanced attine genera that generally rely on living plant tissue for fungus culture. Howard et al. (1989) bring up the interesting question as to whether the more primitive attine genera (unstudied as yet) that do not farm fungi are equally deterred by these compounds. If deterrency is correlated with the use of plant material in fungus cultures, an intriguing hypothesis suggested in earlier studies (Hubbell et al., 1983), the plant has been indirectly defended from ant attack.

Some observational evidence from the study of *Copaifera langsdorfii* in a Brazilian woodland indicates that attine ants avoid trees only with combined high caryophyllene concentrations and caryophyllene oxide (C.A. Macedo, M. Ross, W. Stubblebine and J.H. Langenheim, unpublished data). This suggests that in some species the additive or possibly synergistic effects of the two compounds could be a factor in the deterrence of these highly destructive herbivores.

Effects of Caryophyllene and Caryophyllene Oxide in Mixtures Including Other Terpenoids on Attine Ants. Apart from the direct comparison of differential effects of caryophyllene and caryophyllene oxide, it is interesting to compare several effects of other terpenoids in a mixture against attine ants. Howard et al. (1988) compared the effects of two other terpenoids (nerolidol and kolovenol)

with caryophyllene and caryophyllene oxide in bioassays. They found that the four compounds differed in their effect on deterrence and survival of the ants and in fungus growth. Kolovenol had no effect on the ant or its symbiotic fungus. In contrast to caryophyllene oxide, low concentrations of nerolidol did not significantly reduce survival of adult ants but did inhibit fungal growth more than low concentrations of caryophyllene oxide.

Hubbell and Howard (1984) also showed a decline in the amount of extractable lipophilic compounds (hence potentially terpenoids) that are repellent to *A. cephalotes* from 42 tropical plant species during the latter half of the dry season, one to two months before the dry season began in Costa Rica. They suggested that this decline could be correlated with a reduction in the synthesis of antifungal compounds during the dry season, when the general risk of fungal attack is low. This suggestion again points to an indirect defense of the plant via discouraging the ant from bringing plants with antifungal compounds to their fungal farms.

Role of Quantitative Compositional Variation in Plant Defenses

An important consideration in analyzing the ecological consequences of quantitative compositional variation of a mixture is its advantage in increasing biological activity, apparently without increasing costs.

Relation to Various Specialist Insects. The correlation of high concentrations of an individual defensive compound, such as caryophyllene and γ-muurolene, in conjunction with a high degree of variability of the sesquiterpene mixture with low herbivory in *Hymenaea stigonocarpa* (Langenheim and Hall, 1983) is similar to that reported by Sturgeon (1979). She also found that differential predation by the specialist bark beetle (*Dendroctonus brevicomis*) resulted in frequency-dependent directional selection for high limonene content in *Pinus ponderosa*. Large variation in other monoterpenes, superimposed upon the higher limonene content among individual trees, appeared to substantially reduce the probability of selection of strains of bark beetles capable of detoxifying all compounds. These results are also different from those expressed in the diverse defense and biochemical barrier hypothesis (Jones and Lawton, 1991) in that a single novel chemical is not the basis for deterrence. Rather there has been quantitative variation of compounds in the compositional profile, with one of the compounds predominating. Furthermore, in both *H. stigonocarpa* and *P. ponderosa*, the terpenes in individuals within the population have deterred specialist insects.

Cates et al. (1983), Cates and Redak (1988), and Cates and Zou (1990) also have found terpenes to deter specialist insects in trees, i.e., monoterpenes from *Pseudotsuga menziesii* against spruce budworm (*Choristoneura occidentalis*). They further indicate that success of adapted herbivores is adversely

affected when the terpenoid compositional profile of an individual tree deviates from that of the average profile for the population. Similarly, resistance of some trees within Brazilian woodland populations of *Copaifera langsdorfii* to the adapted *Stenoma* cf. *assignata* was significantly related to the variability of the leaf sesquiterpene mixture among individuals in the populations (Langenheim et al., 1986a; Macedo and Langenheim, 1989a,c).

Iridoid glycosides provide another example of the role of quantitative compositional variation of terpenoids but further indicate its complexity (Bowers, 1991). Plant species that contain iridoid glycosides usually have them in a mixture, in which certain of them may be feeding stimulants for adapted specialist herbivores, whereas others may be toxic or deterrent. Thus attractant, deterrent, or toxic iridoids may occur in the same host plant, such as *Castilleja integra* in Colorado. When six of these iridoid glycosides were incorporated individually into artificial diets and fed to *Euphydryas anicia* (the native herbivore), the larvae grew poorly on some and well on others (Bowers, 1991). Variation in the relative amount (and hence quantitative variation of the mixture) of these attractant versus deterrent iridoid glycosides may determine the relative susceptibility of an individual plant to such a specialist herbivore. Furthermore, Bowers and Puttick (1988) and Puttick and Bowers (1988) predict that such variation may influence larval host plant choice and larval growth rates.

Thus, results from a variety of plants and herbivores in different environments do not support the apparency theories (Feeny, 1976; Rhoades and Cates, 1976), which suggested that terpenoids would be *only* effective in deterring generalist insects. These theories had emphasized the toxic properties of terpenoids and that they generally occurred in low concentrations; they had not considered the possibility of quantitative variation of the components of the mixtures in influencing specialist insect herbivory. Furthermore, they had not been concerned with intraspecific variation. Although it is a truism that individual plants in a population differ from one another in resistance traits to herbivory, it is only relatively recently that ecologists have become interested in the fact that herbivores may behave quite differently on different individuals (Karban, 1992). With a complex resistance provided by variation of the chemical mixture, it is possible that even sublethal doses of toxins could remain effective over long periods. Furthermore, spatial and temporal chemical heterogeneity of different dosage of constituents of the mixtures within the plant as well as within the population can obviously protect certain individuals. Cates and Rhoades (1977), however, do refer to the importance of large quantitative variation in composition among some toxic defensive systems, e.g., alkaloids, among some sympatric as well as subspecies in a plant community.

This is not to deny, of course, that some insects specialize on terpenoid-producing plants, even to the extent of exploiting the terpenoid molecules themselves for various purposes. The relationship, however, may be a complex

one in which the plant benefits at one life stage of the insect, whereas the insect benefits at another stage. This is exemplified by the interaction between conifer diterpene acids and diprionid sawflies, many of which are specialist feeders on conifer foliage. First-year needles of several conifer species contain a mixture of strongly deterrent diterpene acids, such as palustric, levopimeric, dihydroabietic, and neoabietic, in significantly higher concentrations than in older needles (Schuh and Benjamin, 1984). In fact, when such high levels of diterpene acids are added to early instar sawfly diets, toxic effects include reduced growth rate, extended development time and increased mortality (Larsson et al., 1986). However, in contrast to effects on earlier instars, diterpene resin acids had no negative effects on the last larval instar of the European sawfly (*Neodiprion sertifer*). In fact, last-instar larvae of *N. sertifer* appear to seek out tissues high in resin acids to sequester them for use in their own defense. This species accumulates a mixture of monoterpenes and diterpenes while feeding and sequesters them in special pouches in the foregut, which can be ejected on arthropod predators (Eisner et al., 1974). Thus sawflies achieve a compromise between the need to avoid toxic concentrations of diterpenes at one stage of development and to assure later availability of ovipositional and feeding material as well as accumulating sufficient quantity for their own defense at another stage of development.

Monophagous sawflies also provide a noteworthy comparison of deterrence of certain sawfly species on one plant species by a mixture of the terpenoid resin and of another sawfly species on a different plant species by a novel resin acid. In tamarack (*Larix laricina*) a mixture of four diterpene resin acids deterrent to *Pristiphora erichsonii* increases in quantity (up to 10× greater) in the young needles during the growing season but declines toward the end of this period (Ohigashi et al., 1981) However, *N. rugifrons* and *N. swainei* on *Pinus banksiana*, are deterred apparently due to large quantities of a novel compound, rather than a mixture, which occurs in new foliage but only in low quantities in mature foliage (Ikeda et al., 1977).

Relation to a Specialist Mammal. In addition to specialization by insects, a mammal (Abert's squirrel) that is highly specialized on ponderosa pine is primarily influenced in its target tree selection by xylem monoterpene composition and resin flow—both under strong genetic control (Snyder, 1992). The long-term defoliation by these squirrels produces (due to clipping of twigs to feed on the inner bark) sufficiently marked decreases in fitness components such as incremental growth and reproductive capacity of target trees, that Synder (1992, 1993) has assumed that the squirrels exert significant selection pressure on host stands. Despite selective pressures by Abert's squirrels, not all trees that are potentially suitable sources of inner bark have been eliminated. This may partially be explained by other selective pressures on these populations of ponderosa pine. Preliminary evidence has been presented that different kinds of

herbivores (e.g., 200 species of insects have been reported to feed on ponderosa pine) may differentially attack host trees with specific phenotypes (Linhart et al., 1989; Linhart, 1991). Synder (1992) further suggests that differential host selection among species that attack ponderosa pine may generate diversifying selection, thus helping to maintain genetic polymorphisms (such as those of resin composition) within host populations.

Relation to Distribution of Compositional Types in Satureja. A perennial labiate (*Satureja douglasii*)–monoterpene–slug (*Ariolimax dolichopalus*) interaction indicates how the distribution of plants with certain terpenoid compositional profiles or types may be influenced by herbivory (Rice et al., 1978). Several monoterpenoid compositional types characterize the leaves of *S. douglasii* in central coastal California, and these generally occur in certain habitats, e.g., the carvone compositional type occurs predominantly in open dry oak woodland, with relatively high light, low humidity, and high temperatures, whereas the menthone and isomenthone compositional types occur in comparatively more moist, relatively dark, and cooler coastal redwood forests. The carvone type, however, does occur sporadically intermixed with menthone types in more disturbed habitats within the redwood forest. In fact, because of the extensive asexual reproduction of the plant, populations having a particular compositional type may persist in some areas where the forests have been cut or burned (Lincoln and Langenheim, 1979). Experimental studies indicated that these compositional types did not result from differences in the abiotic conditions (Lincoln and Langenheim, 1978, 1979; Gershenzon et al., 1978) and hence indicated a possible role of biotic selection pressures in determining the distribution of these chemical types. Rice et al. (1978) subsequently found in laboratory experiments that the banana slug preferred the carvone compositional type, but under natural conditions the slug did not venture into the open, relatively dry, high-light, and high-temperature habitats where this compositional type was most commonly found. The slug did consume the menthone and isomenthone compositional types that predominated in the usually moist, cool habitat in the redwood forest. However, it was also discovered that the slugs generally prefer young leaves more than mature leaves. In the redwood forest, the young *Satureja* leaves had a mixture dominated by pulegone (the pulegone compositional type), which was highly inhibitory to the slug in laboratory experiments. Therefore, it appeared that the majority of *Satureja* plants in the redwood forest, characterized by menthone–isomenthone compositional types in mature leaves, were able to persist there by the inhibitory nature of the pulegone compositional type in young leaves. On the other hand, the carvone compositional type was only able to persist in abundance by occurring in a habitat, such as the oak woodland, in which the slug could not exist. Furthermore, the carvone type is also more common in the southern, drier portion of the range of *S.*

douglasii. Thus it appears that selective herbivory may have been at least one factor influencing the chemical polymorphism in *S. douglasii*.

Relation to Activity of Foliar Fungal Endophytes in Coastal Redwood. The quantitative variation of the composition of foliar monoterpenoids in possibly controlling the fungal endophytic activity in coastal redwood (*Sequoia sempervirens*) needles has also recently been investigated (Espinosa-Garcia and Langenheim, 1991a,b; Espinosa-Garcia et al., 1993). Fungi with endophytic parts of their life cycle that colonize aerial tissues are ubiquitous in conifers (Carroll, 1986). Although some of these fungal symbionts may be latent pathogens, many do not harm the plant in their endophytic stages. In fact, many fungal endophytes are close relatives of virulent pathogens, and some otherwise harmless endophytes can cause disease when plants are under stress (Carroll, 1988).

Most leaf endophytes in conifers apparently remain inactive after colonizing the leaf, their active growth and reproduction only occurring when the leaf senesces or is damaged (Carroll, 1986). Although the mechanisms by which this apparent inactivity is maintained are not known, Verhoeff (1974) has suggested a potentially important role for secondary compounds in controlling latent pathogens. Espinosa-Garcia and Langenheim (1991a) demonstrated that four redwood essential oil compositional types were uniformly inhibitory to some of the most common endophytic species and differentially so to others, with susceptibility varying widely within and among the fungus species. This diversity of responses suggested that redwood terpenoids may have differential intra- and interspecific importance in regulating pathogenic and nonpathogenic activity in the endophytes occurring within redwood foliage. Since sabinene and γ-terpinene are often important components of redwood essential oils and were the most prominent compounds in the compositional types that were most inhibitory to endophytes, Espinosa-Garcia and Langenheim (1991b) assayed them in vitro both singly and together in the gaseous state. They found that these compounds acted additively on each of six species of redwood endophytic fungi. In another study, Espinosa-Garcia et al. (1993) further discovered that the dose–response curve of the nonpathogenic, host-specific, and suspected redwood mutualist (*Pleuroplacanema* sp.) to essential oils was finely tuned to changes in terpenoid concentrations occurring in mature and senescent needles, being even stimulatory at low dosages. Although terpenoids inhibited the activity of a pathogenic conifer specialist (*Pestalotiopsis funerea*), they did so less than for *Pleuroplacanema* sp. However, dosage effects were particularly striking among isolates of these two endophytes.

Factors Controlling Quantitative Variation of Compositional Profile. We have little evidence as to what is controlling these compositional variations. Bowers and Puttick (1988) have suggested the possibility of mutations leading to variation in enzyme kinetics or blockage of certain biosynthetic steps. Activation of genes controlling the expression of specific cyclases may be significant

in that these enzymes may be the controlling factor over synthesis rates of individual terpenes (Croteau, 1987).

Although Berenbaum and Zengerl (1992) warn that estimates of chemical variation are not necessarily estimates of plant resistance, evidence suggests that compositional types may be selected by biotic pressures in certain environments. For example, the occurrence of varied compositional types or profiles may reduce the incidence of attack of insect herbivores adapted to the average profile of a plant population (Langenheim et al., 1980; Cates et al., 1983). The increased variability in compositional types in *Hymenaea* and *Copaifera* populations in tropical rainforests compared to dry forests was also hypothesized to be related to herbivore pressure (Langenheim and Stubblebine, 1983). Seasonal changes in the relative proportion of certain biogenetically related terpenoids in Douglas fir seem to correlate with spruce budworm feeding patterns (Cates and Redak, 1988; Wagner et al., 1990). Gambliel and Cates (1994) note that low levels of the tricyclene–camphene–bornyl acetate group in coastal Douglas fir may be related to less frequent outbreaks of spruce budworm and reduced selection for this group of terpenes known to be toxic to the insect. Furthermore, the studies of Abert's squirrels that defoliate ponderosa pine are suggestive of biotic selection pressures influencing compositional profiles, i.e., high β-pinene and β-phellandrene being related to nontarget trees (Snyder, 1992).

The possible differential role of different insects on terpenoid compositional variation in different populations of the same plant species is indicated in a comparison of the lack of effects of mountain pine beetle (*Dendroctonus ponderosae*) on Colorado populations of ponderosa pine with strong putative effects of western pine beetle (*Dendroctonus brevicomis*) on California populations of ponderosa pine (Sturgeon and Mitton, 1986). Comparison of the Colorado Front Range populations of ponderosa pine with those in California having a history of western pine beetle infestations revealed large differences in terpenoid variation between the two geographic areas. Sturgeon and Mitton (1986) suggested that one reason why mountain pine beetles do not discriminate among trees with different compositional profiles is because terpenoid variation of ponderosa pines in Colorado is not as great as those in California; in fact, the California populations are three orders of magnitude more variable than the Colorado populations. They further suggested that the Colorado mountain pine beetle may be indifferent to the terpenoid variants in ponderosa pine in this more chemically uniform part of its range. To a mountain pine beetle in Colorado, a host lodgepole pine may be no more different from ponderosa pine than two chemical variants of ponderosa pine to a western pine beetle in California. If mountain pine beetles are discriminating among their several hosts, rather than among variants of one host, diversifying selection would lead to increased chemical distance among hosts and decreased chemical differences among variants within a host (Sturgeon and Mitton, 1982). Hence Sturgeon and Mitton suggest that

the feeding habits of mountain pine beetle may help to maintain patterns of chemical compositional variation found in both ponderosa pine and other host taxa in Colorado.

Marquis (1992) has pointed out that available evidence is insufficient to delineate the *generality* and *strength* of selection exerted by herbivores for plant resistance traits (such as compositional variation) due to the myriad of selective factors that plants face. This is a fact that all botanists are aware of, but it appears that some selective forces may predominate in some populations at particular periods of ecological time—as suggested in the comparison of the effects of different bark beetles in geographically separated populations of ponderosa pine. Furthermore, Synder (1992) has pointed out that there now is preliminary evidence that different herbivores may differentially attack host ponderosa pine trees with specific phenotypes. In *Hymenaea*, it appeared that compositional variation that increased caryophyllene inhibited some kinds of lepidopteran herbivory, whereas increased caryophyllene oxide had more fungistatic properties. However, only with intensive as well as extensive long-term studies, as has been occurring with ponderosa pine, will we have a better idea of the relationship between variation of terpenoids to the variety of selective forces that individual plants face.

Conifer Defense vs. Bark Beetles and Their Vectored Fungi

The terpenoid–bark beetle–fungus interaction is both a complex and fascinating one, which has been studied in considerable detail because of its impact on commercially important conifer forests. This is an excellent example of the complexity of interactions due to the mixture of terpenoids—with some compounds repelling and others attracting herbivores—and the importance of the plant's ability to defend against both insects and their vectoral fungi. In the phytocentric context of this review I can only refer to a few special aspects relating to the success of the plant defenses.

History of Interaction. First, let us look at the history of the terpenoid–fungus, bark beetle–fungus interaction as depicted in the geologic record. Land plants were first probably utilizing terpenoids to protect against microbes. Wood-decaying fungi have been reported in silicified specimens of the late Devonian progymnosperm *Callixylon* and the pattern of decay appears similar to modern-day wood rot (Stubblefield et al., 1985). Furthermore, the associated appearance of "resinous-appearing material" in the wood is thought to be a response to the fungi. Evidence of true terpenoid resins (amber) appears with later arborescent plants during the Carboniferous (Langenheim, 1969, 1990). Several groups of late Carboniferous swamp plants display bored wood, and Scott and Taylor (1983) have reported elaborate networks of connected burrows that suggest the activities of beetles. Direct evidence of the beetles, however, has not been

reported until early Permian, at which time the earliest known vertebrate herbivores also appear. It thus seems that terpenoids, such as resins, could have appeared initially in woody plants as a defensive response to fungal attack rather than to herbivore damage.

Phelan and Stinner (1992) indicate that herbivores such as beetles feeding on woody tissue provide outstanding examples of microbial mediation of plant resources. The scolytid beetles are the best studied group using woody tissue, and also have a history of a relationship with terpenoids and interaction between insects and fungi. Berryman (1989) suggests that primitive scolytids exhibited a saprophagus habit in wood that brought them into intimate contact with fungi and bacteria—and thence the bark beetles formed an association with plant pathogenic fungi.

Berryman et al. (1989) further point out that the need for host plant death for the completion of the bark beetle life cycle puts very heavy selection pressures on conifers for the development of defense, in which terpenoid mixtures have played a prominent role. Some species of bark beetles colonize dead or dying trees, whereas others (*Dendroctonus*, *Ips*, and *Scolytus*) make mass aggregation attacks on trees. Both total resin and individual monoterpenes have been shown to repel beetle attack (e.g., Gollob, 1980; Raffa et al., 1985; Cook and Hain, 1988; Paine and Stephen, 1987), and I previously mentioned the role of monoterpenoid compositional variation in the resistance of ponderosa pine in California to *Dendroctonus brevicomis* (Sturgeon, 1979). Furthermore, the role of the mixture of different terpenoid classes may be important in determining physical as well as toxic properties of resins that may serve as a "first line of defense" against attack of herbivores, such as bark beetles. The ratio of the volatile mono- and sesquiterpenes to the nonvolatile diterpenoids determines the viscosity and the rate of crystallization, which in turn controls the rate of flow in impeding the burrowing or blockage of channels (Hodges et al., 1979; Schuck, 1982; Cook and Hain, 1988; Croteau and Johnson, 1985).

Role of Constitutive and Induced Monoterpenoids in Conifer Defense. Survival of trees attacked by bark beetles not only depends upon constitutive resin, with its specific physical and chemical properties, but also on the general physiological vigor of the tree, number of attacking beetles, and most importantly, the fungal spores that they carry on their bodies. These fungi that are introduced into the tree during attack are often pathogenic to coniferous trees (Strobel and Sugawara, 1986; Rane and Tatter, 1987). This kind of fungal infection apparently aids the success of bark beetle attack by providing an aggressive mechanism for possibly overcoming the tree's resinous defense, weakening the tree and therefore hastening its death. Monoterpenoids have been demonstrated to function in defense against both beetles and their associated fungi (Cobb et al., 1968; Hintikka, 1970; Schuck, 1982; Raffa et al., 1985; Bridges, 1987). The importance of the tree's resistance to the fungal infection is indicated by its

rapid de novo synthesis of resin near the site of infection. This induced resin often has a different monoterpenoid composition than the constitutive resin (Raffa and Berryman, 1982, 1983; Christiansen, 1985; Stephen and Paine, 1985; Raffa, 1991; Raffa and Klepzig, 1992). Generally, those terpenoids in constitutive resins that occur in greatest quantities are induced the least, whereas the rarer monoterpenes are induced in more quantity. In fact, Raffa and Klepzig (1992) have suggested that conifer constitutive monoterpenes function largely to deter feeding or reduce attraction by beetles, thus preventing fungal colonization by orienting the vector elsewhere. Induced chemical defenses, however, have been observed against all insect-vectored tree-colonizing fungi. Responses to mechanical wounds yield less allelochemical accumulation and no proportionate chemical changes (Raffa and Berryman, 1982). As shown in the example from *Abies grandis* phloem monoterpenes (Figure 4), there can be significant qualitative and quantitative changes from the constitutive composition to the reaction or induced composition. In general, there is a disproportionately high increase in those chemicals that have the most deleterious effects on insects and fungi during the induced response (Raffa, 1991).

Conifer monoterpenes are also involved in beetle host-finding as well as intraspecific pheromonal communication, with the aggregation pheromones frequently being allylic oxidation products of host monoterpenes (D.L. Wood, 1982). Although the compounds in the tree's mixture of constitutive monoterpenoids may directly deter the beetles, some may also signal that the tree is an

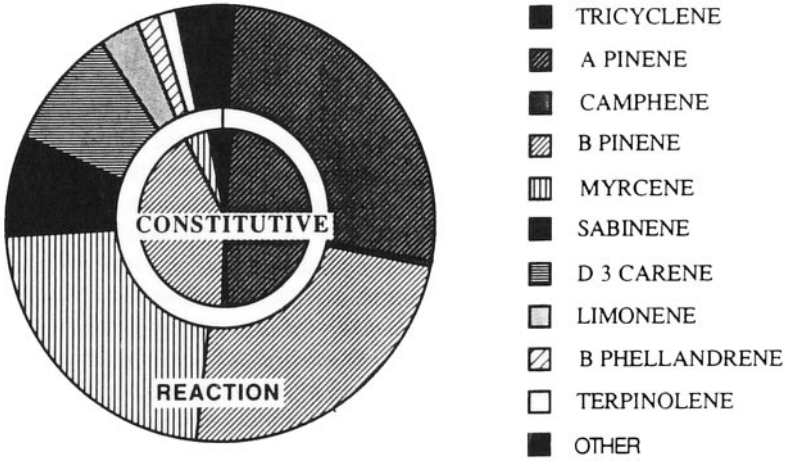

FIG. 4. Comparison between constitutive monoterpenoid composition and composition rapidly induced by *Trichosporium* fungus in grand fir phloem (from Raffa and Klepzig, 1992; with permission from Springer-Verlag).

unfavorable host. For example, the pine shoot beetle, *Tomiscus pinperda*, which feeds on *Pinus sylvestris*, is able to recognize in flight a host tree that is unsuitable for colonization by release of verbenone from a colonized tree. Verbenone is released in increasing quantities as beetle attack progresses, and thence completely inhibits the attractiveness of the host monoterpenes to the beetle (Byers et al., 1989). Klimetzek et al. (1987) also report that the pine shoot beetle is sensitive to a possible synergistic effect of the mixture of terpinolene and α-pinene together with high concentrations of ethanol released from the damaged tissue. I shall not continue with the extensive literature regarding bark beetle pheromone interactions, other than to mention the work of Chararas et al. (1982), in which they indicate the importance of the mixture in comparing the essential oil compositional pattern in ten conifer species with the establishment of six genera of Scolytidae. They concluded that attraction of the bark beetles resulted from synergism of different terpenes, which they thought was necessary for attraction to prevail over the generally "repulsive" properties of some of these terpenes (when they occurred at sufficiently high concentrations). Sturgeon (1979) points out an interesting example of this in ponderosa pine in which western pine beetle preferred trees with low concentrations of the toxic compound limonene but high concentrations of α-pinene, a pheromone precursor.

Defense vs. Vertebrate Herbivores

Some terpenoids that have been implicated in defense against mammals have involved a different kind of interaction with microbes than that vectored by insects. High total amounts of foliar monoterpenes in several plant species have been suggested to deter deer browse (e.g., Nagy and Regelin, 1977; Schwartz et al., 1980; Welch et al., 1981). Some of these researchers interpreted their results on evidence from in vitro studies, which indicate that these dietary monoterpenes could be toxic to ruminants by suppressing the activity of their digestive microorganisms (Oh et al., 1967; Nagy and Tengerdy, 1968; Longhurst et al., 1968). However, some subsequent in vivo research has suggested that monoterpene concentrations in the rumen of at least sagebrush-feeding deer and rabbits appear to be lower than those used in earlier in vitro studies and perhaps too low to interfere with microbial digestion (Cluff et al., 1982; White et al., 1982). Welch et al. (1989) have suggested that the low levels of monoterpenes may result from their volatility; hence a significant amount lost from the diet during mastication may not reach the rumen. However, Foley et al. (1987) indicate that only minor losses of essential oils occur during mastication in the Australian greater glider (*Petauroides volans*) and broadtail possum (*Trichosurus vulpecula*), which feed primarily on eucalyptus leaves that are rich in monoterpenes (such as cineole, limonene, terpineol, piperitone, and β-phellandrene). Foley et al. (1987) have suggested that these animals are able to avoid

deleterious nutritional effects because the microbial populations in their hindgut are protected from terpenes, which are absorbed from the stomach and small intestine and then detoxified by the liver. Thus, these adaptations to dietary terpenes by Australian marsupials provide indirect evidence that terpenes in some cases could inhibit digestion.

Bell and Harestad (1987) suggest that mixtures of monoterpenes, such as α- and β-pinene, limonene, and myrcene, in pine oils are effective as repellents of voles and snowshoe hares because these animals do not become habituated to pine odor. Elliot and Loudon (1987) have also shown that monoterpenoid odors from Sitka spruce (*Picea sitkensis*) and lodgepole pine (*Pinus contorta*) deter feeding of red deer (*Cervis elaphus*). The odors were apparently rejected based on quantity rather than quality of the terpenoids. Reichardt et al. (1990a) have shown that buds, which are the least palatable part of the winter-dormant balsam poplar (*Populus balsamifera*) to snowshoe hares (*Lepus americanus*), are protected by at least three components of a complex mixture of compounds, principally mono- and sesquiterpenes (e.g., cineole and (+)-α-bisabolol).

High concentrations of specific terpenoids have also been shown to deter feeding of hares (Bryant et al., 1992). In Labrador tea (*Ledum groenlandicum*) a sesquiterpene, germacrone, is a major component of the essential oil, which was demonstrated by bioassays to be a potent antifeedant to hares (Reichardt et al., 1990b). Likewise the monoterpene camphor, which occurs in high concentrations of juvenile foliage from white spruce (*Picea glauca*), has been shown to be a specific antifeedant to snowshoe hares (Sinclair et al., 1988). Furthermore, the triterpenoid papyriferic acid has been reported to protect the winter-dormant Alaska paper birch (*Betula papyrifera*) against showshoe hares (Reichardt et al., 1984). In winter, papyriferic acid specifically accumulates on the surface of dormant juvenile twigs, defending the tree only when it is vulnerable to hare attack. The concentrations drop 25-fold in the mature internodes, when herbivory is no longer harmful to the tree. Feeding experiments demonstrate that papyriferic acid is highly distasteful to this major herbivore, with concentrations in the tree being more than sufficient to explain the absence of herbivory at the juvenile growth stages. In older trees, concentration falls off, perhaps because the winter-dormant tissue is out of reach of the hares. In addition to protecting juvenile internodes from the snowshoe hare, Reichardt et al. (1984) indicate that papyriferic acid may also act as a feeding deterrent to moose and certain rodents. Thus a variety of terpenoids appear to be antifeedants to snowshoe hares and possibly other mammals.

Sesquiterpene lactones have been demonstrated to be toxic in a number of plants and poisonous to livestock, including *Hymenoxys* and *Helenium* in the United States and *Geigeria* in South Africa (Gershenzon and Croteau, 1991). Feeding deterrency to rabbits, deer, and voles has been shown (Picman, 1986; Harborne, 1991b). Sesquiterpene lactones have generally been thought to act as

feeding repellents because of the bitter taste to humans. Gershenzon and Croteau (1991) have suggested that bitterness may not be an intrinsic property of these terpenoids but rather a property acquired through natural selection. Herbivores may well have been selected through time for their ability to associate poisonous compounds in potential food plants with unpleasant tastes and thus avoid consuming large amounts of such substances. Thus, for sesquiterpene lactones (as well as other bitter plant terpenoids, such as limonoids and cucurbitacins), bitterness may be an evolutionary consequence of toxicity, which has subsequently led to deterrency.

Contributing Effects of Terpenoid Defense against Herbivory and Disease to Community/Ecosystem Properties

Fox (1988) and Thompson (1988) have pointed out that even low loss of plant tissue by herbivory could be the basis for selection if slight differences in plant growth rate affect competition, which in turn could influence plant species' abundance and diversity (Figure 2). Crawley (1989), however, has warned that a major difficulty in assessing the role of herbivory in plant dynamics lies in demonstrating the impact of herbivore feeding on plant demography rather than on plant performances. He also predicts that vertebrate herbivores *generally* exert more immediate and long-term effects on plant populations and hence on community structure and dynamics than do insects. Ungulate herbivory, for example, has been shown to profoundly alter a landscape where there have been large population increases of these animals (Klein, 1981; Whitney, 1984). Ungulates selectively browse juvenile stages of particular plant species, and sustained herbivory over time results in increases in the less palatable species. Deer exclosure studies in such communities have shown that native old-growth species composition regenerates itself only in the absence of deer (Putman et al., 1989; Tilghman, 1989). Ungulate herbivory can also drive succession in natural systems (Figure 2). Moose that migrated onto Isle Royale over 80 years ago are maintaining new forest growth in an early successional sere by selectively browsing terpenoid-producing balsam fir (*Abies balsamea*) trees in the climax canopy (Brandner et al., 1990). McBride (1974) has shown that native black-tailed deer in the Berkeley hills of California selectively fed on the palatable coastal live oak, *Quercus agrifolia*, thus resulting in the high monoterpenoid-yielding California bay tree, *Umbellularia californica*, becoming the climax species in the woodland areas there. Even more pronounced effects of ungulate herbivory on species abundance and density, and hence on community patterning, have been reported where deer are exotic species (Stewart and Burrows, 1989; Veblan, 1989).

Forest entomologists cite many examples of severe impact on tree growth, survival, and reproduction by both native and introduced insects (Berryman,

1988) but often have not discussed actual effects on plant demography. Myers (1993) has reported that there is surprisingly little study of the impact that cyclic forest defoliations by lepidopterans have on forests, but on the basis of observed defoliation, she thinks that they cause less damage than might be expected. Deciduous trees generally compensate by refoliating; evergreen plants are less able to compensate for the loss of leaves, and hence are more vulnerable to invasion, as demonstrated by the impact of spruce-budworm attacks. Therefore, our discussion of the defensive role of terpenoids in conifers against spruce budworm and bark beetles is significant in terms of effects on community and ecosystem properties.

S.L. Wood (1982) indicates that, taken together, the bark beetles in the genera *Dendroctonus*, *Ips*, and *Scolytus* constitute the most common tree mortality factors in North American conifer forests. Their epidemic outbreaks and resulting tree mortality can present dramatic ecological consequences affecting many elements of the plant community (Figure 2) and as has been shown, terpenoids can play an important contributing role in preventing aggregation of the bark beetles and death of the trees often due to the vectored fungi. Berryman (1986) also points out that when conifer trees are stressed, their defenses against bark beetles are less effective. When these trees are killed, their nutrients are recycled to healthy survivors—thus tending to maximize the rate of biomass accumulation or productivity of the average tree constituting the community. Berryman (1986) further indicates that insect herbivory often plays a significant role in forest succession, either speeding up or slowing down the rate at which it proceeds. For example, when conditions are favorable for an aggressive beetle, such as *Dendroctonus ponderosae* (mountain pine beetle), to undergo extensive population eruptions, lodgepole pine (*Pinus contorta*) mortality often exceeds 90% (Raffa and Berryman, 1987). However, where lodgepole pine is seral, Raffa and Berryman (1987) hypothesize that beetle epidemics and subsequent fires actually favor reestablishment of this pine. Thus the beetle attacks on even-aged old stands, where terpenoid resistance is lower, actually prevent the community from advancing to a more mature status.

PHYTOCENTRIC ROLES OTHER THAN DIRECT DEFENSE FOR TERPENOID MIXTURES EMITTED INTO THE ENVIRONMENT

Protection of Leaf Surfaces

Nonvolatile diterpenoids may be exuded to form coatings of the leaf surface and are particularly prominent in semiarid areas. Dell and McComb (1978) have reported these resin coatings to be very common in perennial plants in the arid areas of Western Australia. In a detailed study of three representative genera, *Beyeria* (Euphorbiaceae), *Newcastelia* (Dicrastylidaceae), and *Eremophila*

(Myoporaceae), they found the major components of the resin to be diterpenoids with some triterpenoids intermixed. Since the resin can comprise 30% leaf dry weight, with half of that occurring in the immature leaf, it would be assumed that considerable costs have been incurred in the production of this resin that has been exuded to the surface of the leaves. Dell and McComb (1978) hypothesize that these hydrophobic nonvolatile and shiny surface coatings may well protect the leaf (particularly the immature leaf) against high evapotranspiration rates, high temperatures, and high insolation found in desert environments. Others, such as Luis (1991), have further questioned whether diterpenoids in members of the Labiatae could represent adaptations to hostile environments with high levels of ultraviolet radiation.

Attraction of Pollinators

Although we have discussed the role of volatile compounds in direct defense, their central importance in numerous other interactions, such as attraction of pollinators, is obvious (Figure 2). Terpenoids and benzenoids are the volatile compounds found most commonly in flower odors (Knudson et al., 1993). Evidence suggests that as a pollinator, attractant scent may even be more ancient than flower color (Harborne, 1988). For example, many primitive beetle-pollinated flowers lack color but have strong odors. Maximum scent production often seems to be coordinated with the maturation of pollen, although insects are sensitive to very small amounts of volatiles that may have been produced previous to the full development of the pollen. In flowers, as in other plant parts, the mixture or blend of volatiles is important, with one component reinforcing several others in producing a characteristic odor (Harborne, 1988). Moreover, the daily rhythmicity of emission of volatile scents is especially pronounced in flowers pollinated by night-flying insects and bats (Dobson, 1993).

Variability of Volatile Mixture. The mixtures of essential oils that attract and guide insect pollinators to flowers have been studied in considerable detail in various species, but mainly in orchids. Thus, in orchids pollinated by euglossine bees in the tropics, more than 60 different volatile compounds have been identified, with each species having a distinct blend, and the relationships between the bees and orchids are often highly specific (Dodson et al., 1969). Flower volatiles that attract pollinator insects may also stimulate their feeding and mating; however, in the case of the orchids and male euglossine bees, the bees visit flowers not to collect food but to gather the floral volatiles. Furthermore, Dobson (1993) points out that the focus on the role of floral scents in attracting pollinators has overshadowed the role that these chemicals may play in the plant's defense against destructive flower-feeding insects and microbial pathogens. When the mixtures that comprise a floral fragrance are tested individually for their activity on insects, these can have differing effects that are not

evident when analyzing the entire mixture. For example, only one of five tested components (linalool) of the alfalfa floral mixture attracted honeybees, whereas the others were considered to be either neutral or repellent (Dobson, 1993).

Although particular floral mixtures characterize certain plant species, inter- and intrapopulational variations of the compositional profiles of the mixtures have also been reported (Bergström and Bergström, 1989). Additionally, Dobson et al. (1990) found that in *Rosa* each floral part has a distinctive compositional profile. Whereas the petals were dominated by terpenoid and benzenoid alcohols, the sepals had a high representation of sesquiterpenoids in addition to some compounds in common with the petals. In contrast, the anthers and pollen had a more diverse representation of compounds, few of which were shared with the perianth. This variation in the composition of the mixture among floral parts may be controlled simply through the biochemistry of organ development or it may conceivably imply the involvement of ecological factors that exert different selective pressures among the floral parts, as has been suggested for vegetative organs (Langenheim et al., 1978; McKey, 1979; Krischik and Denno, 1983).

Most studies of the role of floral volatiles in plant pollination have dealt with particular plant species and their associated groups of insects. However, the mixtures of pollinator-stimulating compounds possess a wide spectrum of volatility and polarity that may affect interactions within the community. Given the diversity of floral blends and the great discriminatory power of the insect olfactory systems, an infinite number of possible signals could occur in gradients through both space and time. Bergström (1987, 1991) has pointed out that there is assortative pollination for numerous flowering plants and their pollinators based only on chemical cues. Therefore, he has queried whether there is competition for pollinators among plants with particular floral mixtures, and hence, whether there is any adaptation by plants to produce particular mixtures or fragrances.

Pollinator–Herbivore Interactions. The situation in which herbivores may not only be unaffected by the plant's terpenoids, but may sequester them to utilize in their own defense, may appear to be detrimental to the plant. However, examples have been presented previously where utilization depends on a particular stage of development of the insect. In another scenario, Rothschild (1985) presents an intriguing hypothesis that in some cases plants offer both food and protective chemicals to their pollinators. *Catalpa speciosa* and its major herbivore, the catalpa sphinx caterpillar, *Ceratomia catalpae*, provide an interesting terpenoid example. This caterpillar is unaffected by the iridoid glycosides from the tree, but obtaining them makes it unpalatable to birds (Bowers and Puttick, 1986). In fact, the larva contains 15% of its dry weight as iridoid glycosides. However, the adult moth, which is a principal pollinator (along with bumblebees and carpenter bees) of *Catalpa* flowers, contains no detectable iridoids. It also is unaffected by those iridoid glycosides (catapol and catalposide) in the nectar,

which deter or incapacitate nonadapted nectar thieves such as ants and the lepidopteran "skipper" (Stephenson, 1982). This obviously demands a delicate relationship between the plant and animal participants.

Attraction of Entomophages

Indirect Plant Defense at the Tritrophic Level. Because insects depend on volatile compounds for social communication, they can become sensitive to molecules similar to those present in flower scents (Harborne, 1988). Flower scents thus can attract and/or arrest potential predators or parasitoids, i.e., natural enemies of herbivores (Whitman, 1988) (Table 2). Since flowers differ intra- and interspecifically in attractive capabilities (and in floral rewards), these differences may determine visitation rates of both pollinators and entomophages.

Many examples have existed for predators being attracted to prey-produced chemicals (Greamy and Hagen, 1981), but until the last decade few have been reported for the predator being attracted to chemicals produced by the host plant (Barbosa and Saunders, 1985; Barbosa and Letourneau, 1988). Dicke et al. (1990) suggest that this lack of information probably has resulted previously from restricted efforts to find the exact source of the attractive chemical with the plant often being dismissed as a primary habitat factor. Letourneau (1988) points out that the previous emphasis on bitrophic levels is understandable in that experiments are easier to manipulate, the data are easier to analyze, and results tend to be more elegant. However, she emphasizes that two-level interactions taken separately cannot adequately describe interactions in three levels because many processes of resource exploitation are coupled and plant-predator-parasitoid interactions involve responses to emergent factors associated with coupling of three trophic levels. Emergent properties appear in plant communities, since the properties of neighboring plants can be beneficial or detrimental to the enemies of the herbivores of a given plant. Price et al. (1980) and Price (1992) have further emphasized that plants are the resource base of the food web and thus influence many aspects of community organization as well as ecosystem functioning. Thus terpenoids that are contributing factors to tritrophic level interactions with predators and parasites also may be contributing significantly to community and ecosystem properties (Figure 2).

Entomophages on Coniferous Trees. Two braconids and a pteromalid species that parasitize larvae of the southern pine beetle (*Dendroctonus frontalis*) are attracted to the host's terpenoids when mixed with certain adult beetle pheromones (Dixon and Payne, 1980). One of the braconids is also attracted to the plant's terpenoids alone. Predaceous dipterans, namely adult *Medetera* whose larvae feed on *D. frontalis* larvae, are attracted to the beetle host tree by certain volatile terpenoids alone or in mixtures with bark beetle pheromones (Dixon and Payne, 1980). The parasitoid *Heydenia unica* is attracted to the host tree

α-pinene after a southern bark beetle attack, but there is also involvement of bark beetle sex pheromones (Camors and Payne, 1971). These examples that implicate pheromones bring up the fact, as Dicke et al. (1990) point out, that many of the terpenoids that function in predator–prey interactions probably originally acted as pheromones for the prey herbivores. Thus, the large body of information regarding bark beetle terpenoid pheromones has provided useful data regarding chemicals that attract predators, even in such varied communities as agroecosystems.

Entomophages on Cotton. Terpenoids in upland cotton (*Gossypium hirsutum*) provide a particularly interesting example of the complicated but significant role that terpenoid mixtures can play in attracting both parasitoids and predators. With the development of glandless cotton (with the goal of increasing cottonseed in animal feed and human food), it was found that these plants were more severely damaged than glanded ones. It has been known that gossypol was a major component in the glands, but studies of 30 cotton species, cultivars, and lines revealed numerous volatile terpenoids as well as nonvolatile terpenoid aldehydes occurring in distinctive volatile profiles (Bell et al., 1987). For example, Egyptian cotton (*G. barbadense*) lacks myrcene, γ-bisabolene, and β-bisabolol, which are major components of upland cotton. Some races of *G. hirsutum* have greatly increased quantities of caryophyllene oxide. These qualitative and quantitative differences in cotton volatile profiles are important in the host location and selection process of parasitoid and predator entomophages.

Although volatile mixtures of sesquiterpenes occur in glands throughout most parts of the plant (Bell et al., 1987), growing terminals, buds, and flowers of cotton are most attractive to the parasitoid, *Campoletis sonorensis* (Elzen et al., 1983). Elzen et al. (1984) report that this ichneumonid wasp is attracted by this terpenoid mixture to parasitize the lepidopteran tobacco budworm, *Heliothis virescens*, that attacks cotton. The terpenoid mixture in the atmosphere above cotton plants also has been shown to contain 50–60% γ-bisabolene and β-bisabolol and 30–40% of a group of other mono- and sesquiterpenoids, including caryophyllene and caryophyllene oxide (Hedin et al., 1978). The terpenes that attracted the parasitic wasp in laboratory bioassays were those similar to the ones found in the atmosphere, i.e., γ-bisabolene, β-bisabolol, as well as α-humulene, spathulenol, gossonorol, caryophyllene oxide, and all terpenes except caryophyllene. In fact, Bell et al. (1987) report the transfer of genes that control the ratio of γ-bisabolene and β-bisbolol to total terpenes and the caryophyllene–caryophyllene-oxide ratio in *G. hirsutum*.

Thus cotton provides another example of the differential role of caryophyllene and caryophyllene oxide within the terpenoid mixture, but at the tritrophic level in an agroecosystem (Table 3). *Campoletis sonorensis* in bioassays responds to caryophyllene oxide, although weakly so compared to the other terpenoids in the mixture, but has no response to caryophyllene. In field tests, caryophyllene

is an attractant for adult predatory green lacewings (*Chrysopa carnea*) whereas caryophyllene oxide is not. In contrast, the predator *Collops vittatus* is attracted by caryophyllene oxide but less so by caryophyllene. These particular tritrophic interactions are beneficial to the cotton plant; however, others may be detrimental (Hedin et al., 1978). For example, tobacco budworms fed high concentrations of the phenolic sesquiterpene dimer, gossypol, were not as favored as hosts for the parasitoid *Campoletis* (Williams et al., 1988), as those with low concentration, pointing again to the importance of quantitative variation of the mixture and of dosage effects. On the other hand, gossypol provides a significant defense in itself, being toxic to a variety of herbivorous insects—such as the tobacco budworm (Stipanovic et al., 1986), Egyptian cotton leafworm (*Spodoptera littoralis*) (Meisner et al., 1977) and the coleopteran boll weevil (*Anthomonus grandis*) (Singh and Weaver, 1972). In another scenario, caryophyllene and caryophyllene oxide again play a role, but in this case are directly toxic to the tobacco budworm; however, caryophyllene oxide, in contrast to caryophyllene, also synergizes the growth-inhibiting effects of higher concentrations of gossypol in the budworm (Gunasena et al., 1988). Thus there appear to be some conflicts between direct and indirect effects of plant defenses, but one or the other type of defense may be most effective under varying circumstances, and its defensive success may depend on the plant's dynamic capacity to adjust.

Entomophages on Other Crop Plants: Terpenoids Induced by Damage. The parasitic wasp *Cotesia marginiventris* that attacks the noctuid lepidopteran corn foliovore (*Spodoptera exigua*) provides another new example of attraction to volatile compounds, including terpenoids released from the plant (Turlings et al., 1991; Turlings and Tumlinson, 1992). In this case, terpenoids are released from both damaged and undamaged parts of the corn plant. The release from the entire plant allows the plant to emit a larger signal than from just the damaged part, and thus the plant increases its chances of attracting wasps. Turlings et al. (1991) suggest that *C. marginiventris* responds to a blend of the volatile chemicals from the host plant and that their response to a particular "odor blend" dramatically increases after a parasitoid experiences it in association with the host "by-product." These findings also imply the existence of some sort of internal communication system whereby damaged portions of the plant relay information to undamaged parts. Thus, Turlings and Tumlinson propose that terpenoids may provide a plant an equivalent to an immune system, an idea further supported by Takabayashi and Dicke (1993). Turlings et al. (1993) indicate that the release of the plant's volatiles (including the terpenoids) appears to be a general response to attack by phytophagous insects and propose that the chemicals serve multifunctional purposes that both directly and indirectly protect plants against herbivores and pathogens.

Dicke et al. (1990) have studied in some detail the role that herbivore-infected leaf volatiles (including seven terpenoids) from Lima bean plants (*Phas-*

eolus lunatus) and cucumber plants (*Cucumis sativus*) have played in attracting the predatory mite (*Phytoseiulus persimilis*) that attacks the spider mite (*Tetranychus urticae*). The study clearly displays the role of the plant in this system. The plant must attract predator mites at an early stage of spider-mite infection. Emission of the volatile mixture (including three terpenoids and an aromatic phenol) predator attractant generally occurs upon spider-mite damage, but similar to the corn scenario, is not restricted to damaged tissue (Takabayaski et al., 1991). Results indicated that these volatile predator-attracting allelochemicals primarily function in plant–predatory-mite interactions with the spider-mite interaction being secondary. Bruin et al. (1992) also found that plants in the neighborhood of the spider-mite-infested plants attract more predatory mites than control plants that have not experienced spider-mite infections, thus influencing the plant population beyond those being attacked.

Vandemeer (1980) hypothesizes that these indirect mechanisms of mutualism are probably more abundant than the traditionally recognized obligate and direct mutualisms. Phelan and Stinner (1992) extend Vandemeer's hypothesis, suggesting that as interactions become more multidimensional, the probability of evolving mutualistic associations increases.

Takabayashi et al. (This issue) summarize some of their research on the spider mites. Their work again shows the importance of mixtures. The different combinations of herbivore-induced terpenoids are shown for five plant species, and the emission of the terpenoids also varies within plant species depending upon: (1) plant cultivar, (2) leaf growth stage, (3) the herbivore species that is attacking, and (4) abiotic conditions (light intensity, time of year, and water stress). Predatory mites cope with this variation by innate recognition as well as temporary specialization to a certain mixture via learning.

Interactions in the Troposphere

The most common biogenetically produced terpenes reported in ambient air are α- and β-pinene and limonene (Table 2). More than a dozen monoterpenes, however, have been identified from agricultural and plant species occupying large areas in the Central Valley of California—some of which are also noted in Table 2 (Arey et al., 1991). Sesquiterpenes (including caryophyllenes) were emitted from one third of the 30 plants analyzed there. These terpenoids are being studied for their role in the transformation and transport of atmospheric ozone and aerosols, since they react rapidly with ozone, as well as hydroxyl and nitrate radicals (Lopez et al., 1988; Grosjean et al., 1992). Additionally, monoterpenes can compete with methane for oxidation by the hydroxyl radical. As monoterpenes increase in abundance, OH available for methane oxidation declines, which leads to an increase in the atmospheric lifetime of methane (Lerdau, personal communication).

Lerdau (1991) indicates that coniferous forests and tropical rainforests, which constitute approximately one quarter of the area of tropical ecosystems and almost one half of the world's net primary productivity, constitute the most important global sources of monoterpenes to the atmosphere. Zimmerman et al. (1988) and Rasmussen and Khalil (1988) further emphasize the role of tropical forests as being a "particularly significant source" globally of biogenically produced hydrocarbons, such as terpenes (including isoprene), that can influence ozone concentrations. The strength of this source changes as a consequence of land clearing: fires volatilize the hydrocarbons, and fast-growing plantation trees (particularly conifers) produce significant amounts of hydrocarbons. Importantly, whether ozone is produced or consumed during hydrocarbon oxidation depends on the concentration of nitric oxide (NO). If NO concentrations are high (greater than 10 parts per trillion by volume), there is net production of ozone (Crutzen and Andreae, 1985), which is generally the case in Amazonia. Vitousek and Mattson (1992) further indicate that the elevated tropospheric ozone concentrations in Amazonia, and other tropical areas with regional influences of biomass burning, can approach those caused by human activity in the eastern United States and northern Europe, where ozone levels are now sufficient to reduce plant growth (of both agricultural crops and forests) significantly. Since ozone affects species differently, Vitousek and Mattson (1992) suggest that alteration of the tropospheric atmospheric chemistry (to which terpenoids contribute) could alter tropical populations and communities and represent an additional stress to endangered tropical biota, even in remote areas. Furthermore, Lerdau (1991) suggests that biogenic monoterpenoids in Amazonia's troposphere could be the source of some of the observed acid deposition in rural areas there. These, of course, are indirect effects of terpenoids (Figure 2). How these indirect effects may impact ecosystem processes is discussed by White in this issue.

Influence on Other Plants and Soil Microbes

Terpenoids may produce effects on seeds and soil microbiota through volatilization, leaching from plants, or decomposition of plant debris (Figure 2). These interactions can significantly affect community and ecosystem properties, although studies of plant–plant chemical interactions have often been controversial because of difficulty in unambiguously demonstrating interference by chemical inhibition rather than through resource competition or other mechanisms (Harper, 1977).

Allelopathic Effects on Seed Germination. Fischer (1991) pointed out that it has long been known that essential oils and individual monoterpenes strongly inhibit seed germination and plant growth, and he listed 14 monoterpenes, various sesquiterpenes (especially sesquiterpene lactones), and a few diterpenoids and triterpenoids, which are implicated as allelopathic agents. Einhellig (1985)

suggested from several lines of evidence that allelopathic inhibition of plant germination typically occurs through combined (additive or synergistic) effects of compounds that alone would be below the threshold of inhibition. This again puts a premium on a mixture of compounds (either among terpenoids or with other classes of chemicals). Bradow and Connick (1990) have further indicated that seeds or seedlings stressed by plant volatiles in the root zone become more susceptible to attack by fungi and insects.

Picman and Picman (1984) have shown that the mixture of two major sesquiterpene lactones (parthenin and coronopilin) from the dry tropical-dwelling *Parthenium hysterophorus* has inhibitory effects on their own seedlings, as well as on older plants, at relatively low concentrations (0.1%). They conclude in this case that allelopathic interactions are involved in population regulation of *Parthenium*. On the other hand, germination inhibition by sesquiterpene lactones can be temporary, with duration determined by both the concentration of the toxins and the moisture in the environment. Thus another factor—control of the timing of germination—can affect a community property such as species abundance. Picman and Picman (1984) further indicate that, since *P. hysterophorus* occurs where rainfall is unpredictable or seasonal, the presence of such germination-controlling autotoxins should be favored by natural selection.

Although Fischer et al. (this issue) summarize a series of allelopathic studies on Florida upland and sandhill pineland communities, I want to emphasize several important points previously reported by them (Fischer et al., 1988) for the action of terpenoids in general: (1) selective action of the same monoterpenoid within a community, e.g., cineole being highly toxic to one grass (*Schizachyrium scoparium*) but not another (*Leptochloa dubia*), (2) seasonal variations in germination and growth inhibition (highest levels occurring during summer months of June and July), (3) high inhibitory effects of terpene mixtures on germination contrasted with stimulatory effects of pure terpene solutions, which suggests strong synergistic effects. Similar dramatic increases in inhibition of germination by terpenoid mixtures (camphor, pulegone and borneol) have also been reported by Asplund (1968, 1969).

Allelopathic Effects on Community Properties. Friedman (1988) has emphasized that allelopathic effects of terpenoids on seed germination and resulting effects on patterning of plant populations within communities have seemed particularly evident in dry regions, i.e., in Mediterranean or desert climates and associated vegetation. Not only are these the regions with the greatest occurrence of plants producing essential oils, but they are also areas where compounds can accumulate in the plant litter, soil, or seeds during the dry period (due to low rates of leaching and reduced activity of the soil microflora). Then the terpenoids may be released by rainfall during the rainy season and become inhibitory to the germinating seed. These conditions particularly affect annual herbs in these environments and may partially be responsible for zones free of these plants.

A dramatic example of zones free of annual herbs, influenced by terpenoids, was demonstrated by the early work by Muller and his students (Muller et al., 1964; Muller and del Moral, 1966) in California chaparral, i.e., those areas surrounding patches of *Salvia leucophylla* (Labiatae) and *Artemisia californica* (Compositae). Although six monoterpenes were identified from the shrubs, camphor and cineole were consistently indicated as possible inhibitors of germinating seeds of the annual plants. It was also assumed that these terpenes were absorbed and concentrated by seeds, as well as in particular kinds of soil, during the dry season and then became inhibitory when the rains induced germination. In this case, Muller and del Moral (1966) and Halligan (1975) hypothesized that there were synergistic relations among toxins and drought, soil types, and also small animal depredation in producing the free zones. In areas of California grassland, Muller (1966, 1969) suggested that allelopathy and cattle grazing were in alliance in conversion of the grasses to shrubland. He also pointed out that there is an apparent reduction in ecosystem properties, i.e., in both total biomass and annual increment of biomass between the young and vigorous outer tiers of shrubs and deteriorated interiors of older stands (Figure 2). Allelopathic effects of shrubs on the grassland species and autotoxic degeneration of old shrub areas thus are involved in shifts in productivity levels.

Annual-herb-free zones also appear around the aromatic shrub *Coridothymus capitatus* in the coastal hills of Israel, although the association of these labiate shrubs and the annual herbs is highly variable. Another interesting factor enters in this case in that Katz el al. (1987) suggest that essential oils (thymol, γ-terpinene, carvarol, p-cymene, and other minor mono- and sesquiterpenes) from the shrubs have inhibitory effects on the annual plants by acting together additively or synergistically with toxic effects of compounds produced by soil actinomycetes.

I again refer to the two co-occurring Florida plant communities whose different response to fire may partially result from allelopathy because of the implications regarding community and ecosystem properties. The sandhill or high pineland is an open woodland with *Pinus palustris* and *P. elliottii* occurring amid dense understory dominated by native grasses and few shrubs, in which surface fires occur every three to eight years. The sand pine scrub, dominated by *P. clausa*, is a fire-sensitive community that has more intense crown fires; however, with a dense endemic scrub layer nearly devoid of grasses or herbs, it only burns every 20–50 years. Richardson and Williamson (1988) have hypothesized that the fire-sensitive scrub community has a longer fire cycle than the pineland because some shrubs release a mixture of terpenoids that inhibit the growth of sandhill grasses—a major fuel for the surface fires. This conclusion as well as the release mechanisms of the allelopathic compounds from the labiate shrubs, their transport to the target species, and allelopathic activation of nontoxic agents are discussed in some detail by Fischer et al. (this issue).

In both the California chaparral and Florida pine scrub, one of the most significant effects of the allelopathy is on successional status of species abundance and diversity relative to the effects of fire, which ultimately affects the the patterning and structure of the community. In the chaparral, fire leads to rejuvenation of the community in the internal portion of the stand as the shrubs age and lose their inhibitory effect; however, in the pine scrub, fire results in the perpetuation of community status quo for longer periods than possible without inhibiting grass growth (Figure 2).

Weidenhamer et al. (1989) suggest that density-dependent toxicity is an additional factor to be considered in allelopathic effects, i.e., plants growing at low density have a larger amount of toxin available for uptake per plant than those at high density, where the toxin is diluted among many plants and each receives a proportionately smaller dose. This, of course, assumes a situation with plants also producing high amounts of allelochemicals, such as in the Florida scrub, some chaparral, and tropical desert communities. Weidenhamer et al. (1989) further suggest that in these communities the overall low plant densities, along with harsh environmental conditions and nutrient stress that intensify allelopathic effects, could increase the effectiveness of the phytotoxins and hence selective pressure for their production. Conversely, the occurrence of greater toxic effects could result in selective pressure on susceptible species to increase seed production and seedling establishment. Because plant species differ in their susceptibility to allelochemicals, the structure of such communities may be controlled by a complex three-way interaction between allelopathy, interspecific competition, and intraspecific density effects (Weidenhamer et al., 1989).

Allelopathic Effects on Crops and Abandoned Cropland. Allelopathic effects in agroecosystems on either crop plants vs. crop plants, weeds vs. crop plants, or crop plants vs. weeds have received considerable attention. Although most of these studies have not implicated terpenoids, some recent reports suggest that this possibility may have been overlooked in the past. For example, Picman (1986) showed the sesquiterpene lactones (parthenin and coronopilin) from *Parthenium hysterophorus*, which has become an aggressive weed in Australia and India, have seriously reduced crop plants such as wheat, peanuts, beans, and tomato there. Dried material or aqueous extracts from roots of *P. hysterophorus* also suppressed growth of three *Rhizobium* species in leguminous plants. The 1980 literature regarding bioassay studies of the inhibitory effects of terpenoids on agroeconomically important weeds has been reviewed by Fischer (1991).

Piqueria trinervia (Compositae) is a pioneer subshrub weed on abandoned cropland and open places in Mexico. In this case, another dimension is added in that monoterpene stereoisomers (piquerol A and B) inhibited germination and growth differentially on six weed species in bioassays (Gonzalez de Parra et al., 1981). The inhibition was dosage-dependent with piquerol A being a more active

root growth inhibitor and piquerol B being a greater stem inhibitor. The ultimate impact of these differences would be expected in the effect on secondary succession (Figure 2).

Impact on Soil Microbial Community and on Ecosystem Nutrient Cycling. Terpenoids in the soil may inhibit some bacteria or provide an energy source for others. Thus these compounds may be influencing the soil microbial community in different ways. For example, 4% of the heterotrophic bacteria in soils beneath *Umbellularia californica* could metabolize the predominant terpenoid umbellulone as a sole source of carbon and energy (S.E. Wood, unpublished data). In another paper in this issue, White discusses how monoterpenoids can influence processes of the nitrogen cycle and emphasizes a proposed mode of action for inhibiting nitrifying bacteria. He further hypothesizes that monoterpenoids may also affect some processes within the carbon cycle, which has similarities with the nitrogen cycle. Thus monoterpenoids may have significant consequences on nutrient cycling (Figure 2), particularly in low diversity forests, such as those dominated by conifers that also produce terpenoids in abundance. These potential effects add to the many other long-known proposed allelopathic influences on other community and ecosystem parameters (Figure 2).

Relatively large masses of polymerized terpenoid mixtures, such as some resins, can remain in the soil and are then found in sediments as a fossilized product, amber (Langenheim, 1990). Interestingly, the most copious production of resins that fossilize occurs from angiosperm and conifer trees in tropical and subtropical forests, where it would have been anticipated that the greatest microbial degradation of them would occur. However, polymerization in some taxa is activated by light and oxygen as these resins exude to the surface, and they rapidly become very resistant to degradation. Even smaller pieces of terpenoid material may occur dispersed in the soil (Simoneit et al., 1986). There is limited evidence that some lower terpenoids pass through the soil column and enter streams through ground water and runoff (S.E. Wood, unpublished data; M.C. Molles, J.R. Gosz, and R. G. Cates, unpublished report). Some terpenoids may even run off into marine estuaries where they may have further effects on the microbial community there (Button, 1984).

SUMMARY AND CONCLUSIONS

After having viewed Figure 2 incrementally, it now serves as a summary view in: (1) pointing to the significance of factors influencing variation of the mixture, (2) indicating various phytocentric allelochemical roles of these higher plant terpenoids, and (3) their contributions to properties of terrestrial communities and ecosystems. I want to emphasize again that in this phytocentric presentation the terpenoids for the producing plant are being considered as allo-

mones only and primarily with their role in ecological time, although obviously the discussion cannot be divorced from evolutionary events. The terpenoid role for defense of roots, for fruit and seed dispersers, etc., are not considered, as such a comprehensive discussion was not possible for this paper.

An emphasis throughout this presentation has been on the qualitative and quantitative variation of the terpenoid mixture that occurs among plant organs as well as during ontogeny and phenology of the plant. These mixtures can be sequestered as constitutive compounds in the plant or possibly be increased and diversified through induction caused by attack by herbivores or microbes. Although total quantities of terpenoids are clearly influenced by abiotic resource availability, variation in the composition of the terpenoid mixtures apparently is under more strongly genetic control. Both total quantity and composition may well be affected by biotic selection pressures determining the survival of the plant (Figure 2). Thus the terpenoid-producing plant is a highly complex, dynamic mosaic of variable chemistry.

Because individual components of a terpenoid mixture can affect many interactions, their effects can be considered broad spectrum. On the other hand, these effects can be selective—inhibiting one organism but not others within the same community or among communities. Furthermore, specificity of the compounds is increased by dosage effects, some of which also require additive or synergistic action to attain effective ecological impact. This kind of variation in terpenoid mixtures may well be significant in partially meeting the multidemands that the plant faces for defense.

The overall spatial and temporal patterns of distribution of these terpenoid mixtures within the plant are consistent with the defensive role attributed to them. Direct bitrophic-level plant defense against herbivores has been considered so important (especially in a coevolutionary plant–insect herbivore context) that it frequently has been the primary interaction studied and considered in chemical defense theories. In fact, this type of direct defense has often been considered to be the *raison d'être* of compounds, such as terpenoids (excepting those involved in primary plant metabolism). However, it has been suggested that a volatile compound that once served as direct plant defense against insects may subsequently serve in indirect defense, i.e., by attracting both predators and parasites. The increasing evidence for the importance of these tritrophic interactions in both natural and agroecosystems direct us to think again about the role of the plant in producing these chemicals. Additionally, increasing attention is being given to incorporating the importance of phytopathogens in discussion of plant resistance (e.g., Fritz and Simms, 1992). The fossil record, in fact, indicates that terpenoids were probably initially defensive against microbes in arborescent plants and subsequently became important in defense against fungi vectored by insects in early coniferous trees. Today induced resins in conifers appear to be more effective against the fungi vectored by bark beetles

than constitutive compounds (Raffa and Klepzig, 1992). The general topic of terpenoids as phytoalexins, however, is too extensive to be undertaken in this presentation.

In focusing on evidence for the qualitative and quantitative variation of the terpenoid mixture, I have pointed out that the defensive role of this kind of variation has not received adequate attention in the apparency models (Feeny, 1976; Rhoades and Cates, 1976) or the resource availability model (Coley et al., 1985). In fact, terpenoids have been considered in the apparency model to have an "all-or-nothing" ("qualitative") response and in the resource availability model are considered as "mobile" compounds; in both cases the effects were not thought to be dosage dependent. The lack of recognition of the compositional variation of terpenoid mixtures is particularly interesting in that Rhoades (1983) indicates that "any factor reducing defensive chemical variation within plant communities, between plants within a species or between different parts of the plant is likely to have a negative effect on plant defensive capabilities . . . ." Furthermore, he recognized that the study of variation was likely to include "the most exciting and potentially rewarding areas of current research in plant defense." The lack of inclusion of the role of compositional variation in chemical defense theories may result partially from the difficulty in quantifying this kind of variability. Although it obviously is easy to quantify the relative proportions of the mixture, the possible additive and synergistic effects are not as easily quantified (although multivariate statistical procedures are increasingly providing means of attacking this conundrum).

Recently, attempts have been made to integrate concepts regarding resource acquisition and allocation in higher plants for a better understanding of the production of plant defensive chemicals. This has led to assessment of the growth–differentiation balance hypothesis (Herms and Mattson, 1992; Tuomi, 1992), which is based on the premise that there is a trade-off between growth and differentiation (which includes production of defensive compounds). Thus, Lerdau et al. (1994) point out that this model is source driven, being solely concerned with relative availability of resources and not on either intrinsic or extrinsic demands on the plant. Lerdau et al. (1994), therefore, recognize that demands on the plant alter the patterns of allocation to defense. Their view is that demand-side processes complement those that are source driven by exerting additional controls over the flow of carbon to differentiated products, such as terpenoids. Growth–differentiation balance additionally does not explain why changes in resource availability or biotic pressures should trigger changes in the qualitative or quantitative composition of the mixture. It seems clear that future research will need to search for answers regarding genetic, biochemical, and ecological controls of the intraplant, and intra- and interpopulation variation in these terpenoid mixtures. Although Jones and Firn (1991) are concerned with diversification of all defensive secondary compounds, they have provocatively

suggested that "without detailed knowledge over controls of diversification, it may not be possible to guess the likely path of chemical evolution." This may well be true in just considering terpenoid variation.

Although terpenoids as semiochemicals may have evolved primarily as defense compounds, another focus in this presentation has been to direct attention to the other roles that this largest and most diverse group of chemicals play and their consequent contributing influence on plant community and ecosystem properties. First, certain terpenoids in the mixture may be recycled into the plant's primary metabolism during leaf senescence (following the assumed time of primary need for defense). Secondly, other compounds are emitted into the environment, either through volatilization, leaching, or decomposition of plant litter or by exudation onto the surface of organs, with volatilized compounds playing a major role in numerous interactions (Figure 2). Thus, these terpenoids may contribute significantly to community/ecosystem properties. I strongly emphasize again that terpenoids are only being considered as a contributing factor to the *many other* abiotic and biotic factors that determine plant community properties and ecosystem functions.

Although some ecologists may still question the impact of herbivory and disease on plant demography and community properties, others have presented evidence to support this (e.g., Crawley, 1989; Simms and Fritz, 1990; Fritz and Simms, 1992) (Figure 2). Evidence of the impact of terpenoids on insects and their vectored fungi and hence of their role on community and ecosystem dynamics in conifer forests is compelling. Increasing evidence regarding the impact of vertebrate herbivores also appears significant. Furthermore, the effect of terpenoids in tritrophic level indirect defense can affect not only plant community properties but also the food web in the ecosystem. Variability of terpenoid floral scents that attract pollinators, essential to the existence of many flowering plants, may affect competition for pollinators and hence plant species abundance and diversity.

The potential contributing effects of allelopathy on community and ecosystem properties has long been noted (Figure 2), despite studies that have been fraught with problems in distinguishing allelopathy from resource competition. The interference with germination of either their own progeny or unrelated plants by some terpenoids has been shown in certain cases to result in changes in the plant population structure and hence community patterning. It also has influenced successional dynamics, which in turn may relate to different levels of ecosystem productivity. Allelopathy additionally often seems to be related to the successional role of fire, but with effects varying in different communities. Terpenoid mixtures may also affect nutrient cycling by being a possible contributing factor to the inhibition of nitrifying bacteria in ecosystems with dominance of terpenoid-producing trees; they can further influence the soil microbial community by providing a carbon source for heterotrophic bacteria. In most

cases, however, where community or ecosystem effects have been noted, there appear to be interacting effects of the terpenes with some other conditions, such as drought, soil types, small animal depredation, interspecific competition, or intraspecific diversity effects.

In the troposphere, the lower terpenoids can be the source of some acid deposition, can interact with reactive gases to produce ozone, and their increase can also lead to an increase in the atmospheric lifetime of methane. Such changes in tropospheric composition can result in indirect effects of terpenoids on plant species diversity and thus on community structure (Figure 2). These tropospheric changes are often in response to human alteration of native vegetation. Furthermore, in plants subject to air pollution and increases in CO_2, the production of terpenoids is affected, which may have attendant effects on plants in communities and ecosystems—a topic which again cannot be addressed in this paper.

I predict that we shall see more explicit recognition of the kind of contributions that terpenoids make to community properties and ecosystem functions, as our knowledge increases regarding the kinds of interactions that terpenoids mediate. At this stage in the development of the field of chemical ecology, I have only attempted to integrate a discussion of major phytocentric roles of terpenoids by pointing out pathways of possible contributing effects at the plant community and ecosystem levels of ecological organization.

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METABOLIC COSTS OF TERPENOID ACCUMULATION IN HIGHER PLANTS

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Abstract—The net value of any plant trait can be assessed by measuring the costs and benefits associated with that trait. While the other contributors to this issue examine the possible benefits of terpenoids to plants, this article explores the metabolic costs of terpenoid accumulation in plants in the light of recent advances in terpenoid biochemistry. Terpenoids are more expensive to manufacture per gram than most other primary and secondary metabolites due to their extensive chemical reduction. The enzyme costs of making terpenoids are also high since terpenoid biosynthetic enzymes are apparently not shared with other metabolic pathways. In fact, plant cells may even possess more than one set of enzymes for catalyzing the basic steps of terpenoid formation. Terpenoids are usually sequestered in complex, multicellular secretory structures, and so storage costs for these substances are also likely to be substantial. However, not all of the processes involved in terpenoid accumulation require large investments of resources. For instance, the maintenance of terpenoid pools is probably inexpensive because there is no evidence that substantial quantities of terpenes are lost as a result of metabolic turnover, volatilization, or leaching. Moreover, plants may reduce their net terpenoid costs by employing individual compounds in more than one role or by catabolizing substances that are no longer needed, although it is still unclear if such practices are widespread. These findings (and other facets of terpenoid biochemistry and physiology) are discussed in relation to the assumptions and predictions of several current theories of plant defense, including the carbon–nutrient balance hypothesis, the growth–differentiation balance hypothesis, and the resource availability hypothesis.

Key Words—Terpenoid biosynthesis, terpenoid storage, secretory structures, metabolic turnover, volatilization, catabolism, carbon–nutrient balance hypothesis, growth–differentiation balance hypothesis, resource availability hypothesis.

INTRODUCTION

As the largest group of organic substances in the plant kingdom, terpenoids play a multitude of important physiological and ecological roles in higher plants. Terpenoid primary metabolites serve as hormones, membrane components, photoprotective pigments, and membrane-bound sugar carriers in glycoprotein and polysaccharide biosynthesis (Gershenzon and Croteau, 1993). Terpenoid secondary metabolites have been implicated in defense against herbivores and pathogens, allelopathic interactions, nutrient cycling, and attraction of pollinators, dispersers, and entomophages, as detailed by the other contributors to this issue. However, from an ecological perspective, any enumeration of the benefits of terpenoids to plants should be balanced by a consideration of their cost. Indeed, several lines of evidence indicate that the accumulation of terpenoids may entail substantial costs in terms of energy and nutrients. First of all, terpenoids are sometimes present in plants at very high concentrations, for example, 10–13% of dry weight in the leaves of *Eucalyptus dives* (Morrow and Fox, 1980) and 9–16% of dry weight in the juvenile twig internodes of *Betula resinifera* (Reichardt et al., 1984). Second, terpenoids are usually stored in complex secretory structures, such as resin ducts, glandular trichomes, or laticifers (Fahn, 1979). Third, certain terpenoids have been reported to undergo rapid metabolic turnover in plants (Seigler and Price, 1976), implying that continual synthesis may be necessary to maintain a fixed concentration. Fourth, inverse correlations sometimes exist between terpenoid accumulation and growth (Adzet et al., 1992; Hanover, 1966; Mathur et al., 1988). In *Cymbopogon winterianus*, for instance, a strong negative relationship was demonstrated between monoterpene content and aboveground biomass among a group of over 200 individuals (Mathur et al., 1988). Finally, there are indications that terpenoid defense compounds are reduced when there is less need for them. On the island of Puerto Rico, for example, where a certain specialist seed predator found on the Central American mainland is absent, the tropical legume *Hymenaea courbaril* has lower amounts of terpenoid resins in its seed pods than do conspecifics growing on the mainland (Janzen, 1975). Thus, costs are widely believed to influence the amounts of terpenes and other secondary metabolites found in plants and may at least partially explain terpenoid distribution patterns among species, individuals, and organs (Chew and Rodman, 1979; Coley et al., 1985; Fagerstrom, 1989; Gulmon and Mooney, 1986; Krischik and Denno, 1983; Rhoades, 1979).

The costs of terpenoid accumulation can be evaluated in an evolutionary context in terms of reproductive fitness or in a metabolic context in terms of resources, such as fixed carbon or nutrients. While fitness is a more relevant currency for assessing ultimate costs and benefits (Simms and Rausher, 1987), it is difficult to measure under natural conditions (Chapin, 1989). Costs expressed in terms of resources, sometimes referred to as metabolic costs, direct costs

(Gulmon and Mooney, 1986; Zangerl and Bazzaz, 1992), or allocation costs (Simms, 1992), may be easier to quantify and should help to identify the major physiological processes contributing to cost (Lerdau, 1993). This paper surveys the metabolic costs of terpenoid accumulation in higher plants in the light of recent progress in terpenoid biochemistry. The approach taken is, of necessity, quite speculative, since very little research has been directed specifically towards establishing the costs of terpenoid accumulation. However, within the last few years substantial advances have been made in our general understanding of terpenoid metabolism, yielding a wealth of new information on the pathways and enzymes of biosynthesis, and the sequestration, turnover, and catabolism of various terpenoids. These findings afford fresh insights into various aspects of the costs of terpenoid accumulation. In this review, I examine the costs of terpenoid biosynthesis, storage, and maintenance, and outline a number of mechanisms by which terpenoid costs may be reduced. Several current theories of plant defense are also discussed in the context of what is known about terpenoid costs.

BIOSYNTHETIC COSTS

Costs of Substrates and Cofactors

Terpenoids are formed from the fusion of five-carbon units having a branched, isopentanoid skeleton. Each unit is constructed from three molecules of acetyl-coenzyme A (acetyl-CoA) via the mevalonic acid pathway, a process that utilizes three molecules of ATP and two molecules of NADPH (Gershenzon and Croteau, 1993; Goodwin and Mercer, 1983). Following the initial condensation of five-carbon units, terpenoids undergo many types of cyclizations, couplings, and rearrangements to generate the basic representatives of each skeletal class. These products are then subject to a variety of ATP- or NADPH-mediated transformations, including oxidations, reductions, and conjugations, that eventually give rise to the many thousands of different terpenoid metabolites found in plants. Thus, terpenoid biosynthesis requires ample supplies of the substrate acetyl-CoA and the cofactors ATP and NADPH as raw materials.

Nearly 20 years ago, a young Dutch ecologist named F.W.T. Penning de Vries, developed a straightforward approach to calculating the costs of the raw materials involved in the synthesis of plant metabolites, based on procedures for estimating growth yield and efficiency in microorganisms (Penning de Vries et al., 1974). In these methods, which were later refined by Mooney and coworkers (Chiariello et al., 1989; Gulmon and Mooney, 1986; Merino et al., 1984; Williams et al., 1987), cost is determined by computing the amount of glucose required to provide all the substrates and cofactors consumed in biosynthesis. Glucose or another simple carbohydrate seems a suitable unit of currency for

such calculations, since carbohydrates are the usual storage and transport forms of fixed carbon in plants and can be readily respired to generate ATP and NADPH. Using this methodology, I have calculated the substrate and cofactor costs for the construction of a variety of plant terpenoids based on the latest available biosynthetic information. The results are presented in Table 1 as the quantity of glucose in grams needed to manufacture a gram of each terpene. In preparing this list, I attempted to choose only substances whose formation is fairly well understood, since the validity of these computations obviously depends on the accuracy of our biosynthetic knowledge. Considerable progress has recently been made in the study of terpenoid metabolism in plants (Cane, 1990; Croteau, 1987; Gershenzon and Croteau, 1993; Towers and Stafford, 1990). Nevertheless, for several classes of terpenes, it was difficult to find representatives whose biosynthetic pathway had been completely worked out, and so, for some of the compounds selected, inferences regarding intermediates and cofactor requirements had to be made based on analogies to better-studied compounds.

The calculated substrate and cofactor costs in Table 1 range from 3.54 g glucose/g for α-pinene and several other compounds to 1.99 g glucose/g for stevioside, a diterpene glycoside (Figure 1). Cost varies inversely with the degree of oxygenation, with highly oxygenated terpenes being much cheaper to make than terpenes with little or no oxygenation. For instance, among the monoterpenes, the highly oxygenated iridoid glycosides aucubin (2.39 g glucose/g, $C_{15}H_{22}O_9$) and antirrhinoside (2.32, $C_{15}H_{22}O_{10}$) are much less expensive to construct than the nonoxygenated olefins α-pinene, limonene, and myrcene (all 3.54, $C_{10}H_{16}$). Thus, on a weight basis, it may be cheaper for a plant to accumulate greater amounts of oxygenated terpenoids than nonoxygenated terpenoids, as long as the costs of catalyzing the additional enzymatic transformations are not excessively high. Of course, from a functional point of view, oxygenated and nonoxygenated terpenoids may have substantially different biological properties and are not necessarily interchangeable (Langenheim, this issue).

The overall costs of providing substrates and cofactors for terpenoid biosynthesis depend not only on the particular types of compounds produced, but also on the final concentration of terpenoids attained in plant tissue (Gulmon and Mooney, 1986). For example, among diterpenes, stevioside has a lower calculated cost per gram than abietic acid (1.99 g glucose/g vs. 3.26), so 1 g of stevioside is considerably cheaper to make than 1 g of abietic acid. However, the actual stevioside concentration in *Stevia rebaudiana* leaves is 3–8% of dry weight (Metivier and Viana, 1979), which is much greater than the 0.1% concentration of abietic acid found in the needles of *Larix laricina* (Ohigashi et al., 1981). Therefore, on a tissue basis, the cost of stevioside production in *S. rebaudiana* (60–160 mg glucose/g leaf) is about 20–50 times as high as that of abietic acid in *L. laricina* (3.3 mg glucose/g needles). Clearly, the outlay of

TABLE 1. SUBSTRATE AND COFACTOR COSTS FOR BIOSYNTHESIS OF SOME PLANT TERPENOIDS[a]

Compound (and corresponding number in Figure 1)	Molecular formula	Cost (g glucose/g)	References to its biosynthesis[b]
Monoterpenes			
α-Pinene (5)	$C_{10}H_{16}$	3.54	Gambliel and Croteau, 1984; Lewinsohn et al., 1992
Limonene (3)	$C_{10}H_{16}$	3.54	Alonso et al., 1992; Kjonaas and Croteau, 1983
Myrcene (1)	$C_{10}H_{16}$	3.54	Gambliel and Croteau, 1984
Menthone (4)	$C_{10}H_{18}O$	3.37	Croteau and Venkatachalam, 1986
Linalool (2)	$C_{10}H_{18}O$	3.12	Croteau and Gershenzon, 1994
Camphor (6)	$C_{10}H_{16}O$	3.10	Croteau and Shaskus, 1985; Dehal and Croteau, 1987
Iridoid glycosides			
Aucubin (7)	$C_{15}H_{22}O_9$	2.39	Jensen, 1991; Damtoft et al., 1993b
Antirrhinoside (8)	$C_{15}H_{22}O_{10}$	2.32	Jensen, 1991; Damtoft et al., 1993a
Sesquiterpenes			
Caryophyllene (9)	$C_{15}H_{24}$	3.54	Dehal and Croteau, 1988
Capsidiol (13)	$C_{15}H_{24}O$	3.43	Threlfall and Whitehead, 1991
Caryophyllene oxide (10)	$C_{15}H_{24}O$	3.35	
Germacrone (11)	$C_{15}H_{22}O$	3.34	Cane, 1990
Patchoulol (12)	$C_{15}H_{26}O$	3.25	Croteau et al., 1987a
Diterpenes			
Casbene (14)	$C_{20}H_{32}$	3.54	Dueber et al., 1978
Pachydictyol A (15)	$C_{20}H_{32}O$	3.35	
Manool (16)	$C_{20}H_{34}O$	3.32	West, 1981
Abietic acid (17)	$C_{20}H_{30}O_2$	3.26	Seaman et al., 1990; Funk and Croteau, 1994
Stevioside (18)	$C_{38}H_{60}O_{18}$	1.99	Graebe, 1987
Triterpenes			
β-Amyrin (19)	$C_{30}H_{50}O$	3.46	Goodwin and Mercer, 1983
Bryonolic acid (20)	$C_{30}H_{48}O_3$	3.34	Tabata et al., 1993
Cucurbitacin B (21)	$C_{32}H_{46}O_8$	2.87	Balliano et al., 1983
Papyriferic acid (22)	$C_{35}H_{56}O_8$	2.72	
Polyterpenes			
Rubber (23)	$(C_5H_8)_n$	3.54	Cornish, 1992; Patterson-Jones et al., 1990

[a] Costs were calculated as the quantity of glucose required to make all the starting materials, reactants, and cofactors necessary for biosynthesis using methods developed by Penning de Vries et al. (1974), Merino et al. (1984), and Williams et al. (1987). See Gershenzon (1994) for further details. The results are sometimes at variance with costs computed by other authors for the same compounds (Gulmon and Mooney, 1986; Lambers and Rychter, 1989; Williams et al., 1987) due to the use of newer biosynthetic information in the present treatment and to slight variations in some of the assumptions made regarding cofactor formation.

[b] General references to terpenoid biosynthesis are: Gershenzon and Croteau (1993), Porter and Spurgeon (1981), and Towers and Stafford (1990).

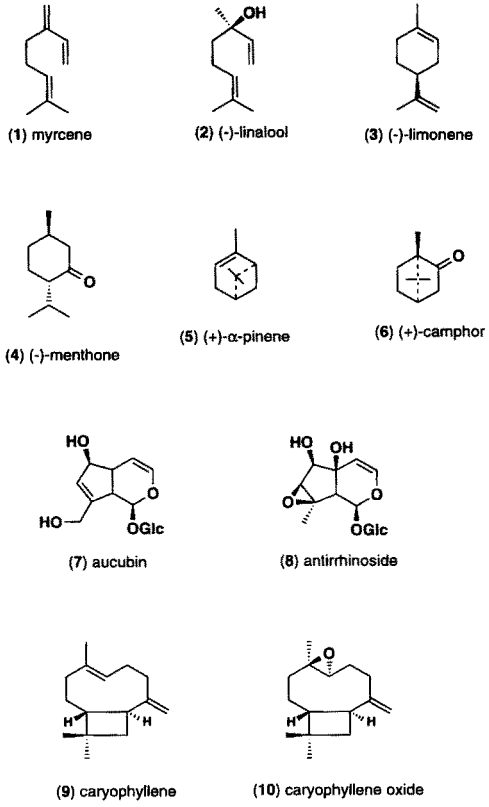


FIG. 1. Structures of compounds listed in Table 1.

resources to terpenoid biosynthesis is very strongly controlled by the actual concentration present in the plant.

Another factor that could affect the raw material costs of terpenoid formation is the type of cell in which biosynthesis occurs. In computing the glucose requirements in Table 1, terpenoid biosynthesis was assumed to occur in heterotrophic cells with NADPH arising from glucose via the pentose phosphate pathway and ATP being derived from glycolysis and the citric acid cycle coupled to mitochondrial electron transport (Gershenzon, 1994). However, in photosynthetic cells, both of these cofactors can be produced in the chloroplast by light-driven electron transport and transferred to other subcellular compartments by metabolic shuttles and membrane-bound translocators (Heldt and Flugge, 1987). Therefore, cofactor supply may be much less expensive in green tissue than in nongreen tissue (Chiariello et al., 1989; McDermitt and Loomis, 1981), and

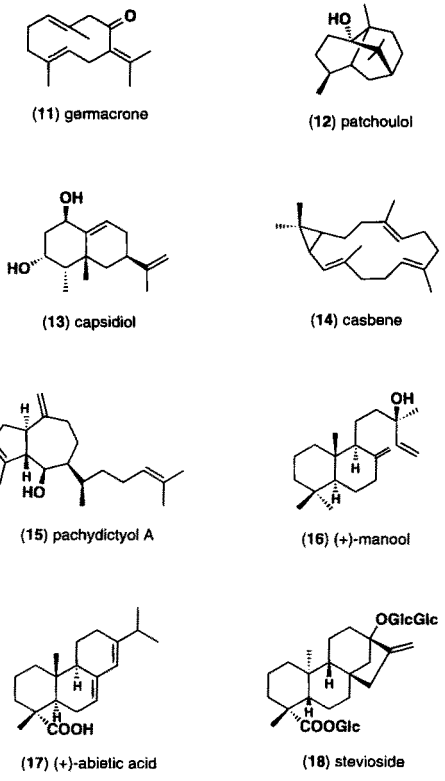
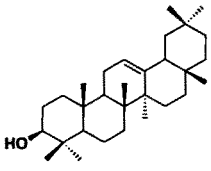


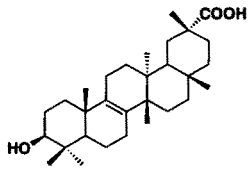
FIG. 1. Continued

hence terpenoids made in photosynthetic cells, such as carotenoids, phytol, or the diterpenes of *Nicotiana tabacum* (Keene and Wagner, 1985), could cost considerably less to manufacture than the estimates in Table 1 would indicate.

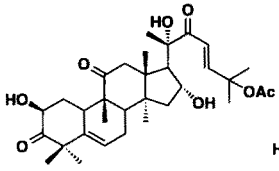
In comparison to other classes of plant secondary metabolites, terpenoids generally have greater raw materials costs due to their high degree of chemical reduction. The average cost of all the terpenoids listed in Table 1 (3.18 g glucose/g) is greater than the average costs of both a representative group of plant phenolics (2.11) and a selected group of nitrogen-containing secondary metabolites (2.27), but similar to the average cost of a representative group of alkaloids (3.24) (Table 2). Terpenoids are also more expensive than nearly all types of primary metabolites, including carbohydrates, organic acids, amino acids, and nucleotides. However, fatty acids, which, like terpenoids, are biosynthesized from acetyl-CoA units and have a high level of chemical reduction, are equally costly. It is important to note that all of these comparisons of raw



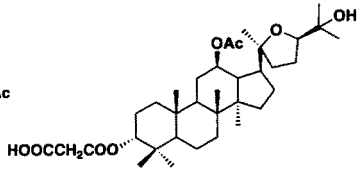
(19) β-amyrin



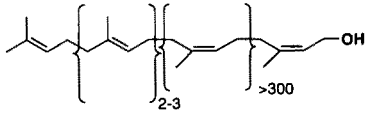
(20) bryonolic acid



(21) cucurbitacin B



(22) papyriferic acid



(23) rubber

FIG. 1. Continued

materials costs are based on the use of glucose as a standard currency. When the supply of nitrogen limits growth, nitrogen may be a more appropriate currency for evaluating costs (Chapin, 1989), and under such conditions nitrogen-containing metabolites could well become significantly more expensive to manufacture than terpenoids (Gulmon and Mooney, 1986).

Costs of Biosynthetic Enzymes

In addition to substrates and cofactors, the formation of terpenoids requires specific enzymes to catalyze the reactions of the biosynthetic pathway. Enzyme costs will depend, first of all, on the total number of enzymes needed, a quantity that varies with the length of the pathway and the extent to which enzymes are shared among several pathways. Enzyme costs are also contingent upon the characteristics of individual enzymes, such as their molecular weight, amino acid composition, catalytic efficiency, and turnover rate.

TABLE 2. AVERAGE SUBSTRATE AND COFACTOR COSTS FOR TERPENOIDS AND VARIOUS OTHER CLASSES OF PLANT PRIMARY AND SECONDARY METABOLITES[a]

Class	N	Cost (g glucose/g)	
		Mean	Range
Terpenoids	23	3.18	1.99–3.54
Primary metabolites			
Fatty acids	2	3.10	3.01–3.18
Amino acids	20	2.09	1.23–2.82
Nucleotides	4	1.59	1.27–1.80
Carbohydrates	5	1.07	1.00–1.11
Organic acids	4	0.73	0.61–0.87
Secondary metabolites			
Alkaloids	5	3.24	2.89–3.62
Other nitrogen-containing compounds[b]	8	2.27	1.70–2.83
Phenolics	9	2.11	1.28–3.39

[a] Raw data for terpenoids are from Table 1. Raw data for other classes of compounds are from Gershenzon (1994). Each class includes representatives of a variety of different skeletal types.
[b] Includes cyanogenic glycosides, glucosinolates, nonprotein amino acids, and proteinase inhibitors.

Length of Pathway. Terpenoids exhibit great variability in the overall length of their biosynthetic pathways. Using the examples in Table 1, the number of enzymatic conversions involved in terpenoid formation (starting from acetyl-CoA) ranges from nine for the sesquiterpenes caryophyllene and patchoulol, the diterpene casbene, and several monoterpenes to 23 for the iridoid glycoside antirrhinoside. In general, substances with a lesser degree of oxygenation require fewer steps, which may offset their higher substrate and cofactor costs (Table 1). Unfortunately, not enough is known about the properties of terpenoid biosynthetic enzymes to estimate the costs of additional enzymatic steps in a meaningful way. The expense of additional steps may be reduced if enzymes are not specific to one metabolic pathway but instead are shared among several pathways.

Enzyme Sharing among Different Pathways of Terpenoid Biosynthesis. The formation of all terpenoid metabolites begins with the seven basic reactions of the mevalonate pathway, from acetyl-CoA to dimethylallyl pyrophosphate (Gershenzon and Croteau, 1993). Hence, in theory a cell could employ a single set of mevalonate pathway enzymes for producing all of its terpenoid constituents. Nevertheless, recent research suggests that plant cells possess multiple sets of mevalonate pathway enzymes distributed among different subcellular compartments, although this is still a somewhat contentious issue (Bach, 1986; Gray,

1987; Kleinig, 1989; Schulze-Siebert and Schultz, 1987). Each compartment involved in terpenoid formation appears to produce a distinctive complement of terpenoid metabolites. Chloroplasts, for instance, synthesize carotenoids, tocopherols, and the phytol side chain of chlorophyll (Kleinig, 1989), while other types of plastids seem to be responsible for carrying out monoterpene and diterpene biosynthesis (Dudley et al., 1986; Gleizes et al., 1983). Elsewhere in the cell, the Golgi vesicles are thought to be the site of plastoquinone and ubiquinone formation (Swiezewska et al., 1993), while the cytoplasm and endoplasmic reticulum together produce sesquiterpenes, triterpenes, and sterols (Berlingheri et al., 1988; Kleinig, 1989). Whether all the steps of terpenoid formation beginning with acetyl-CoA actually occur in each location is still uncertain. Nevertheless, plants clearly do not economize by using a single set of biosynthetic enzymes to make all the terpenoids they require. Perhaps it is more critical for them to partition the various branches of terpenoid biosynthesis among separate subcellular locations so that the formation of different end products can be independently regulated.

Enzyme Sharing among Different Branches of Plant Metabolism. Enzyme sharing is also a possibility for certain enzymes of terpenoid biosynthesis that catalyze general reactions, such as the insertion of a hydroxyl group, the reduction of a carbon–carbon double bond, or the formation of glucoside linkage. Each of these reaction types could in theory be mediated by a single enzyme of low substrate specificity that participates in many different pathways, including those outside the realm of terpenoid metabolism. However, plants do not appear to reduce their biosynthetic costs in this manner. Most well-characterized enzymes of terpenoid biosynthesis that catalyze general types of reactions have high substrate specificities. For example, the hydroxylase that transforms (+)-sabinene to (+)-*cis*-sabinol during the biosynthesis of the monoterpene thujone in *Salvia officinalis* (garden sage) is very selective for its substrate (Karp et al., 1987). Eleven monoterpenes that are structurally related to (+)-sabinene were tested as possible substrates for this enzyme, but none was hydroxylated at a detectable rate (Figure 2). Several other hydroxylases of monoterpene biosynthesis have been extensively characterized, and these also appear to utilize only a limited range of substrates (Karp et al., 1990). In addition to hydroxylases, other classes of terpenoid biosynthetic enzymes exhibit high degrees of substrate specificity as well, including dehydrogenases (Dehal and Croteau, 1987; Kjonaas et al., 1985), keto-reductases (Kjonaas et al., 1982), and glucosyltransferases (Kalinowska and Wojciechowski, 1988; Paczkowski and Wojciechowski, 1985; Zimowski, 1991, 1992). Thus, most enzymes involved in terpenoid formation seem unlikely to participate in multiple metabolic pathways.

Turnover Rate. Plant proteins are subject to continuous degradation in vivo, with the half-lives of specific enzymes ranging from less than an hour to several weeks (Vierstra, 1993). Rapid turnover is thought to benefit an organism by

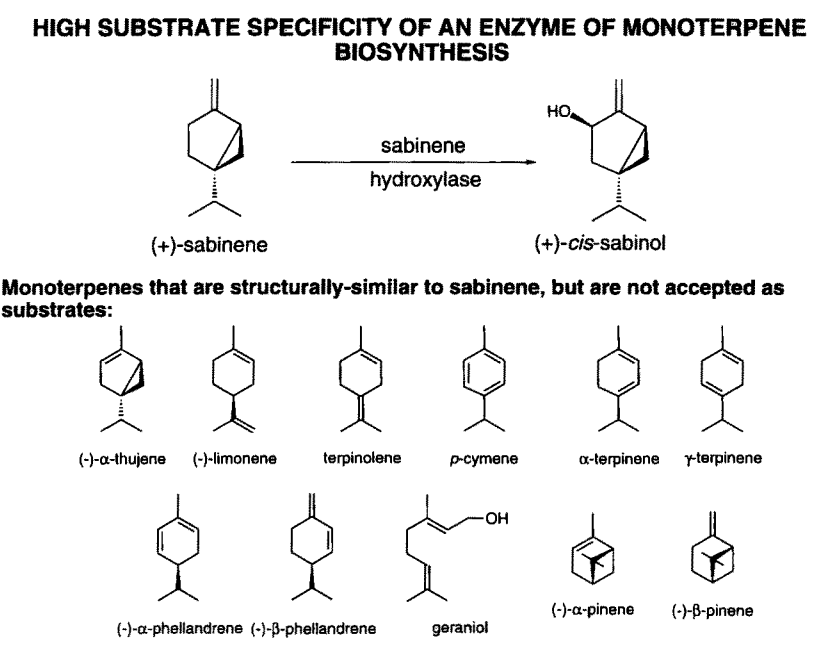


FIG. 2. Substrate specificity of a monoterpene biosynthetic enzyme. Sabinene hydroxylase converts (+)-sabinene to (+)-*cis*-sabinol, an intermediate in thujone formation. This enzyme catalyzes a general type of reaction (allylic hydroxylation), but displays great selectivity for its natural substrate, (+)-sabinene. When the 11 structurally related monoterpenes shown were tested as possible substrates, none was hydroxylated at a rate approaching that of (+)-sabinene (Karp et al., 1987).

allowing its biochemical machinery to quickly adapt to changing environmental conditions (Hawkins, 1991; Vierstra, 1993). However, this process could significantly raise enzyme costs. Unfortunately, no information is yet available on the turnover rate of any enzyme of terpene biosynthesis, except 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR), a well-studied enzyme of the mevalonate pathway that has been shown to have a PEST amino acid sequence in its structure (Bach et al., 1991; Caelles et al., 1989; Chye et al., 1992; Monfar et al., 1990). Such a sequence, named for its high content of proline (P), glutamine (E), serine (S) and threonine (T) residues, is thought to target proteins for rapid degradation (Rogers et al., 1986). It would not be surprising if HMGR was actually found to have a higher turnover rate than most other plant proteins, since this enzyme has been postulated to catalyze an important, rate-controlling step in terpenoid biosynthesis (Chappell et al., 1991); Gershenzon and Croteau, 1990, 1993). The rapid turnover of regulatory proteins should

be especially important in facilitating prompt metabolic adjustment to changing conditions (Hawkins, 1991; Vierstra, 1993).

Accurate measurements of enzyme turnover rates are essential for making realistic estimates of the cost of terpenoid biosynthesis in plants. However, in the absence of such information, it may be instructive to examine patterns of change in enzyme occurrence. If enzymes are only present in a plant for a short period of time, there will be less opportunity for them to undergo turnover, and their potential cost will be lower. We recently investigated the formation of monoterpenes in *Mentha* × *piperita* (peppermint) in relation to leaf development and found that monoterpene biosynthesis only occurs for a brief interval during the first two to three weeks of leaf ontogeny (J. Gershenzon and R. Croteau, unpublished results) (Figure 3A). When assays were carried out for the eight enzymes of the monoterpene pathway (Figure 4) between dimethylallyl pyrophosphate and menthone, the principal monoterpene in maturing peppermint leaves, it was discovered that these activities are uniformly high during the first few weeks of leaf development, but then decline to very low levels (Figures 3B and 3C). If it is assumed that changes in enzyme activity reflect changes in the amount of enzyme protein, the enzymes of monoterpene biosynthesis in *M.* × *piperita* are only present for a brief span of leaf development, thus minimizing the opportunity for turnover with its attendant costs of replacement.

Many terpenoid compounds are not constitutively present in plants, but are produced only in response to herbivore or pathogen attack (Puritch and Nijholt, 1974; Takabayashi et al., 1991; Tallamy and Raupp, 1991). The enzymes involved in the biosynthesis of these induced substances are also found to occur in plants for only restricted periods of time. They are readily apparent following herbivory or infection, but are usually not detectable in undamaged plants (Croteau et al., 1987b; Dudley et al., 1986; Gijzen et al., 1992; Vogeli and Chappell, 1990). For instance, *Abies grandis* (grand fir) produces large quantities of monoterpenes after wounding that have a different composition than the monoterpenes present constitutively in this species. These compounds serve as a defense against fungi and bark beetles (Gershenzon and Croteau, 1991). The enzymatic machinery responsible for making these wound-induced monoterpenes is not active in uninjured trees, and was only discernible several days after wounding (Gijzen et al., 1992). Another example of induced terpenoid synthesis is seen in tobacco cell cultures, where the application of a fungal elicitor preparation triggers the formation of capsidiol, a sequiterpene phytoalexin. Detailed studies of the enzyme that catalyzes the first committed step of capsidiol biosynthesis, 5-*epi*-aristolochene synthase, showed that this protein is not detectable at all by assay or immunoblotting in unelicited cultures, but is only observed after elicitor treatment (Vogeli and Chappell, 1990). Thus, many enzymes of terpenoid biosynthesis appear to be only transiently present in plants,

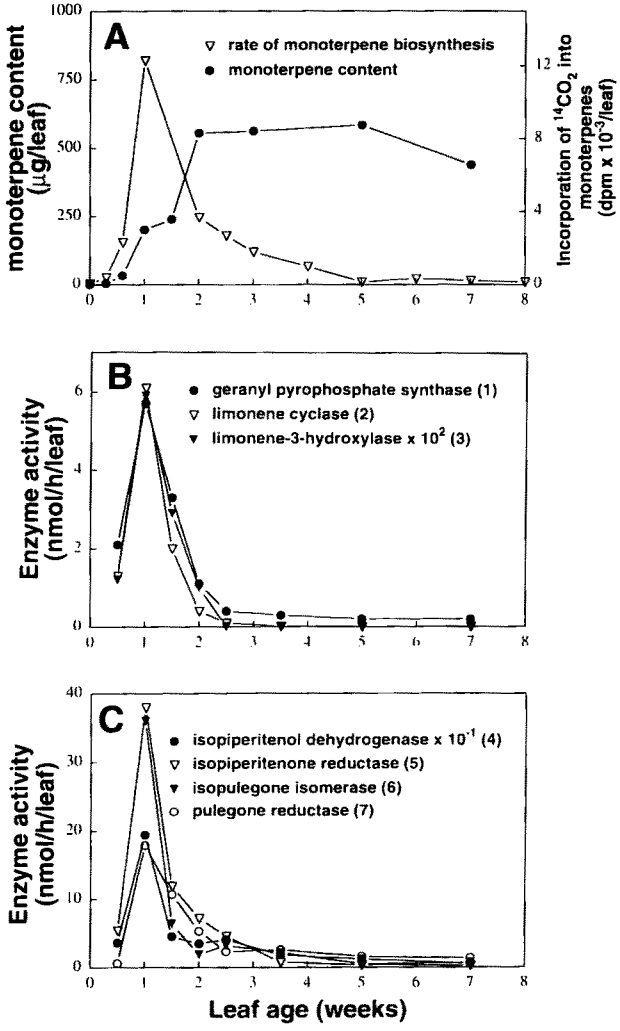


FIG. 3. (A) Changes in monoterpene content (left axis) and the rate of monoterpene biosynthesis from $^{14}CO_2$ (right axis) during leaf development in peppermint (*Mentha* × *piperita*). Leaves are fully expanded when two weeks old (see Figure 12). (B and C) Changes in the activities of monoterpene biosynthetic enzymes over the same time period. High rates of monoterpene biosynthesis and high levels of monoterpene biosynthetic enzyme activities are almost completely confined to a brief period during early leaf development. The pathway of menthone biosynthesis from isopentenyl pyrophosphate and dimethylallyl pyrophosphate is given in Figure 4, and the numbers after each enzyme refer to the reactions in this figure. (J. Gershenzon and R. Croteau, unpublished results.)

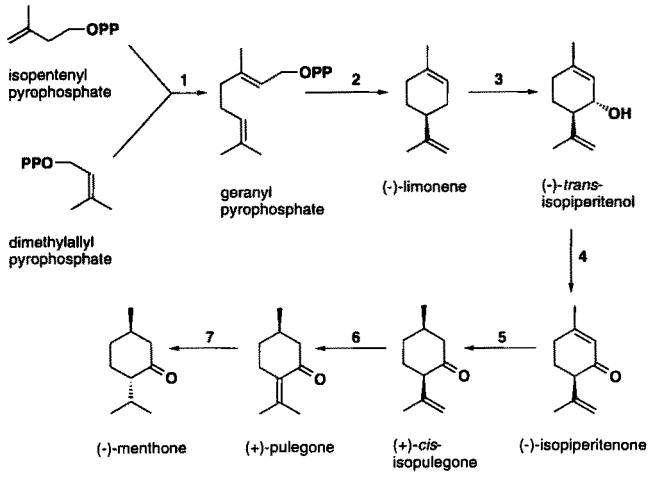


FIG. 4. The pathway of menthone biosynthesis in *Mentha* × *piperita* (peppermint) from isopentenyl pyrophosphate and dimethylallyl pyrophosphate. The names of the enzymes are given in Figure 3.

which reduces the likelihood of turnover and, consequently, minimizes the need for resources to be expanded on replacement.

Enzymes that Make Mixtures. Most terpenoid-accumulating plant species produce complex mixtures of structurally related terpenoid substances, rather than just a few individual compounds. For instance, the essential oils of mints (monoterpenes), the leaf pocket resins of *Hymenaea* (sesquiterpenes), and the irritant oils of members of the Euphorbiaceae (diterpenes) are all present as multicomponent blends of terpenoid metabolites (Lawrence, 1992; Langenheim et al., 1978; Evans and Soper, 1978). Mixtures have several important ecological advantages over the equivalent amount of a single substance, as discussed by Langenheim (this issue) and Gershenzon and Croteau (1991), but would also appear to possess distinct disadvantages due to the cost of the extra enzymes needed to generate the increased number of structures. However, in several terpene biosynthetic sequences, the final steps are catalyzed by enzymes that produce multiple products, indicating that the formation of mixtures need not always require additional enzymes. For example, several monoterpene cyclases, enzymes that convert geranyl pyrophosphate to various cyclic products, yield mixtures of monoterpenes (Alonso and Croteau, 1991; Croteau et al., 1987c; Gambliel and Croteau, 1984; Hallahan and Croteau, 1988; Lewinsohn et al., 1992; Rajaonarivony et al., 1992). (−)-Pinene cyclase from *Salvia officinalis* (garden sage) produces an assortment of five monoterpenes; (−)-camphene, (−)-α-pinene, (−)-β-pinene, myrcene, and (−)-limonene (Gambliel and Cro-

teau, 1984). γ-Terpinene synthase from *Thymus vulgaris* (thyme) forms significant amounts of eight other monoterpenes in addition to γ-terpinene (Alonso and Croteau, 1991) (Figure 5). With regard to sesquiterpene biosynthesis, an enzyme from *Pogostemon cablin* (patchouli) generates the alcohol patchoulol, an important raw material in perfumery, as well as the structurally related olefins α- and β-patchoulene, α-bulnesene, and α-guiaene (Munck and Croteau, 1990). In many of these cases, experiments with specifically deuterated substrate analogs have provided evidence for isotopically sensitive branching (Croteau et al., 1987c; Rajaonarivony et al., 1992; Wagschal et al., 1991), demonstrating that the ability to make mixtures is not due to the fortuitous copurification of several similar enzymes, but to the actual synthesis of multiple products by a single enzyme. The presence of such enzymes in plants may be a direct result of natural selection for the capacity to make mixtures or simply an accident of the reaction mechanism employed. Regardless of how they arose, multiproduct enzymes could certainly lower the costs of producing mixtures of terpenoids.

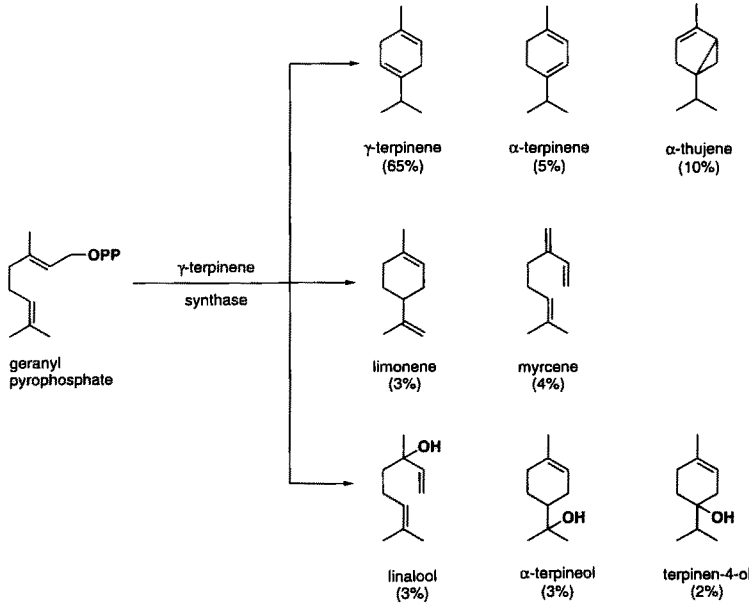


FIG. 5. Products of γ-terpinene synthase, a monoterpene cyclase from *Thymus vulgaris* (thyme) that synthesizes a mixture of monoterpenes from the substrate geranyl pyrophosphate. The numbers in parentheses represent the proportion of each product in the total mixture (Alonso and Croteau, 1991). Multiproduct enzymes of this type could lower the costs of producing mixtures of terpenoids.

STORAGE COSTS

Terpenoid accumulation in plants is generally restricted to specialized secretory structures of which there are several different types: (1) Glandular trichomes are multicellular epidermal hairs, found in species of the Lamiaceae, Asteraceae, Solanaceae, and other families, that secrete terpenes into an extracellular cavity situated at the apex of the trichome (e.g., Kelsey et al., 1984; Meyberg et al., 1991; Werker et al., 1993) (Figure 6A). In some species, the cuticle covering the extracellular cavity ruptures, allowing the secreted terpenoids to volatilize or to exude onto the plant surface and form a resinous coating (Dell and McComb, 1975; Werker et al., 1985). (2) Secretory cavities and secretory pockets are internal structures in which terpenes accumulate in large spherical intercellular spaces lined by a layer of specialized secretory cells (Langenheim et al., 1982; Russin et al., 1988; Thomson et al., 1976) (Figure 6B). These structures are common in the Leguminosae, Myrtaceae, and Rutaceae, among other families. (3) Resin ducts are similar to secretory cavities, but the intercellular spaces are extensively elongated (Bhatt, 1987; Charon et al., 1986; Stahl-Biskup and Wichtmann, 1991) (Figure 6C). Terpenoid-containing resin ducts are known from many families, including the Pinaceae, Burseraceae, and Apiaceae. (4) Laticifers represent another type of duct system derived from an elongated cell or series of interconnected cells in which terpenoids are part of a specialized cytoplasmic suspension known as latex (de Fay and Jacob, 1989; Mahlberg et al., 1987; Roy and De, 1992). Terpene-accumulating laticifers have been described in members of the Apocynaceae, Asclepiadaceae, Euphorbiaceae, and Asteraceae. (5) Secretory idioblasts, in contrast to the multicellular structures discussed so far, are individual, terpene-accumulating cells. Found in species of the Magnoliaceae, Lauraceae, Piperaceae, and related families, these cells are typically much larger than their neighbors, and contain an oil drop that nearly fills the cell at maturity (Bakker et al., 1991; Mariani et al., 1989; Platt and Thomson, 1992).

The metabolic costs of terpenoid storage are difficult to quantify precisely, but the construction and upkeep of such complex secretory structures clearly require substantial amounts of resources. First of all, the structures involved in terpenoid storage are usually composed of a large number of cells comprising several different types. For example, in the secretory cavities of *Tagetes erecta* (African marigold), the lumen of the terpenoid-filled cavity is lined by a ring of epithelial cells that synthesize and release the accumulated substances (Russin et al., 1992) (Figure 6B). The epithelial cells are in turn surrounded by two to three layers of tightly packed, radially flattened cells that form a sheath, which serves to confine the secretion and strengthen the cavity against collapse. A second factor contributing to high terpenoid storage costs is that, in *T. erecta* and most other terpenoid-accumulating species, the cells of secretory structures

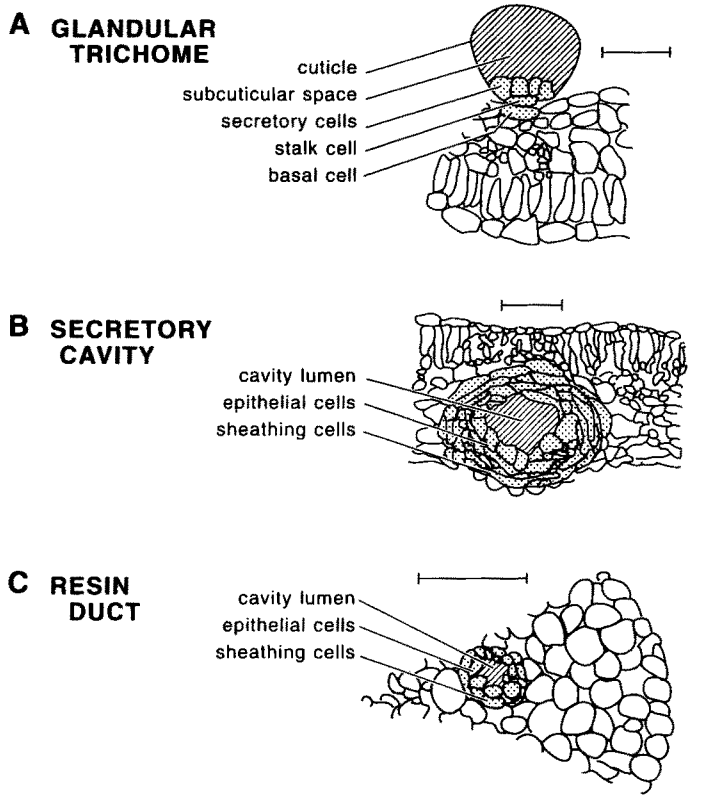


FIG. 6. Sketches of three types of specialized, terpenoid-accumulating secretory structures illustrating the specialized cells associated with each structure (dotted region) and the location of the terpene secretion product (hatching). (A) Transverse section of a *Mentha* × *piperita* (peppermint) leaf showing a peltate glandular trichome. This structure accumulates principally monoterpenes, which are synthesized by the secretory cells and deposited in the subcuticular space. The stalk cell supports the trichome, and the basal cell anchors it to the epidermis. Length of the bar is 50 μm. (Drawn from a photograph by J. Gershenzon and M. Maffei.) (B) Transverse section of a *Tagetes erecta* (African marigold) leaf showing a secretory cavity. This structure accumulates a secretion consisting principally of indole and two monoterpenes (Russin et al., 1988). Synthesis occurs in the epithelial cells, the first layer of cells surrounding the cavity lumen. The sheathing cells function to strengthen the cavity wall. Length of the bar is 100 μm. (Redawn from Russin et al., 1992.) (C) Cross section of a young stem segment of *Artemisia dracunculus* (French tarragon) showing a resin duct. This structure accumulates an oil made up of both monoterpenes and phenylpropanoid constituents. The specialized cells shown have roles similar to those described for the specialized cells of the secretory cavity. Length of the bar is 50 μm. (Redrawn from Cotton et al., 1991.)

are seldom capable of photosynthesis, and so need a continuous infusion of fixed carbon for growth and maintenance. Third, the individual cells of secretory structures are usually highly differentiated, possessing very specialized morphological features. In the glandular trichomes of *Mentha* × *piperita* (peppermint), for instance, the outer walls of the stalk cells are thickened and highly cutinized, and so have been suggested to function in supporting and strengthening the gland (Amelunxen, 1965) and in restricting the apoplastic loss of water (Schnepf, 1974) (Figure 6A).

Given that terpenoid storage in complex secretory structures appears to impose significant costs on plants, it is appropriate to consider the possible benefits of this practice. The various modes of terpenoid storage seem to have been selected for their ability to further the ecological roles of these substances. For example, for terpenoids that serve as antiherbivore defenses, accumulation in glandular trichomes on the plant surface may represent an adaptation that provides increased protection against small herbivores, such as insects or mites. High concentrations of defensive substances on the surface could present a very effective barrier against small herbivores, deterring feeding before any significant damage occurs. Deposition in glandular trichomes may also facilitate the volatilization or leaching of terpenoids into the environment, and so enhance the ability of these compounds to attract entomophages and pollinators or act in allelopathy. Turning to other types of storage compartments, the utilization of secretory cavities, resin ducts, or lactifers appears to increase the effectiveness of terpenoids in antiherbivore defense because the contents of these secretory structures are usually stored under pressure. Thus, when an herbivore severs a cavity, duct, or laticifer, the contents flow toward the cut surface, creating a high concentration of terpenoid defenses at the point of attack (Dussourd and Denno, 1991).

Terpenoid storage may also serve to limit the risk of toxicity to the plant itself, since there is evidence that many terpenoids are potentially poisonous to plant tissues. For example, when secretory structures containing monoterpenes are disrupted and release their contents, significant injury sometimes occurs to surrounding cells (Loveys et al., 1992; Shomer and Erner, 1989). Injury was also found to result when certain sesquiterpenes and triterpenes were artificially applied to leaves during tests of their ability to deter herbivore feeding (Asakawa et al., 1988; Polonsky et al., 1989). Further indications of terpene toxicity to plants come from the multitude of allelopathic investigations that have demonstrated that monoterpenes and sesquiterpenes prevent seed germination and inhibit root and shoot growth (Fischer, 1986, 1991). These activities have been ascribed to several factors, including the disorganization of cellular membranes and the inhibition of respiration (Muller, 1986). Consequently, the sequestration of terpenoids in specific compartments away from sensitive metabolic processes may be essential for avoiding any harmful effects. Among the secretory structures

discussed, glandular trichomes, secretory cavities, and resin ducts all secrete terpenoids outside the plasmalemma into extracellular spaces (Fahn, 1979). In secretory idioblasts, the whole cell is surrounded by a special suberized wall that prevents leakage into neighboring cells via symplastic or apoplastic routes (Bakker and Baas, 1993). Moreover, in all types of secretory structures, terpenoids appear to be synthesized by the cells of the structure itself (Gershenzon et al., 1989; Keene and Wagner, 1985) rather than being imported from elsewhere in the plant, which should help reduce exposure to these potential toxins. Therefore, the synthesis and localization of terpenoids in secretory structures appear to help plants accommodate the presence of these potentially toxic substances in their tissues.

MAINTENANCE COSTS

Terpenoid levels in plants may decline as a result of metabolic turnover, emission to the atmosphere, or the leaching action of rainfall or dew. If any of these processes lead to substantial losses of terpenoids, it may be essential for plants to produce extra quantities of terpenoids in order to retain an effective concentration in their tissues or maintain a suitable rate of volatilization. The need for such additional synthesis could significantly increase the cost of terpenoid accumulation. In this section, I survey the incidence of terpene losses from living plants due to metabolic turnover, volatilization, and leaching, and assess the possible need for additional synthesis.

Metabolic Turnover

A variety of plant terpenoids appear to be metabolically transformed or degraded at some time after their initial accumulation. Evidence for such "metabolic turnover" comes chiefly from pulse-chase studies in which the radioactivity incorporated into terpenes from administered precursors is lost, sometimes within a very short time following the initial pulse (e.g., Banthorpe and Ekundayo, 1976; Breccia and Badiello, 1967; Francis and O'Connell, 1969). In addition, reports of large diurnal variations in terpenoids (Adams and Hagerman, 1977; Adzet et al., 1992; Hopfinger et al., 1979) and of sizable decreases in terpenoid accumulation at various stages of plant development (Croteau, 1988; Dolman et al., 1992; Flesch et al., 1992) have also been taken as evidence that these substances are metabolically labile.

Turnover may have a sizable impact on the metabolic costs of terpenoid accumulation. First of all, the degradation of terpenoids may allow plants to recoup some of the resources originally invested in the synthesis and storage of these substances, and so could reduce net costs (see under "Catabolism," below). On the other hand, in many species, rapid terpene turnover has been observed

to occur at a time when there is no decline in the total amount of terpenes present (Burbott and Loomis, 1969; Croteau and Loomis, 1972; Croteau et al., 1972a), implying that pools of these substances are in a state of flux, subject to continual degradation and resynthesis. Turnover of this sort could significantly increase the expense of maintaining a particular concentration of terpenes, and, in fact, several authors have theorized that rapid metabolic turnover is a major component of the cost of plant defenses in general (Coley et al., 1985; Fagerstrom, 1989; Fox, 1981; Gulmon and Mooney, 1986; Skogsmyr and Fagerstrom, 1992).

The most frequently cited example of rapid terpene turnover is the synthesis and loss of monoterpenes in *Mentha* × *piperita* (peppermint) stem cuttings (Burbott and Loomis, 1969; Croteau and Loomis, 1972; Croteau et al., 1972a). In this tissue, up to 90% of the monoterpenes labeled with a pulse of radioactive CO_2, glucose, or mevalonic acid are observed to be lost within a 10-hr chase period, even though the total quantity of monoterpenes present does not appreciably change. We recently repeated these experiments using both *M.* × *piperita* cuttings and rooted plants to check whether the metabolic lability of monoterpenes in cuttings differs from that in intact plants (Mihaliak et al., 1991). While the monoterpenes of stem cuttings were found to be turned over rapidly following a single brief pulse of $^{14}CO_2$, as previously reported, rooted plants did not exhibit any significant losses of radiolabeled monoterpenes during the 29 hr time course of the experiment (Figure 7). These results indicate that rapid monoterpene turnover probably does not normally occur in *M.* × *piperita* shoots, but is an artifact seen only in detached cuttings.

Next, we performed similar experiments using only rooted *M.* × *piperita* plants, but extending the sampling period to six weeks after the $^{14}CO_2$ pulse (J. Gershenzon and R. Croteau, unpublished results). While the weight and monoterpene content of leaves increased steadily over this period (Figure 8), there was no significant change in the radioactivity of extractable monoterpenes after the initial increase of the first two days. Hence, in intact *M.* × *piperita*, monoterpenes do not undergo any measurable turnover for at least 40 days after synthesis. Additional evidence for this metabolic stability comes from the changes in monoterpene content and biosynthesis observed during *M.* × *piperita* leaf development (Figure 3). As leaves age, the rate of monoterpene biosynthesis from $^{14}CO_2$ and the activities of biosynthetic enzymes drop to very low levels, but, since monoterpene content shows no significant change, the rate of turnover must be extremely low.

More recently, we have extended our studies on intact plants to several additional species: *Melaleuca alternifolia* (Australian tea tree, Myrtaceae), *Tanacetum vulgare* (common tansy, Asteraceae), *Salvia officinalis* (garden sage, Lamiaceae), and *Pinus contorta* (lodgepole pine, Pinaceae) (Gershenzon et al., 1993). These species were chosen to span a range of taxonomically distant,

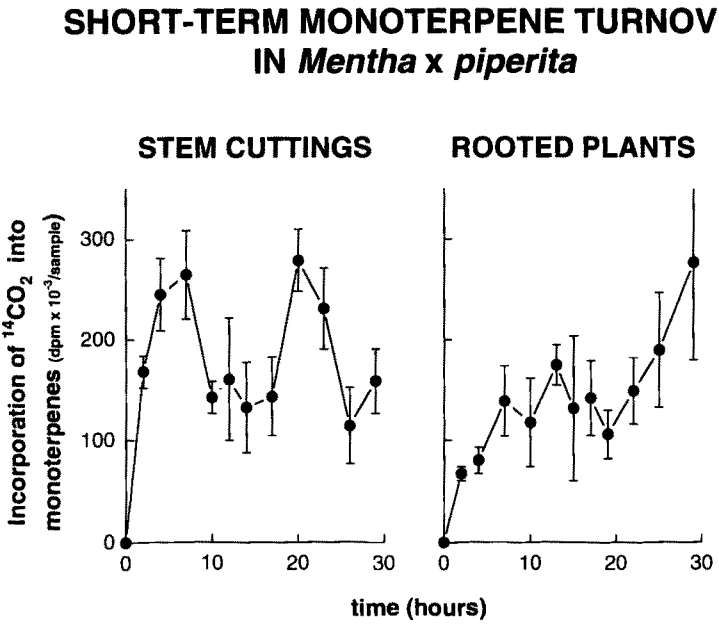


FIG. 7. Short-term incorporation of $^{14}CO_2$ into the leaf monoterpenes of *Mentha* × *piperita* (peppermint) stem cuttings and rooted plants. Plants were exposed to $^{14}CO_2$ for 5 min in a sealed Plexiglas chamber under high intensity lights. After the pulse, samples were harvested periodically over the next 29 hr and subjected to simultaneous steam distillation–pentane extraction to isolate the monoterpenes. Extracted monoterpenes were quantified by gas–liquid chromatography, and the incorporation of ^{14}C was measured by liquid scintillation counting. For both cuttings and rooted plants, there were no significant alterations in leaf monoterpene content during the course of the experiment (data not shown). However, dramatic changes were observed in the level of ^{14}C found in the monoterpene pool. In stem cuttings, monoterpenes exhibited pronounced metabolic turnover. There were two episodes of ^{14}C incorporation and loss, with peaks of incorporation (at 7 and 20 hr) differing significantly (Tukey's studentized range test, $P < 0.05$) from subsequent sampling intervals (10–17 and 26–29 hrs, respectively). Rooted plants, on the other hand, showed only a steady increase in ^{14}C incorporation into monoterpenes over the time span of the experiment with no significant losses of radioactivity ($P > 0.05$). Each point represents the mean (bars indicate standard error) of three samples, each consisting of the apical tip of five separate plants (Mihaliak et al., 1991).

terpene-accumulating plant families, to include plants that contain sesquiterpenes and diterpenes as well as monoterpenes, and to encompass taxa that use a variety of different types of secretory structures for terpene storage. As before, each of these species was subjected to a single, brief pulse of $^{14}CO_2$, but in this case

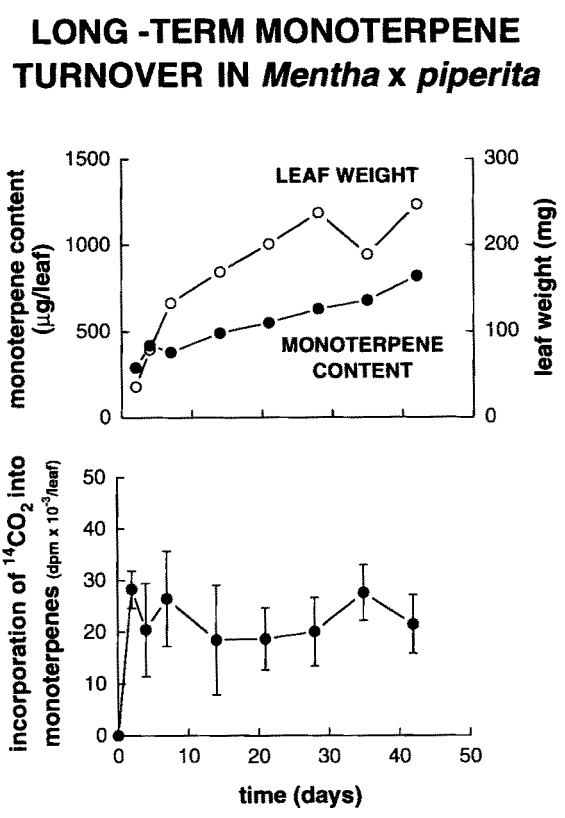


FIG. 8. Long-term incorporation of $^{14}CO_2$ into the leaf monoterpenes of rooted *M.* × *piperita* plants. This experiment was performed in a similar fashion to that depicted in Figure 7, except that after the 5-min pulse of $^{14}CO_2$, plants were sampled for six weeks. Upper panel: Leaf weight and monoterpene content steadily increased over the course of the experiment. Lower panel: ^{14}C incorporation into monoterpenes did not change significantly (Tukey's studentized range test, $P > 0.05$) after the initial increase of the first two days, indicating the lack of any measurable turnover. Each point represents the mean (bars indicate standard error) of three samples, each consisting of a pair of leaves from a single stem (J. Gershenzon and R. Croteau, unpublished results).

the incorporation of radioactivity into terpenes was followed over a period of 10–14 days. No significant terpene turnover was detected in any of these species (Figure 9) regardless of taxonomic affinity, anatomical features, or the types of terpenes accumulated.

Our results contrast with those of many other investigations in which terpene turnover has been measured. Most previous pulse-chase labeling studies

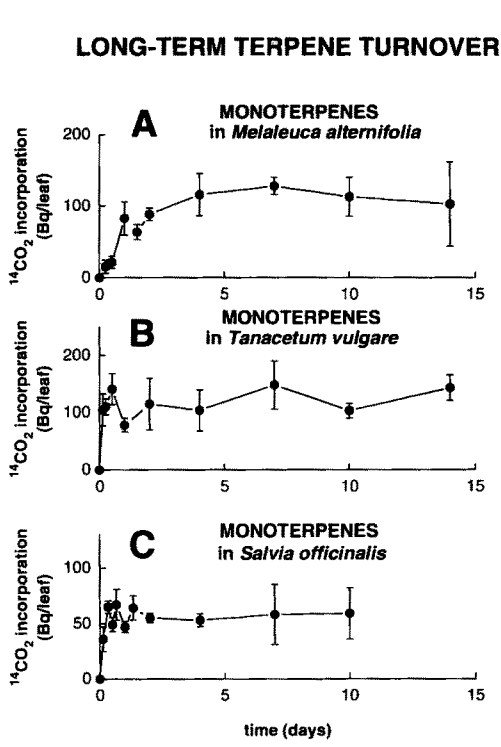


FIG. 9. Long-term incorporation of $^{14}CO_2$ into (A) the monoterpenes of *Melaleuca alternifolia* (Australian tea-tree), (B) the monoterpenes of *Tanacetum vulgare* (common tansy), (C) the monoterpenes of *Salvia officinalis* (garden sage), (D) the monoterpenes of *Pinus contorta* (lodgepole pine), (E) the sesquiterpenes of *S. officinalis* (garden sage), and (F) the diterpenes of *P. contorta*. Experimental methods were as in Figures 7 and 8, except that the diterpene resin acids of *P. contorta* were isolated by soaking in *tert*-butyl methyl ether for two days at room temperature and analyzed by gas–liquid chromatography after methylation with diazomethane. The level of ^{14}C did not decline significantly (Tukey's studentized range test, $P > 0.05$) over the course of these experiments for any of the species or terpene types studied. Hence, none of the substances investigated appears to undergo metabolic turnover. Each point represents the mean of at least three samples (bars indicate standard errors) (Gershenzon et al., 1993).

of terpenes have reported rapid rates of turnover, with the compounds of interest having half-lives of less than 24 hr, assuming first-order exponential decay and a complete mixing of metabolic pools (Banthorpe and Ekundayo, 1976; Breccia and Badiello, 1967; Burbott and Loomis, 1969; Croteau and Loomis, 1972, 1973; Croteau et al., 1972a; Francis and O'Connell, 1969; Hefendehl et al., 1967; Njar et al., 1989; Scora and Mann, 1967). However, the majority of

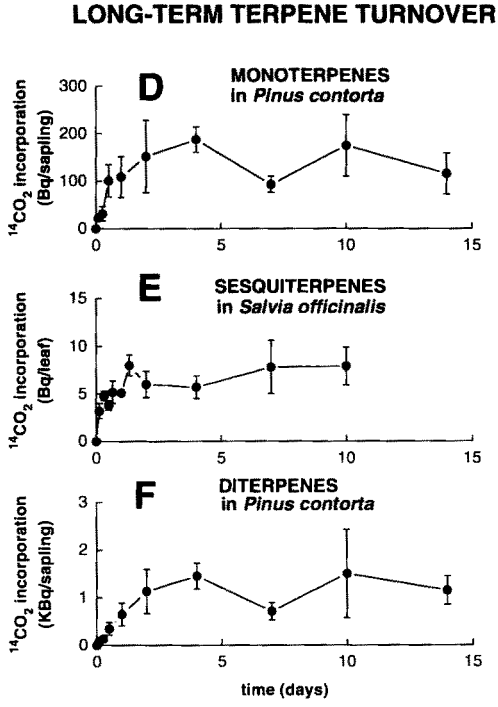


FIG. 9. Continued

these experiments were carried out with detached shoots, foliage, or flowers, rather than intact plants, and so may have limited validity, since the rapid monoterpene turnover previously described in detached *M.* × *piperita* shoots was shown to be an artifact that does not occur in rooted plants (Mihaliak et al., 1991). If one only considers studies in which pulses were administered to intact plants (Mihaliak and Lincoln, 1989; Regnier et al., 1968; Sukhov, 1958), terpene turnover appears to proceed at a rate that is either slow (half-life of 5–170 days) or undetectable, in general agreement with the results of our own investigations. Thus, terpenoid pools do not appear to be readily turned over in intact plants. Since most terpenoids accumulate in extracellular compartments of secretory structures, away from living cytoplasm (as discussed above under "Storage Costs"), their metabolic stability is not surprising. However, many additional species and types of these substances must be surveyed to confirm this trend.

Volatilization

Many monoterpenes and sesquiterpenes are quite volatile and so could be continuously released from plant foliage at a substantial rate. However, the magnitude of volatilization from living tissue seems to be quite small. With the exception of emission from flowers, the rates of terpenoid volatilization from foliage recorded under natural conditions (e.g., Janson, 1993; Lamb et al., 1987; Tingey et al., 1991) are much too low to cause significant declines in overall plant terpenoid levels. For example, in two species of conifers, *Picea abies* (Norway spruce) and *Pseudotsuga menziesii* (Douglas-fir), extrapolation from volatilization rates measured over short periods suggests that only 4–5% of the total monoterpenes present in the plant are emitted into the atmosphere over a typical six month growing season (Schindler and Kotzias, 1989). Among herbaceous species, *Salvia mellifera* (black sage) appears to volatilize just a little over 1% of its leaf terpene pool each month (Tyson et al., 1974).

Leaching

Another possible route of terpenoid loss from plants is the leaching of metabolites from aerial parts due to rainfall, mist, fog, or dew. Although terpenoids are generally thought to be highly insoluble in water, this generalization does not apply to all substances of this class. Among the monoterpenes, for instance, glycosides, including the large family of iridoid glycosides, show pronounced water solubility, while many simple oxygenated compounds are soluble in water at levels greater than 500 ppm (0.5 g/liter) (Fisher et al., this issue; Smyrl and LeMaguer, 1980; Weidenhamer et al., 1993). Such compounds could be readily leached from plants in high concentrations if they are present in apoplastic compartments on or near the surface. Unfortunately, terpenoid leaching rates from higher plants have not been accurately determined, and so it is difficult to estimate the extent of losses due to this process.

In summary, there is currently no strong evidence that metabolic turnover, volatilization, or leaching actually cause significant losses of terpenoids from higher plants, and as a consequence there is no apparent need for additional synthesis to replenish depleted stores. Hence terpenoid maintenance costs are likely to be minimal. (It is worth noting that low rates of terpenoid volatilization or leaching from individual plants do not rule out the possibility that, in certain plant communities, large quantities of terpenes could be released into the environment if terpenoid-accumulating plants are especially abundant or dominant.)

ADAPTATIONS FOR REDUCING COSTS

While the maintenance of terpenoid pools in plants does not seem to demand a substantial outlay of resources, previous sections of this review have demonstrated that both terpenoid biosynthesis and storage require sizable metabolic

expenditures. A survey of phenolics, alkaloids, cyanogenic glycosides, and other plant defense compounds suggests that the synthesis and storage of these substances also exacts significant metabolic costs (Gershenzon, 1994). In terms of reproductive fitness, however, the costs of plant chemical defenses are not always apparent. A number of studies have failed to detect any fitness costs for plant defense (Rausher, 1992; Simms, 1992; Simms and Fritz, 1990; Zangerl and Bazzaz, 1992), implying that such costs may be quite low or only evident at particular developmental stages (Baldwin et al., 1990; Briggs and Schultz, 1990) or under specific environmental conditions (Herms and Mattson, 1992; Yates and Peckol, 1993). To reconcile the high metabolic costs involved in the synthesis and storage of plant defenses with the frequent absence of measurable fitness costs for defense as a whole, it can be proposed that plants have evolved various mechanisms for reducing costs (Simms, 1992). Several metabolic adaptations that could reduce terpenoid costs have already been mentioned earlier in this article, including the sharing of biosynthetic enzymes among multiple pathways, the minimization of enzyme turnover, and the use of a single enzyme to make a mixture of products. Here, I consider two other cost-reducing adaptations: the catabolism of terpenoids that are no longer needed and the use of individual terpenoid metabolites for more than one function.

Catabolism

Terpenoids sometimes accumulate to concentrations of at least 5% of plant dry weight (Dell and McComb, 1975, 1977; Morrow and Fox, 1980; Reichardt et al., 1984), and so may represent important storage forms of fixed carbon. As long as these substances are not released to serve as allelopathic agents and not volatilized to attract pollinators or entomophages, they could be degraded when no longer needed to permit recovery of some of the costs originally invested in their synthesis and storage. To facilitate cost recovery, degradative reactions must be coupled directly to the production of ATP or reduced pyridine nucleotides, or must generate simple metabolites, such as acetyl-CoA or TCA cycle intermediates, that can undergo further oxidation via basic respiratory pathways or serve as raw materials for the biosynthesis of other needed metabolites.

Croteau and coworkers have established that the monoterpenes found in the leaves of certain species of the Lamiaceae are catabolized at late stages of plant development. For example, in *Mentha* × *piperita* (peppermint), approximately 50–75% of the monoterpenes present in the mature leaves are degraded at the time of flowering (Croteau, 1988). The ketone menthone, which is the principal monoterpene in this species, is reduced to approximately equal amounts of the stereoisomeric alcohols menthol and neomenthol (Croteau and Winters, 1982; Kjonaas et al., 1982) (Figure 10). While the menthol produced remains in the leaves, the neomenthol becomes glucosylated and is transported to the

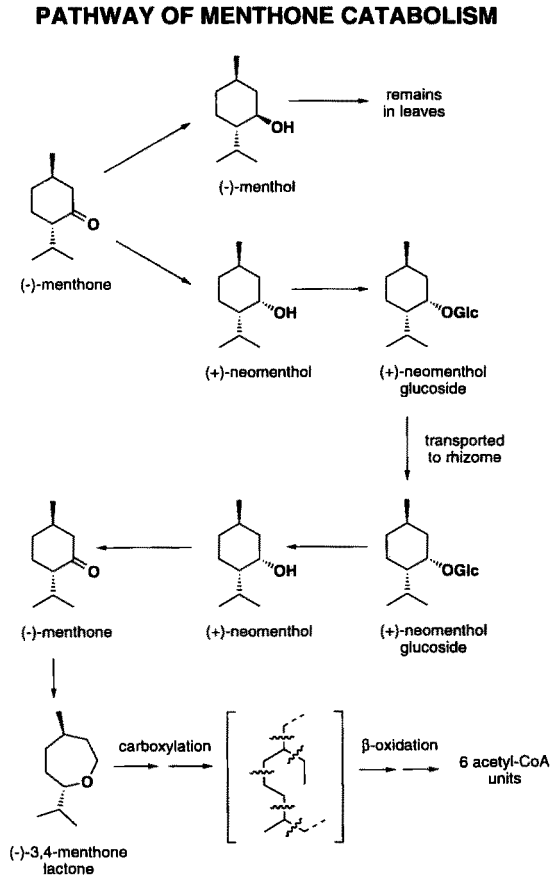


FIG. 10. Menthone, a principal monoterpene of the mature leaves of *Mentha* × *piperita* (peppermint), is metabolically degraded at the time of flowering according to the pathway illustrated (Croteau and Sood, 1985; Croteau and Winters, 1982; Croteau et al., 1984a; Kjonaas et al., 1982). The proposed β-oxidation scheme is shown in brackets with the dotted lines indicating likely sites of carboxylation and the wavy lines depicting the bonds cleaved to form units of acetyl-CoA. The acetyl-CoA produced is incorporated into sterols and acyl lipids, while the reduced pyridine nucleotides formed are used in carbohydrate formation.

rhizome (Croteau et al., 1984a). Once in the rhizome, neomenthol glucoside is sequentially hydrolyzed and oxidized to reform menthone, which is then converted to menthone lactone by an unusual ring-opening reaction (Croteau et al., 1984a). Finally, the menthone lactone produced is subjected to a modified

β-oxidation sequence generating six units of acetyl-CoA (Croteau and Sood, 1985). An analogous process occurs in the mature leaves of *Salvia officinalis* (garden sage) where, during flowering, the monoterpene camphor is converted to a water-soluble transport derivative that is exported to the root and oxidatively degraded to acetyl-CoA (Croteau et al., 1984b, 1987d). The early steps of camphor catabolism in *S. officinalis* have recently been established by feeding experiments conducted in cell culture (Funk et al., 1992). Camphor was shown to be converted, in sequence, to 6-hydroxycamphor, 6-oxocamphor, α-campholonic acid, and 2-hydroxy-α-campholonic acid (Figure 11). These studies not only reveal that monoterpenes are mobilized prior to leaf senescence and then degraded, but also demonstrate that the fixed carbon and reducing equivalents released by degradative reactions are recycled back into primary metabolic processes. When labeled monoterpenes were applied to either *M.* × *piperita* rhizomes or *S. officinalis* roots, the acetyl-CoA units derived from β-oxidation were found to serve as substrates for the formation of sterols and acyl lipids, and the reduced pyridine nucleotides produced were observed to participate in the biosynthesis of sugars and starch (Croteau and Sood, 1985; Croteau et al., 1987d).

Apart from these examples, few other well-documented cases of terpenoid catabolism are known in plants. A number of investigations have reported terpenoid degradation in detached organs (e.g., Banthorpe et al., 1972; Njar et al., 1989) or in cell cultures (e.g., Berger et al., 1990; Threlfall and Whitehead, 1991). However, these transformations are of uncertain significance and may only represent detoxification or salvage-type reactions that are not of catabolic

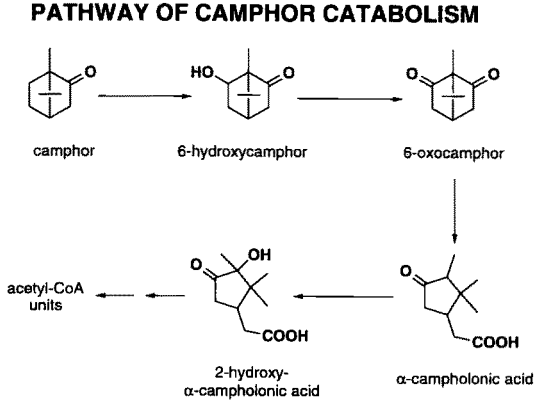


FIG. 11. The early steps of camphor catabolism in *Salvia officinalis* (garden sage) (Funk et al., 1992). Camphor, like menthone, is a principal monoterpene of mature leaves that is metabolically degraded at the time of flowering (Croteau et al., 1984b, 1987d).

importance in the intact plant. Nevertheless, a great variety of sesquiterpenes (Carlton et al., 1992; Dolman et al., 1992), diterpenes (Flesch et al., 1992), triterpenes (Henry et al., 1991; Marner and Kerp, 1992), and other monoterpenes (Dudai et al., 1992; Russin et al., 1988) are known to be lost from foliage late in development, and so terpenoid catabolism could well be widespread in plants. Further research in this area is urgently needed.

Multiple Roles

Terpenoids as a class are thought to play an assortment of roles in higher plants. If specific terpenoid compounds serve in more than one role, this should reduce the need for other metabolites that perform the same functions, and hence decrease the overall cost to the individual plant. Terpenoids have been implicated in both ecological and physiological roles. Proposed ecological roles include antiherbivore defense, antimicrobial defense, allelopathy, attraction of pollinators and entomophages, and alteration of the rate of nutrient cycling, all described in detail in the other contributions to this issue (see especially Langenheim, Table 2). Much less is known about the possible physiological roles of terpenoids in plants. For example, the diterpenoid resins coating the leaves of certain species native to arid regions have been suggested to limit water loss (Dell and McComb, 1978a). A tetracyclic triterpene from the roots of *Alnus glutinosa* could be essential for the endophytic growth of the nitrogen-fixing symbiont *Frankia* (Quispel et al., 1989). Saponins stored in seeds might act as germination inhibitors (Zambou et al., 1993), while leaf monoterpenes may serve as antioxidants that quench free radicals generated by light-activated processes (Deighton et al., 1993). One can readily envision individual terpenes serving in more than one capacity, either simultaneously or sequentially. A monoterpene molecule, for instance, might act first as a defense against both herbivores and pathogens (and perhaps also serve as an antioxidant). Then, following its release from living tissue or litter, the same molecule might function as an allelopathic agent or as a modulator of nutrient cycling processes in the soil. Unfortunately, we still lack sufficient information regarding many of these proposed functions, and so it is difficult to decide how often individual plants actually employ terpenoids in multiple roles. Nevertheless, this tactic could well have been selected for in order to reduce the overall costs of terpenoid accumulation, and hence could be widespread in plants.

TERPENOID COSTS AND CURRENT THEORIES OF PLANT DEFENSE

The vast majority of plant terpenoids are believed to function principally as antiherbivore defenses. Thus, having examined the metabolic costs of terpenoid accumulation in plants, it now seems appropriate to consider some current

ideas regarding the cost of plant defense against herbivory. The cost of antiherbivore defense has been a frequent topic of discussion in plant-herbivore studies for many years, and costs are generally thought to impose significant constraints on the types and amounts of antiherbivore defenses produced (e.g., Chew and Rodman, 1979; Fox, 1981; Krischik and Denno, 1983; Zangerl and Bazzaz, 1992), despite the fact that a number of studies have failed to detect any measurable costs for defense (Rausher, 1992; Simms, 1992; Zangerl and Bazzaz, 1992). The concept of cost has been incorporated into several theories that attempt to explain intraspecific and interspecific patterns of plant defense (Bryant et al., 1992; Coley et al., 1985; Feeny, 1976; Herms and Mattson, 1992; Lorio, 1988; Rhoades, 1979). In this section, I assess the validity of some of these theories in the light of the major trends we have noted in our survey of the metabolic costs of terpenoids.

The Carbon–Nutrient Balance Hypothesis

Variations in environmental conditions often modify the quantities of terpenoids and other antiherbivore defenses found in plants (Gershenzon, 1984; Gershenzon and Croteau, 1991; Waterman and Mole, 1989). These modifications can frequently be explained by the carbon–nutrient balance hypothesis, which attributes phenotypic variations in the concentration of defenses to changes in the relative availability of resources for growth and defense (Bryant et al., 1983, 1992; Tuomi et al., 1988). The carbon–nutrient balance hypothesis was founded on the observation that nutrient deficiencies limit the rate of growth more than they limit the rate of photosynthesis (Chapin, 1980). Hence, when nutrients are curtailed (creating a high carbon–nutrient ratio), a plant may decrease its growth while still photosynthesizing at an undiminished rate, leading to an accumulation of carbohydrate in excess of what is needed to support immediate growth. This buildup of carbohydrate is believed to provide additional substrate for the production of nonnitrogenous secondary metabolites that act in defense, such as terpenoids. Conversely, under low-light conditions, when growth is limited by the availability of fixed carbon rather than nutrients (creating a low carbon–nutrient ratio), carbohydrate reserves are expected to decline, leading to a reduced formation of terpenoids and other nonnitrogenous (carbon-based) defenses.

The carbon–nutrient balance hypothesis correctly predicts many of the variations in plant antiherbivore defense levels that have been reported under different nutrient and light regimes (e.g., Bryant et al., 1992; Fajer et al., 1992; Reichardt et al., 1991). However, changes in terpenoid levels do not conform to this hypothesis as well as do changes in the levels of other carbon-based compounds, such as tannins and miscellaneous phenolic substances. In particular, changes in the terpenoid levels of woody plants show only a weak corre-

spondence with the expectations of carbon–nutrient balance theory. A number of recent investigations have indicated that neither low-nutrient (Bjorkman et al., 1991; Lapinjoki et al., 1991; McCullough and Kulman, 1991; Muzika et al., 1989; Reichardt et al., 1991; Rousi et al., 1993) nor high-light regimes (Kelsey and Vance, 1992) consistently result in higher terpenoid levels in woody plants. On the other hand, among terpenoid-bearing herbs, both low-nutrient (Emongor and Chweya, 1992; Mihaliak and Lincoln, 1989; Ross and Sombrero, 1991; V.P. Singh et al., 1989; Spencer et al., 1993; Van Wassenhove et al., 1990) and high-light conditions (Dudai et al., 1992; Reynolds and Wardle, 1989; Tanaka et al., 1989; Yamaura et al., 1989) generally stimulate increases in terpenoid concentration, in agreement with the tenets of the theory. Curiously, when plants are grown in an atmosphere of elevated carbon dioxide concentration (which presumably raises their carbon–nutrient ratio), terpenoid concentrations are not observed to increase in either woody species (Johnson and Lincoln, 1990, 1991) or herbaceous species (Fajer, 1989; Fajer et al., 1989, 1992; Lincoln and Couvet, 1989). Some of these apparent failures of the carbon–nutrient model may be due to the unusual responses of very young seedlings to fertilization (Bryant et al., 1992; Rousi et al., 1993), while other inconsistencies have been attributed to the rapid metabolic turnover of defenses, which could make it difficult to detect changing rates of synthesis (Reichardt et al., 1991). However, the rapid turnover of terpenoids is probably not a widespread phenomenon in plants, as we have seen previously under "Maintenance Costs."

The failure of the carbon–nutrient balance hypothesis to reliably predict the response of terpenoids and other carbon-based defenses to alterations in light and nutrient supply may be related to the assumptions this theory makes concerning the cost of defense. The production of terpenoids and other carbon-based defense compounds is not anticipated to be costly for plants, according to the theory, since these substances are postulated to be manufactured primarily from excess carbohydrate. However, this line of reasoning fails to take into account the nutrient costs of terpenoid accumulation. Construction of both biosynthetic enzymes and multicellular storage structures requires adequate supplies of nutrients, such as nitrogen, sulfur, and phosphorus. Thus, terpenoid accumulation may proceed at only a limited rate under conditions of nutrient scarcity regardless of the availability of excess carbohydrate. Bjorkman et al. (1991) recently observed that *Pinus sylvestris* foliage from unfertilized trees had lower concentrations of diterpene resin acids than foliage from trees fertilized with nitrogen, a result they attributed to the decreased size and number of resin ducts in unfertilized trees.

Another weakness of the carbon–nutrient balance hypothesis is its supposition that the manufacture of plant defenses is influenced chiefly by changes in the supply of carbohydrate. In fact, substrate levels appear to be just one of several factors regulating the biosynthesis of defense compounds. We have seen

above (Figure 3) that the levels of monoterpene biosynthetic enzymes are very closely correlated with the rate of monoterpene biosynthesis in developing *Mentha* × *piperita* leaves, suggesting that variations in enzyme activity could well control the formation of monoterpenes in this species. Changes in enzyme activity (Chappell et al., 1991; Dudley et al., 1986), subcellular compartmentation (Gray, 1987; Kleinig, 1989), morphological differentiation (Gershenzon et al., 1989; Keene and Wagner, 1985), and substrate levels (Croteau et al., 1972b; N. Singh et al., 1991) may all contribute to the regulation of terpenoid biosynthesis in plants (Gershenzon and Croteau, 1990, 1993) Thus, at least for terpenoids, the predictions of the carbon–nutrient balance hypothesis may only hold in cases where substrate supply is the major factor limiting biosynthesis, and some threshold level of nutrients is available to meet the costs of enzymes and storage structures.

Not all facets of terpenoid costs covered here are inconsistent with the carbon–nutrient balance paradigm. As we have noted under "Costs of Substrates and Cofactors" above, the costs of providing cofactors for terpenoid biosynthesis may be significantly lower in photosynthetic than in nonphotosynthetic tissue because ATP and NADPH can be cheaply produced by light-driven electron transport in chloroplasts. The carbon–nutrient balance hypothesis also predicts that terpenoids and other carbon-based secondary metabolites should be more readily produced by photosynthetic cells than nonphotosynthetic cells because carbon fixation would be expected to raise the carbon–nutrient ratio.

Growth–Differentiation Balance Hypothesis

The growth–differentiation balance hypothesis is of older origin (Loomis, 1932) than the carbon–nutrient balance hypothesis, but has been applied to plant defense theory more recently (Lorio, 1986, 1988; Herms and Mattson, 1992). This hypothesis also asserts that the supply of resources controls the distribution of defenses in plants, but invokes shifts in development, rather than changing resource ratios, as the mediating factors. The growth–differentiation balance hypothesis divides development into two distinct processes: (1) growth, which includes cell division and enlargement, and (2) differentiation, which includes cell maturation, specialization, and the production of defensive compounds. Both growth and differentiation are thought to draw on the same pool of resources, and so are negatively correlated with growth predominating under favorable environmental conditions and differentiation predominating when resources are limited. As in the carbon–nutrient balance hypothesis, declines in resource availability are believed to inhibit growth before they affect photosynthesis. Consequently, photosynthesis is expected to continue under adverse conditions, leading to a build-up of fixed carbon that serves as a source of raw materials for differentiation processes. The implication then is that defenses are synthesized when

stores of fixed carbon are not being used for growth. Hence, the growth–differentiation balance hypothesis makes the same assumptions as the carbon–nutrient balance hypothesis: (1) that the synthesis of defenses is not costly since it does not compete directly with growth processes for resources, and (2) that changes in substrate supply closely regulate the formation of defenses. One may therefore offer the same criticisms as those directed against the carbon–nutrient balance hypothesis: Both of these hypotheses fail to consider the nutrient costs of the enzymes and storage structures involved in the accumulation of defenses and neglect to acknowledge that factors other than substrate supply may control the biosynthesis of defensive compounds.

Further consideration of the growth–differentiation balance theory in relation to the biochemistry and physiology of terpenoid manufacture in plants reveals that, in many species, terpenoid biosynthesis occurs primarily in young, rapidly growing tissue. Thus, often no trade-off exists between terpenoid biosynthesis (a differentiation-associated process) and growth, a finding inconsistent with the growth–differentiation balance concept. This conclusion is based on several lines of evidence. First, in many species, the rate of terpenoid synthesis from basic precursors, such as CO_2 or sucrose, has been shown to be much greater in expanding leaves and other rapidly growing organs than in mature plant tissue. For example, in *M.* × *piperita* leaves, monoterpene biosynthesis is almost completely confined to the first two weeks of leaf ontogeny, a period during which the leaves attain over 85% of their final mass (J. Gershenzon and R. Croteau, unpublished results) (Figure 12). The same trend holds for many different classes of terpenes in taxonomically diverse plant families, such as

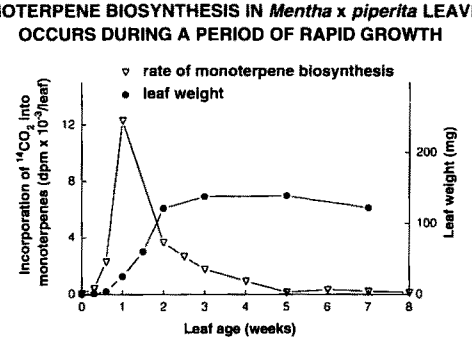


FIG. 12. Changes in the rate of monoterpene biosynthesis from $^{14}CO_2$ (left axis) and leaf weight (right axis) during leaf development in *Mentha* × *piperita* (peppermint) (J. Gershenzon and R. Croteau, unpublished results). The rate of monoterpene biosynthesis is much more rapid in young, expanding leaves than in mature leaves, in contrast to the predictions of the growth–differentiation balance hypothesis.

monoterpenes in *Cymbopogon flexuosus*—Poaceae (Singh and Luthra, 1987; N. Singh et al., 1990), *Heterotheca subaxillaris*—Asteraceae (Mihaliak and Lincoln, 1989), *Pinus pinaster*—Pinaceae (Bernard-Dagan et al., 1982; Bernard-Dagan, 1988), and *Salvia officinalis*—Lamiaceae (Croteau et al., 1981); sesquiterpenes in *H. subaxillaris*—Asteraceae (Mihaliak and Lincoln, 1989); diterpenes in *Newcastelia viscida*—Dicrastylidaceae (Dell and McComb, 1978b); and triterpenes in *Euphorbia lathyris*—Euphorbiaceae (Groeneveld et al., 1987; Koops and Groeneveld, 1991). Second, the occurrence of terpenoid formation in young tissue is suggested by numerous cell-free studies, which have shown that many enzymes of terpenoid biosynthesis are significantly more active in extracts prepared from young, actively-growing tissue than in extracts derived from mature tissue (e.g., Croteau et al., 1981; Hallahan and Croteau, 1988; Munck and Croteau, 1990). Third, ultrastructural studies have demonstrated that the cells comprising the specialized secretory structures in which terpenoids are synthesized and stored are usually metabolically active only in young tissue (e.g., Charon et al., 1986; Dell and McComb, 1977; Russin et al., 1988; Werker and Fahn, 1981). In *M.* × *piperita* leaves, for instance, the cells of the glandular trichomes involved in producing monoterpenes are fully developed at a very early stage in leaf ontogeny. At a time when the leaf is less than 0.5 cm long (final length: 5–10 cm) and fully differentiated chloroplasts are not yet evident in the mesophyll cells, the secretory cells of *M.* × *piperita* glandular trichomes contain abundant plastids and mitochondria, an extensive network of smooth endoplasmic reticulum, and a profusion of osmiophilic droplets assumed to be newly manufactured monoterpenes (Amelunxen, 1965; Gershenzon and Croteau, unpublished results, see also Figure 3 in Lerdau et al., 1994). Fourth, literature reports indicate that most terpenoid-producing taxa contain higher concentrations of terpenoid constituents in young, rapidly growing organs than in mature, fully developed organs (Gershenzon and Croteau, 1991), although a number of woody species show the opposite pattern (e.g., Putievsky et al., 1984; Tisdale and Nebeker, 1992). While high concentrations in young tissue could conceivably arise as a result of transport from older tissue, there is little evidence at present for the intercellular movement of terpenes into young tissue [although certain types of triterpenes, including limonoids (Hasegawa et al., 1986) and phytoecdysteroids (Grebenok and Adler, 1991), may be exceptional in this regard]. Thus, it appears that terpenoid formation and accumulation are frequently associated with growing tissue, in apparent contradiction of the central postulate of the growth–differentiation balance hypothesis. In a thoughtful appraisal of this hypothesis in relation to the patterns of monoterpene distribution in plants, Lerdau et al. (1994) suggested that the predictive value of the growth–differentiation balance model might be enhanced if allocation to the competing sinks of growth and differentiation were at least partially based on the demand for these processes, rather than solely on the availability of resources. A demand-

based model might attribute the high rate of terpenoid synthesis in young foliage to the urgent need for defensive substances in these organs, arising from the high value of young foliage to the plant in terms of reproductive fitness and the high risk of herbivore attack on tissue rich in water and nutrients (Krischik and Denno, 1983; McKey, 1979; Rhoades, 1979).

Resource-Availability Hypothesis

As its name indicates, the resource-availability hypothesis also asserts the importance of light, nutrients, and other resources in determining the patterns of antiherbivore defenses in plants (Coley et al., 1985). However, unlike the theories previously discussed, the resource-availability hypothesis is concerned with evolutionary rather than ecological time scales and attempts to explain interspecific differences in the type and magnitude of defensive compounds present. According to the hypothesis, species native to resource-rich environments usually have rapid growth rates and short leaf lifetimes and are therefore expected to employ low concentrations of mobile defensive substances, such as alkaloids and cyanogenic glycosides, that can be recovered from leaves prior to senescence. The mobility of these substances is thought to be a consequence of their rapid rate of metabolic turnover. On the other hand, species of resource-poor habitats typically have slow growth rates and long leaf lifetimes and hence are predicted to protect themselves with high concentrations of defensive compounds that are immobile, such as condensed tannins. In long-lived leaves, a one-time investment in immobile defenses should be less expensive than the task of continually replacing mobile defenses as they are rapidly turned over, and so immobile defenses are expected to predominate in the foliage of species growing in unfavorable environments.

The resource-availability theory classifies terpenoids as mobile defense compounds based on the numerous reports of terpenoid turnover found in the older literature (Seigler and Price, 1976). However, as we have seen above (under "Maintenance Costs") terpenoids do not actually appear to turn over rapidly in intact plants. Nevertheless, it may be still correct to consider many terpenoids mobile defenses because several investigations have documented terpenoid catabolism in older leaves (see above under "Adaptations for Reducing Costs"). Thus, in contrast to the assumptions of the resource availability hypothesis, the mobility of defensive compounds does not necessarily require a high rate of metabolic turnover, and so mobility is not inherently costly. Hence, terpenoids may be appropriate defenses for either long-lived or short-lived leaves since they are cheap to maintain and can be mobilized prior to leaf senescence.

CONCLUSIONS

Students of plant–herbivore interactions have maintained a long-standing interest in the costs of terpenoids and other plant antiherbivore defense compounds. However, no consensus has been reached on the biological importance

of these costs. Theoretical treatments have commonly assumed that costs are high enough to significantly constrain the accumulation of defensive metabolites in plants (e.g., Coley et al., 1985; Fagerstrom, 1989; Gulmon and Mooney, 1986; Krischik and Denno, 1983), but the fitness costs of plant chemical defense are not always detectable when attempts are made to measure them empirically (e.g., Briggs and Schultz, 1990; Brown, 1988; Simms, 1992; Simms and Rausher, 1987), suggesting that such costs may sometimes be quite low. This survey of terpenoid metabolic costs provides support for both of these points of view.

We have seen that several processes involved in terpenoid accumulation demand large outlays of plant resources. For example, the raw materials costs of synthesizing terpenoids are higher than the raw materials costs for making most other primary and secondary metabolites because terpenoids are chemically very reduced. The enzyme costs of terpenoid synthesis are also high since there seems to be little sharing of enzymes with other branches of plant metabolism, and many plant cells appear to contain multiple sets of terpenoid biosynthetic enzymes. Furthermore, large amounts of resources are allocated to the construction of the complex secretory structures in which terpenoids are usually sequestered. Thus, the accumulation of terpenoids incurs substantial metabolic costs that might be expected to result in substantial fitness costs.

However, a number of factors may alleviate the high costs of terpenoid accumulation. For instance, maintenance expenses for terpenoids are low since neither volatilization, leaching, nor rapid metabolic turnover seem to cause significant losses of terpenoids from plants. In addition, the costs of producing mixtures of terpenoids are reduced by the presence of enzymes that make more than one product. Finally, the overall costs associated with terpenoid accumulation may be considerably diminished if terpenoids serve in multiple roles or if these substances are catabolized when they are no longer needed. Such cost-reducing strategies may sufficiently decrease the net expenditures on terpenoids so that no fitness costs are evident.

Clearly, much more additional information is needed before we can accurately assess the metabolic costs associated with terpenoid accumulation. New insights on the costs of biosynthesis should continue to emerge from ongoing research programs on terpenoid formation in plants. However, to round out what we know about costs, more attention must be devoted to the turnover, catabolism, and transport of plant terpenoids. In addition, the frequency with which terpenoids play multiple roles in individual plants needs to be established. Plants that have been genetically transformed to modify their terpenoid phenotype may be especially useful for such studies. A more extensive knowledge of terpenoid costs will undoubtedly improve our understanding of the ecology and evolution of these substances in higher plants.

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VOLATILE HERBIVORE-INDUCED TERPENOIDS IN PLANT-MITE INTERACTIONS: VARIATION CAUSED BY BIOTIC AND ABIOTIC FACTORS

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Abstract—Plants may defend themselves against herbivores by enhancing the effectiveness of natural enemies of herbivores. This is termed "indirect defense," which may be induced by herbivore damage. An important aspect of induced indirect defense is the attraction of the herbivore's natural enemies to infested plants by the plant emitting so-called "herbivore-induced synomone" (HIS) in response to herbivore damage. In this paper, we review the role of terpenoids in the induced indirect defense of plants against herbivorous mites. HIS are emitted from both damaged and undamaged areas of infested plants, and the composition of HIS varies among different plant species. The emission of HIS may also vary within a plant species, depending upon: (1) plant cultivar, (2) leaf growth stage, (3) the herbivore species that is attacking, and (4) abiotic conditions (light intensity, time of year, and water stress). Predatory mites cope with this variation of HIS by innate recognition as well as temporary specialization to a certain HIS via learning.

Key Words—Tritrophic interactions, volatile terpenoids, induced defense, indirect defense, Acari, Tetranychidae, Phytoseiidae, mites, herbivore-induced synomones, abiotic conditions.

INTRODUCTION

Plants produce toxins, digestibility reducers, repellents, and other secondary products to directly defend themselves against herbivores. In addition to such

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direct defense mechanisms, plants may also defend themselves indirectly by enhancing the effectiveness of natural enemies of herbivores (Price, 1981; Dicke and Sabelis, 1988a). This may be done by providing carnivores with shelter or food, but it may also be done by attracting the foraging natural enemies of the herbivores through infochemicals (*sensu* Dicke and Sabelis, 1988b).

The latter aspect has received increasing interest in the past 10 years and it appears that an important aspect of indirect plant defense through infochemicals involves the emission of herbivore-induced plant volatiles that attract the enemies of herbivores (Sabelis and Dicke, 1985; Dicke and Sabelis, 1988a; Dicke et al., 1990a,b; Turlings et al., 1990b; Takayabashi et al., 1991a,b). When allelochemicals are adaptive to both emitter and receiver, they are classified as synomones (Dicke and Sabelis, 1988b). Thus the plant volatiles that are produced upon herbivore damage and recruit carnivorous natural enemies are called "herbivore-induced synomone" (HIS) (Vet and Dicke, 1992).

Many volatile HIS consist of terpenoids (Dicke et al., 1990a; Turlings et al., 1990a,b; Dicke 1994). In this paper, we examine the role of terpenoids as mediators of induced indirect defense in tritrophic systems consisting of plants, herbivorous mites, and predatory mites. We focus on the chemical variation in herbivore-induced synomones emitted by different plants, and also by plants of the same species, and the influence of this variation on predator behavior.

EVIDENCE FOR PLANTS RESPONDING TO HERBIVORE DAMAGE BY PRODUCING HERBIVORE-INDUCED SYNOMONES

Carnivores are known to discriminate between plants with and without herbivores on the basis of volatiles (Vinson, 1975; 1976; Sato, 1979; Nordlund et al., 1981; Loke et al., 1983; Sabelis and van de Baan, 1983; Vet and Dicke, 1992). Recent chemical analyses of volatiles from infested plants have shown that plants start emitting volatile infochemicals that attract the natural enemies of herbivores upon herbivore damage (Vet and Dicke, 1992 and citations therein).

For example, the predatory mite *Phytoseilius persimilis* prefers the odor of Lima bean leaves infested by *Tetranychus urticae* to the odor of uninfested Lima bean leaves in a Y-tube olfactometer (Sabelis and van de Baan, 1983). When the spider mite *T. urticae* and all visible products were removed from the infested leaves, these leaves remained preferred by the predaroty mites for at least a few hours. The removed spider mites, their exuviae, or their webbing were not attractive, and the removed feces were preferred by the predatory mites only very slightly (Sabelis et al., 1984).

The intensity of the predator response is correlated with the amount of feeding damage inflicted by the spide mites (Sabelis et al., 1984). Mechanical damage of Lima bean leaves with carborundum (aimed at imitating feeding

damage), however, did not result in the predatory mites preferring the damaged leaves to undamaged leaves (M. Dicke, unpublished data, 1984). We suspect that the saliva of *T. urticae* is responsible for triggering the HIS production. However, it has been impossible to collect spider-mite saliva because of the small size (ca. 0.8 mm) of the mites.

Chemical analyses of the headspace composition of uninfested, mechanically damaged, and spider-mite infested Lima bean leaves showed qualitative and quantitative differences. Spider-mite infested leaves emit relatively large amounts of five compounds: four terpenoids, and one phenolic compound (Figure 1).Two of these compounds [(3*E*, 7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene and methyl salicylate] were not found at all, and (*E*)-β-ocimene, linalool, and (3*E*)-4,8-dimethyl-1,3,7-nonatriene were found in minute amounts in the headspace of uninfested Lima bean leaves and artificially damaged Lima bean leaves (Dicke et al., 1990a; J. Takabayashi, M. Dicke, and M. A. Posthumus, unpublished data, 1989). No volatiles have been recorded from spider mites that were removed from a plant (M. Dicke and M. A. Posthumus, unpublished data). Among the volatiles emitted by spider-mite infested Lima bean leaves, four compounds, (*E*)-β-ocimene, (3*E*)-4,8-dimethyl-1,3,7-nonatriene, linalool, and methyl salicylate, were found to be preferred by the predatory mites (hereafter called predator attractants) (Dicke et al., 1990a).

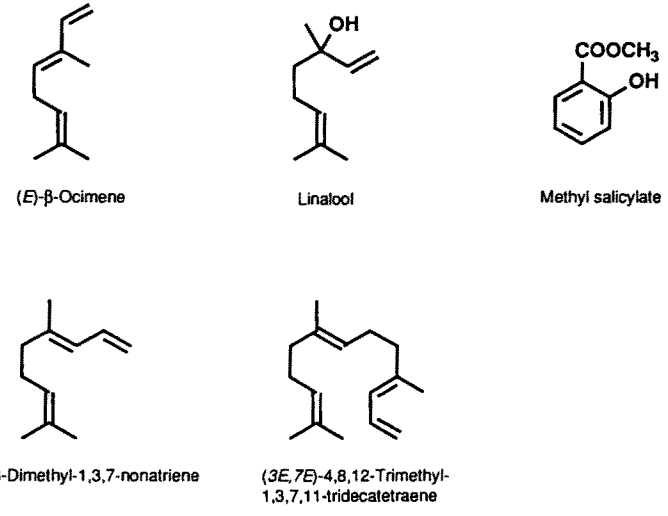

FIG. 1. Chemical structure of herbivore-induced volatiles emitted from Lima bean leaves infested by *T. urticae*. (3*E*)-4,8-Dimethyl-1,3,7-nonatriene, linalool, (*E*)-β-ocimene, and methyl salicylate are predator attractants.

However, one of the induced chemicals, (3*E*, 7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene, was not preferred by the predatory mites (Dicke et al., 1990a).

Thus, the above data suggest that the predator attractants may be produced by the plant, or, alternatively they may be plant chemicals that are taken by the herbivores, modified by herbivore enzymes, and deposited on the plant by the herbivore. However, the second possibility seems highly unlikely as Takabayashi et al. (1991b) reported that not only the infested part of an infested Lima bean leaf but also the uninfested part emits two HIS components: (*E*)-β-ocimene and (3*E*)-4,8-dimethyl-1,3,7-nonatriene. The uninfested part had never experienced spider mite activity, such as being infested or walked on. The emission of HIS by the uninfested part may reflect adsorption of (*E*)-β-ocimene and (3*E*)-4,8-dimethyl-1,3,7-nonatriene produced in the infested part onto the uninfested part, from which second-hand release occurs, but this seems unlikely, because of the high amounts of volatiles found in the uninfested part. Attempts to adsorb synthetic (*E*)-β-ocimene volatile to uninfested Lima bean leaves by placing the chemical next to the uninfested leaves also were not successful (J. Takabayashi and M. Dicke, unpublished data, 1989). It seems more likely that the two terpenoids are produced by the uninfested part of the infested leaves. Recently, an elicitor mediating the systemic production of HIS in Lima bean plants has been extracted from spider-mite infested leaves; upon feeding this elicitor to uninfested leaves, it induces the production of synomone (Dicke et al., 1993). Adsorption of synomones produced by spider-mite-infested leaves was not possible in their set up.

As physical damage imitating spider mite infestation alone does not increase the amount of predator attractants, it is the spider mite itself that acts as a catalyst, causing the plant to produce more of them (ca. more than 100 times).

VARIATION IN HERBIVORE-INDUCED SYNOMONE COMPOSITION WITHIN AN INDIVIDUAL PLANT

The finding that plants produce HIS in response to herbivory evokes the question of whether or not the response of the plant is affected by the plant's developmental stage. To a plant, different leaves may differ in value; young upper leaves are more important than old lower leaves with respect to plant growth and reproduction. In an investigation of the production of HIS in cucumber leaves of different developmental stages, we found that the predatory mite *P. persimilis* responded to young *T. urticae*-infested leaves, but not to old *T. urticae*-infested leaves in the Y-tube olfactometer (Takabayashi et al., 1994).

A chemical analysis of the headspace of *T. urticae*-infested young and old leaves showed that terpenoids are among the major spider-mite-induced plant volatiles (Table 1). Among these terpenoids, the predator attractants (*E*)-β-

TABLE 1. COMPOUNDS IDENTIFIED IN HEADSPACE VOLATILES OF CUCUMBER LEAVES WITH DIFFERENT TREATMENT (Data from Takabayashi et al. 1994)

	Relative amount (mean %)			
	Undamaged leaves ($N = 2^a$)	Leaves damaged with carborundum ($N = 2$)	Leaves infested by *T. urticae*	
			Young ($N = 2$)	Old ($N = 2$)
Aldehydes				
Pentanal	0.2			
2-Pentenal	0.2	0.4		
2-Hexenal	1.7	6.5		
Hexanal	1.2	1.6		
Heptanal	0.1	0.1		
Decanal	0.3	0.2		
Nonanal	0.2	0.9		
Alcohols				
(*Z*)-3-Hexen-1-ol	34.9	36.0	0.4	0.6
(*E*)-3-Hexen-1-ol		0.1		
1-Hexanol	1.4	2.4		
2-Ethyl-1-hexanol	0.2	0.2		0.4
1-Butanol	0.1			
1-Penten-3-ol	0.1	tr[b]		
1-Pentanol	0.1			
Benzyl alcohol	0.6			
Ketones				
3-Octanone	2.7			
1-Penten-3-one	0.4	0.3		
6-Methyl-5-heptene-2-one	0.2	0.1		
2-Nonanone	0.9			
Esters				
Butyl acetate	0.7	0.1		
Hexyl acetate	0.3	1.1	1.1	
iso pentyl acetate				0.6
(*Z*)-3-Hexen-1-yl acetate	53.0	47.2	1.2	1.0
3-Hexenyl formate		tr		
Terpenoids				
Limonene	0.4	tr	tr	
(*Z*)-β-Ocimene			0.3	1.0
(*E*)-β-Ocimene			24.9	38.4
(3*Z*)-4,8-Dimethyl-1,3,7-nonatriene			2.5	0.1
(3*E*)-4,8-Dimethyl-1,3,7-nonatriene			53.8	19.8
Linalool	0.1			
(*E*, *E*)-*a*-Farnesene			4.0	1.9
(3*E*, 7*E*)-4,8,12-Trimethyl-1,3,7,11-tridecatetraene			3.4	0.6

TABLE 1. CONTINUED

	Relative amount (mean %)			
	Undamaged leaves ($N = 2^a$)	Leaves damaged with carborundum ($N = 2$)	Leaves infested by *T. urticae*	
			Young ($N = 2$)	Old ($N = 2$)
Nitriles				
2-Methylpropanenitrile			0.1	
2-Methylbutanenitrile			0.8	
3-Methylbutanenitrile			2.5	2.4
Oximes				
2-Methylbutanal *O*-methyloxime			0.8	0.5
3-Methylbutanal *O*-methyloxime			3.8	32.0
Unknown oxime			0.1	0.8
Others				
2-Ethylfuran	0.2	0.2		
Caproic acid	0.8	0.7		
Methyl salicylate			tr	
Unidentified minor peaks	1.2	2.4	0.8	

[a] Number of replications of GC–MS analysis.
[b] Compounds that were found less than 0.1%

ocimene and (3*E*)-4,8-dimethyl-1,3,7-nonatriene (Figure 1) were found. In addition, three oximes and three nitriles were found in spider-mite-infested leaves but not in uninfested or artificially damaged leaves (Table 2) (Takabayashi et al., 1994). Among these nitrogenous compounds, two oximes (3-methylbutanal *O*-methyloxime and an unknown oxime) were much more abundant in the headspace of infested old cucumber leaves than in the headspace of infested young cucumber leaves (Table 1). These two oximes may mask the two predator attractants emitted by old cucumber leaves. Indeed, the response of *P. persimilis* towards volatiles of infested young leaves was neutralized in the presence of volatiles of infested old cucumber leaves when compared with clean air in the Y-tube olfactometer (Takabayashi et al., 1994).

This difference in attractiveness of old and young *T. urticae*-infested leaves reflects the different value of young and old leaves to the plant. Young leaves at the growing tip of the plant are of high value (Edwards et al., 1992). Therefore, it is very important for a plant that predatory mites are directed to the growing points. It remains unknown whether this preference for young leaves is also advantageous for predators. For a more elaborate discussion of this intriguing phenomenon, see Takabayashi et al. (1994).

TABLE 2. COMPOUNDS IDENTIFIED IN HEADSPACE VOLATILES OF APPLE LEAVES OF DIFFERENT CULTIVAR INFESTED BY *T. urticae* (Data from Takabayashi et al. 1991a)

	Relative amount (mean %)	
	cv. Summer Red leaves infested by *T. urticae* ($N = 3^a$)	cv. Cox Orange Pippin leaves infested by *T. urticae* ($N = 2$)
Aldehydes		
2-Methyl-2-propenal	tr[b]	
Hexanal	<0.4[c]	tr
2-Hexenal	0.7	13.3
Alcohols		
Butanol		tr
2-Butanol		0.2
1-Pentene-3-ol	<0.5	1.4
3-Pentanol		1.0
2-Pentene-1-ol		tr
1-Hexanol	0.3	3.7
(*Z*)-3-Hexen-1-ol	0.5	0.4
2-Hexene-1-ol	<0.5	18.3
1-Octene-3-ol	tr	
Ketones		
2-Butanone	tr	tr
2-Pentanone	tr	tr
6-Methyl-5-heptene-2-one	<0.3	
Esters		
2-Hexen-1-yl acetate		11.1
(*Z*)-3-Hexen-1-yl acetate	<0.3	14.7
2-Hexene-1-ly butyrate	<0.3	3.9
(*Z*)-3-Hexene-1-yl butyrate		0.4
2-Hexene-1-yl isovalerate		0.7
Methyl benzoate	tr	
Ethyl benzoate	2.6	
3-hexen-1-yl benzoate	tr	tr
Terpenoids		
Myrcene	0.2	<0.1
Limonene	tr	
(*Z*)-β-Ocimene	tr	
(*E*)-β-Ocimene	23.8	6.0
(3*Z*)-4,8-Dimethyl-1,3,7-nonatriene	<0.1	0.9
(3*E*)-4,8-Dimethyl-1,3,7-nonatriene	8.5	0.5
Linalool	0.3	0.5
β-Cubebene	0.3	<0.5
β-Caryophyllene	0.6	0.9
Germacrene-D	1.9	1.5
(*E*, *E*)-α-Farnesene	54.7	6.3
(3*E*, 7*E*)-4,8,12-Trimethyl-1,3,7,11-tridecatetraene	0.2	tr

TABLE 2. CONTINUED

	Relative amount (mean %)	
	cv. Summer Red leaves infested by *T. urticae* ($N = 3^a$)	cv. Cox Orange Pippin leaves infested by *T. urticae* ($N = 2$)
Nitriles		
2-Methylbutanenitrile	<0.6	
3-Methylbutanenitrile	<1.3	tr
Phenylacetonitrile	<1.2	2.6
Oximes		
3-Methylbutanal *O*-methyloxime	<0.3	
Others		
Methyl salicylate	<0.2	0.6
2-Methylfuran	tr	
Unidentified minor peaks	2.0	

[a] Number of replications of GC–MS analysis.
[b] Compounds that were found less than 0.1%.
[c] The maximum value (expressed as <max. value) for compounds that were found in more than one, but not in all samples.

VARIATION IN HERBIVORE-INDUCED SYNOMONES BETWEEN CULTIVARS

The discovery that the plant produces the HIS also raises the question of whether different genotypes differ in HIS composition and/or abundance. A first step in the comparison of genotypes is to compare different cultivars of a plant species.

Data about the effect of the plant cultivar on the composition of herbivore-induced plant volatiles has been reported for apple foliage infested by *T. urticae* (Takabayashi et al., 1991a) (Table 2). The blends emitted by leaves of two different apple cultivars infested by spider mites varied in their composition (Table 2). When *T. urticae* infested leaves of apple cv. Summer Red, the relative percentages of (*E*)-β-ocimene and (3*E*)-4,8-dimethyl-1,3,7-nonatriene were markedly higher than when this spider mite infested apple leaves of cv. Cox Orange Pippin. Large differences were also observed for several other volatile components. 2-Hexanal, (*Z*)-2-hexen-1-yl acetate, 2-hexen-1-yl acetate, 2-hexen-1-ol, and phenylacetnitrile were present in larger proportions and (*E*, *E*)-α-farnesene was present in smaller proportions in the blend emitted by infested Cox Orange Pippin leaves. The other components exhibited smaller differences. No data on the absolute emission rates of the two cultivars are available.

In comparison of the response of *P. persimilis* to the HIS emitted by two different bean plant (*Phaseolus vulgaris*) cultivars, leaves of different cultivars with the same level of *T. urticae* infestation showed significantly different attractiveness to *P. persimilis* (Dicke et al., 1990c). It remains unknown whether this was due to differences in the amount of HIS or to differences in HIS composition.

All recent research on herbivore-induced synomones has been conducted with agricultural plants that have undergone selective breeding for yield and pest resistance but not for HIS. Perhaps in these derived cultivars, some HIS potential has been lost.

VARIATION IN HERBIVORE-INDUCED PLANT VOLATILES BETWEEN PLANT SPECIES

Plant species differ greatly in chemical composition, and it is no surprise that there are large qualitative and quantitative differences in HIS composition among them (Takabayashi and Dicke, 1993; J. Takabayashi, M. Dicke, and M. A. Posthumus, preliminary data, 1988). The infested leaves of the four plant species in Table 3 were preferred to uninfested leaves by the predatory mites *P. persimilis* (Dicke and Sabelis, 1988b; Takabayashi et al., 1994; Takabayashi and Dicke, 1983).

Of the four plant species listed in Table 3, only *S. luteum* has as effective direct defense mechanism against spider mites. The *S. luteum* leaf has glandular hairs on its surface from which viscous materials are secreted. Such glandular hairs hamper mites (Haren et al., 1987). In fact, we were not able to maintain the colony of *T. urticae* on this plant species. However, when we introduced *T. urticae* on *S. luteum* leaves, momentary damage occurred. Interestingly, *S. luteum* leaves infested by *T. urticae* emitted the simplest blend of HIS among the four plant species; the infested leaves only produced (3*E*)-4,8-dimethyl-1,3,7-nonatriene as a major HIS component and (*E*)-β-ocimene as a minor HIS component (Table 3). The same phenomenon was also found in the case of tomato plants. The tomato plant also has a direct defense mechanism against *T. urticae* with glandular hairs. However, when we introduced a large number of *T. urticae* on the tomato leaves, feeding damage occurred, and the infested leaves started to attract the predatory mite *P. persimilis*. Headspace analyses showed that the tomato leaves produced only one HIS component, methyl salicylate (Takabayashi et al., in preparation). We hypothesize that a plant that has an effective direct defense mechanism invests less energy in the production and emission of herbivore-induced synomones. In order to test this hypothesis, further studies on the herbivore-induced volatiles emitted from a plant that has a highly effective direct defense mechanism against *T. urticae* are necessary. This should preferably be done with a wild plant species.

TABLE 3. HERBIVORE-INDUCED CHEMICALS EXTRACTED IN HEADSPACE VOLATILES OF LEAVES OF FOUR PLANT SPECIES

	Relative amount (mean %)			
	Lima bean ($N = 2^a$)	Cucumber (young leaves) ($N = 2$)	Apple (cv. Summer Red) ($N = 3$)	*S. luteum* ($N = 1$)
Terpenoids				
Linalool	3.6			
Limonene			tr[b]	
(*E*)-β-Ocimene	15.3	24.9	23.8	1.1
(*Z*)-β-Ocimene		0.3	tr	
(3*E*)-4,8-Dimethyl-1,3,7-nonatriene	12.9	53.8	8.5	59.4
(3*E*, 7*E*)-4,8,12-Trimethyl-1,3,7,11-tridecatetraene	5.7	3.4	0.2	
(*E*, *E*)-α-Farnesene		4.0		
Germacrene D			1.9	
Esters				
Methyl salicylate	2.7	tr	<0.2	
Ethyl benzoate			2.5	
Nitriles				
2-Methylpropanenitrile		0.1		
2-Methylbutanenitrile		0.8	<0.6[c]	
3-Methylbutanenitrile		2.5	<1.3	
Phenylacetonitrile			<1.2	
Oximes				
2-Methylbutanal *O*-methyloxime		0.8		
3-Methylbutanal *O*-methyloxime		3.8	<0.3	
Unknown oxime		0.1		

[a] Number of replications of GC–MS analysis.
[b] Compounds that were found less than 0.1%.
[c] The maximum value (expressed as <max. value) for compounds that were found in more than one, but not in all samples.

VARIATION IN HERBIVORE-INDUCED SYNOMONE COMPOSITION ACCORDING TO ATTACKING HERBIVORE SPECIES

Several reports indicate that different herbivore species elicit different HIS blends. For example, the predatory mites *Amblyseius andersoni* (=*A. potentillae*) and *A. finlandicus* responded to apple foliage infested by the European red spider mite *P. ulmi* (Sabelis and van de Baan, 1983), which is a suitable prey species (Dicke et al., 1988). However, they did not respond to apple foliage infested by the two-spotted spider mite *T. urticae*, which is not a suitable prey

species to these predators (Sabelisa and van de Baan, 1983). In contrast, satiated females of *P. persimilis* responded to volatiles emitted from apple leaves (Cox Orange Pippin) infested by *T. urticae*, but not to volatiles emitted from apple leaves infested by *P. ulmi* (Sabelis and van de Baan, 1983; Sabelis and Dicke, 1985). Starved females of *P. persimilis*, however, responded to apple leaves infested both by *T. urticae* and by *P. ulmi* over the uninfested apple leaves. When apple leaves infested by *T. urticae* and those infested by *P. ulmi* were compared simultaneously in a Y-tube olfactometer, starved females clearly preferred to walk towards the arm containing the volatiles of *T. urticae*-infested apple leaves (Sabelis and Dicke, 1985). This preferential response parallels the reproductive success of *P. persimilis* on these species: *T. urticae* is a more suitably prey species than *P. ulmi*. Such differential responses may be based upon the chemical differences recorded by Takabayashi et al. (1991a).

The composition of the volatile blends emitted by apple leaves of cv. Summer Red when infested by *T. urticae* or *P. ulmi* showed many quantitative differences (Table 4) (Takabayashi et al., 1991a). The four chemicals that are known to attract *P. persimilis*, (*E*)-β-ocimene, (3*E*)-4,8-dimethyl-1,3,7-nonatriene, linalool, and methyl salicylate, were found in the headspace of apple leaves infested by both spider mite species. However, the relative percentages differed between the two blends. This was especially true for (*E*)-β-ocimene and (3*E*)-4,8-dimethyl-1,3,7-nonatriene. The latter compound made up ca. 50% of the blend emitted by apple leaves infested by *P. ulmi* but only ca. 5–9% of the blend emitted by apple leaves infested by *T. urticae* or uninfested apple leaves. Although the relative percentage of (3*E*)-4,8-dimethyl-1,3,7-nonatriene is similar between uninfested leaves (6.7%) and leaves infested by *T. urticae* (8.5%), the infested leaves emit larger amounts of it (approximately more than 100 times) than the uninfested leaves. For linalool and methyl salicylate, the differences between blends were minor. (*E*, *E*)-α-farnesene made up more than 50% of the blend emitted by leaves infested by *T. urticae*, ca. 16% of the blend emitted by leaves infested by *P. ulmi*, and ca. 28% of the blend of uninfested leaves.

Other examples of predatory mites that are differentially attracted by volatiles emitted by plants of the same species that are infested by different herbivore species have been recorded by Dicke (1988) and Dicke and Groeneveld (1986).

It is likely that the natural enemies' physiological constraints on the sensory processing of this wide variety of chemical information makes it difficult for them to pick up the most beneficial message. Natural enemies may solve such constraints by temporary specialization through, e.g., learning (see section "How Do Predatory Mites Cope with Variation in Herbivore-Induced Synomone?" below).

TABLE 4. COMPOUNDS IDENTIFIED IN HEADSPACE VOLATILES OF LEAVES OF APPLE CV. SUMMER RED INFESTED BY *T. urticae* OR *P. ulmi*
(Data from Takabayashi et al. 1991[a])

	Relative amount (mean %)		
	Uninfested leaves ($N = 2^a$)	Leaves infested by *T. urticae* ($N = 3$)	Leaves infested by *P. ulmi* ($N = 4$)
Aldehydes			
2-Methyl-2-propenal		tr[b]	1
2-Pentenal			tr[b]
Hexanal	2.5	<0.4[c]	<0.1
2-Hexenal	3.5	0.7	
Nonanal	tr		
Alcohols			
1-Butanol	0.6		tr
2-Butanol			
1-Pentene-3-ol	0.9	<0.5	<1
3-Pentanol			tr
1-Hexanol	tr	0.3	
(Z)-3-Hexen-1-ol		0.5	1.5
2-Hexene-1-ol		<0.5	
1-Octene-3-ol		tr	
2-Ethyl-1-hexanol	8.3		0.6
Ketones			
2-Butanone	tr	tr	<1
1-Pentene-3-one	tr		
2-Pentanone		tr	tr
6-Methyl-5-heptene-2-one		<0.3	<0.2
Esters			
(Z)-3-Hexen-1-yl acetate	14.9	<0.3	6.3
2-Hexene-1-ly butyrate		<0.3	
(Z)-3-Hexene-1-yl butyrate			<2.5
Methyl benzoate		tr	
Ethyl benzoate		2.6	
3-Hexen-1-yl benzoate		tr	tr
Terpenoids			
Myrcene		0.2	0.1
Limonene		tr	tr
(Z)-β-Ocimene		tr	<0.2
(E)-β-Ocimene	3.8	23.8	10.1
(3Z)-4,8-Dimethyl-1,3,7-nonatriene		<0.1	0.9
(3E)-4,8-Dimethyl-1,3,7-nonatriene	6.7	8.5	49.4
β-Bourbonene	6.4		1.8
Linalool	0.6	0.3	1.3
β-Cubebene	tr	0.3	<0.5

TABLE 4. CONTINUED

	Relative amount (mean %)		
	Uninfested leaves ($N = 2^a$)	Leaves infested by *T. urticae* ($N = 3$)	Leaves infested by *P. ulmi* ($N = 4$)
Terpenoids			
β-Caryophyllene	1.5	0.6	0.9
β-Farnesene			<3.5
Germacrene-D	tr	1.9	tr
(*E*, *E*)-α-Farnesene	27.6	54.7	15.7
(3*E*, 7*E*)-4,8,12-Trimethyl-1,3,7,11-tridecatetraene		0.2	<0.5
Nitriles			
2-Methylbutanenitrile		<0.6	
3-Methylbutanenitrile		<1.3	
Phenylacetonitrile		<1.2	
Oxime			
3-Methylbutanal *O*-methyloxime		<0.3	
Others			
2-Methylfuran		tr	
2-Ethylfuran	tr		
Rosefuran			tr
Methyl salicylate	1.3	<0.2	1.5
Unidentified minor peaks	3.2	1.1	2.0

[a] Number of replications of GC–MS analysis.
[b] Compounds that were found less than 0.1%.
[c] The maximum value (expressed as <max. value) for compounds that were found in more than one, but not in all samples.

VARIATION IN PLANT VOLATILES ACCORDING TO ABIOTIC FACTORS

Because environmental conditions greatly influence plant physiology, we examined the effect of several abiotic factors (light, season, and water stress) on HIS production.

Light Intensity

Lima bean plants were reared from seeds in a greenhouse (15 days) at 20–30°C and subsequently placed in a climate room at 25°C, 50–70% relative humidity at different light intensities for six days prior to the olfactometer test. Uninfested leaves of Lima bean plants placed under high light intensity were

more attractive than leaves of Lima bean plants placed under low light intensity (Takabayashi et al., 1990) (Figure 2).

Chemical analysis showed that the headspace of leaves under high light intensity was characterized by relatively higher amounts of the synomone component (*E*)-β-ocimene (20.9%) than leaves under low light intensity (5.4%) (Table 5). This may explain why the leaves under high light intensity were more attractive to the predators than the leaves under low light intensity. The presence of higher amounts of (*E*)-β-ocimene in the volatiles of the leaves under high light intensity may be partly because: (1) plants under high light intensity received more photolytic energy input to produce more plant synomone and/or (2) plants under high light intensity may suffer water stress that triggers the production of plant synomone (see section "Water Stress" below).

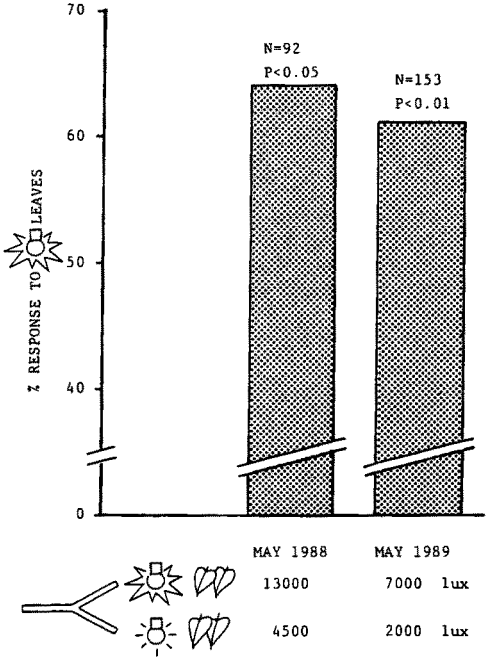

FIG. 2. Response of satiated female *P. persimilis* in a Y-tube olfactometer when offered Lima bean leaves that have been exposed to different light intensities for six days prior to the experiment. Experiments were done in May 1988 and May 1989 at Department of Entomology, Wageningen Agricultural University, The Netherlands.

TABLE 5. COMPOUNDS IDENTIFIED IN THE HEADSPACE OF UNINFESTED LIMA BEAN LEAVES UNDER HIGH LIGHT CONDITION AND THOSE UNDER LOW LIGHT CONDITION (J. Takabayashi, M. Dicke and M. A. Posthumus, unpublished data, 1989)

	Relative amount (mean %)	
	High light condition (N = 3[a])	Low light condition (N = 4)
Aldehydes		
Hexanal	2.2	2.0
2-Hexenal	2.7	1.5
Heptanal	0.6	
Octanal	0.8	0.7
Decanal		4.7
Nonanal	3.3	4.8
Alcohols		
1-Butanol	0.4	
(Z)-3-Hexen-1-ol	21.1	16.0
1-Hexanol	2.1	1.2
1-Penten-3-ol	0.3	
1-Octen-3-ol	3.6	2.1
2-Ethyl-1-hexanol	5.6	16.1
Ketones		
3-Pentanone	0.3	0.3
2-Butanone		2.8
Esters		
1-Butyl acetate	0.4	
Hexyl acetate	tr[b]	0.5
(Z)-3-Hexen-1-yl acetate	11.8	17.8
(Z)-3-Hexen-1-yl butyrate	5.2	1.7
3-Hexen-1-yl isovalerate	0.7	0.8
Terpenoids		
Limonene	1.9	4.4
(Z)-β-Ocimene	0.7	0.5
(E)-β-Ocimene	20.9	5.4
(3E)-4,8-Dimethyl-1,3,7-nonatriene	6.0	5.2
Linalool	2.8	1.7
(3E, 7E)-4,8,12-Trimethyl-1,3,7,11-tridecatetraene	1.1	1.0
Others		
Methyl salicylate	3.2	3.2
Unidentified minor peaks	2.4	5.4

[a]Number of replications of GC-MS analysis.
[b]Compounds that were found less than 0.1%.

Season

The same experiment as described above was carried out at different times of the year. In the greenhouse, artificial light (SON-T 400 W, 20,000 lux) was switched on when the light intensity dropped below 500 lux so as to provide plants with at least 16 hr of light per day all year. However, it is obvious that this constant photoperiod did not remove seasonal environmental differences (e.g., natural light intensity and natural light composition). The effect of light intensity experienced by the plants during the six days prior to the experiment on predator response depended on the time of the year at which plants were reared. When growth conditions were relatively good (from April to August 1989), predators differentiated between plants of different light regimes (Figure 3). In contrast, when growth conditions were relatively poor (from November to February 1988 and from September to November 1989), predators did not differentiate between plants of different light regimes (Figure 3). Thus, whether Lima bean plants reared in high light conditions are more attractive to their herbivores' natural enemies than Lima bean plants reared in low light conditions depends on the time of year (Takabayashi et al., 1990).

Water Stress

Since plants damaged by spider mites lose large amounts of water by transpiration (Tomczyk and Kropczynska, 1985), water stress is one of the physiological conditions of spider-mite damaged plants. To examine the influence

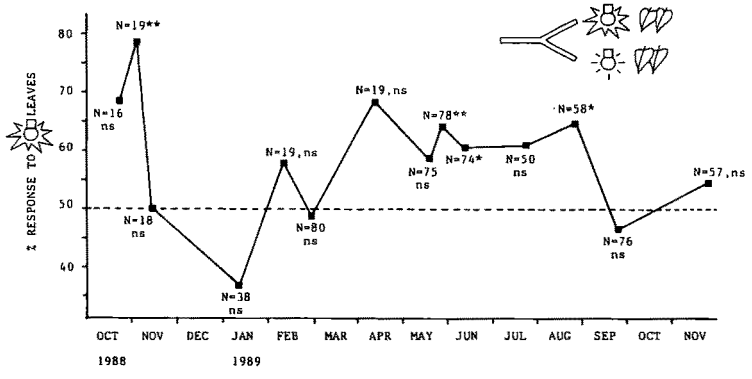

FIG. 3. Effect of time of the year on the response of *P. persimilis* to odors of Lima bean leaves from plants that have been exposed to different light intensities for six days prior to the experiment. Experiments were carried out at Department of Entomology, Wageningen Agricultural University, The Netherlands.

of water stress on HIS production, the soil was rinsed from 20 Lima bean seedlings of 20 cm height, and the plants were replanted in a sandy loam soil (600-cm^3 pots). Ten plants were reared under pF (moisture level) 1.5–1.8 (non-water stress condition) and the other ten were reared under pF 3–3.5 (water stress condition) for three weeks in a greenhouse (20–30°C, 60–70 relative humidity) where artificial light was switched on when the light intensity dropped below 500 lux so as to provide at least 16 hr of light per day. Leaves of uninfested plants reared under these two pF conditions were cut and used for the Y-tube olfactometer bioassay. About 20 g of leaves from water-stressed plants and 20 g from non-water-stressed plants were used simultaneously in each arm of the Y-tube olfactometer. The results show that the predatory mites preferred the odor of water-stressed Lima bean plants over non-stressed plants (Figure 4) (J. Takabayashi and M. Dicke, unpublished data, 1989).

We also compared the volatiles of leaves from water-stressed uninfested Lima bean plants with the volatiles of leaves from uninfested Lima bean plants reared under normal conditions (non-water-stressed condition, pF value unknown) (Table 6) (J. Takabayashi, M. Dicke, and M. A. Posthumus, preliminary data, 1989). The headspace of leaves from water-stressed Lima bean plants was characterized by relatively higher amounts of synomones [linalool, (*E*)-β-ocimene, (3*E*)-4,8-dimethyl-1,3,7-nonatriene, and methyl salicylate] than leaves from non-water-stressed Lima bean plants (Table 6). This may explain why the leaves from water-stressed Lima bean plants were more attractive to the predators than the leaves from non-water-stressed Lima bean plants.

All these data on abiotic effects were obtained from uninfested plants. It would be interesting to investigate to what degree abiotic conditions affect the emission of HIS by spider-mite-infested plants.

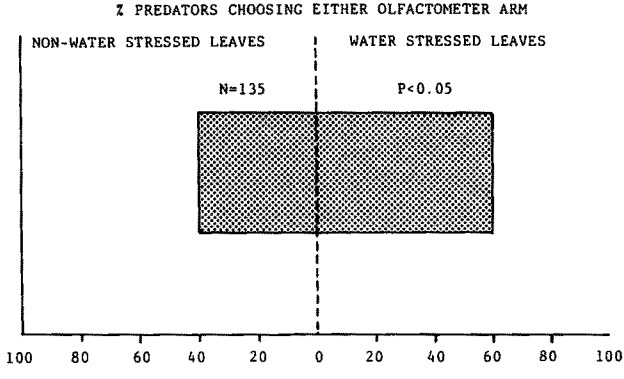

FIG. 4. Response of satiated female *P. persimilis* in a Y-tube olfactometer when offered water-stressed Lima bean leaves vs. non-water-stressed Lima bean leaves.

TABLE 6. COMPOUNDS IDENTIFIED IN HEADSPACE OF UNINFESTED LEAVES FROM WATER-STRESSED AND NON-WATER-STRESSED LIMA BEAN PLANTS (J. TAKABAYASHI, M. DICKE AND M.A. POSTHUMUS, UNPUBLISHED DATA, 1989)

	Relative amount (mean %) in leaves from	
	Water-stressed Lima bean ($N = 2^a$)	Non-water-stressed Lima bean ($N = 2$)
Aldehydes		
1-Hexenal		1.3
2-Hexenal		0.9
Alcohols		
1-Butanol	0.2	
(Z)-3-Hexen-1-ol	8.6	31.4
1-Hexanol	0.3	1.8
2-Ethyl-1-hexanol	13.6	0.7
1-Octene-3-ol		6.0
3-Octanol		2.8
Ketones		
2-Butanone	1.5	6.0
3-Pentanone	0.6	2.9
3-Octanone		2.0
Esters		
(Z)-3-Hexen-1-yl acetate	2.0	29.2
(Z)-3-Hexen-1-yl butyrate		3.6
3-Hexene-1-yl propionate		0.4
3-Hexene-1-yl isopropionate		0.4
Terpenoids		
β-Phellandrene		0.2
Limonene	0.4	2.9
(E)-β-Ocimene	6.5	2.1
(3E)-4,8-Dimethyl-1,3,7-nonatriene	33.5	2.6
(3Z)-4,8-Dimethyl-1,3,7-nonatriene	0.5	
Linalool	4.1	2.7
(3E, 7E)-4,8,12-Trimethyl-1,3,7,11-tridecatetraene	3.7	
Others		
Methyl salicylate	24.5	

[a] Number of replications of GC–MS analysis.

HOW DO PREDATORY MITES COPE WITH VARIATION IN HERBIVORE-INDUCED SYNOMONE?

Many herbivorous spider mites are polyphagous. Thus, predatory mites such as *P. persimilis* that feed on spider mites, will encounter a wide variety of HIS compositions. In addition to this variation, the previously recorded variation within a plant species or an individual plant adds more variation in HIS composition. Furthermore, even relative specialists such as *P. persimilis* feed on several prey species, depending on their starvation level.

Predator fitness is affected by both the prey species and the plant species that has been fed upon by the herbivore (Sabelis and Dicke, 1985; Dicke and Sabelis, 1988a). Therefore, an important question is how do the predators cope with all this variation in HIS and how do they select the most profitable HIS? Three factors influence predator response:

1. Starvation. The predator *P. persimilis* responds to the HIS of a wide range of plants that are infested by *T. urticae* (Dicke and Sabelis, 1988a). The predator is only attracted to a plant infested by a much less suitable prey species such as the spider mite *P. ulmi* after starvation, but even then, the predator prefers the HIS associated with its suitable prey species *T. uritcae* (Sabelis and Dicke, 1985).

2. Dietary Needs. The predatory mite *A. andersoni* is not attracted by plants infested by secondary prey species such as *T. urticae* or the rust mite *Aculus schlechtendali* when the predator has been feeding on a carotenoid-containing food source (Dicke et al., 1986; Dicke and Groeneveld, 1986). The carotenoids are essential nutrients for diapause induction and possibly also for other functions (Overmeer and van Zon, 1983; Dicke et al., 1989). If the predators are reared on a food source that lacks carotenoids but that appears to be a rewarding food source in terms of offspring production, the predators broaden their response to HIS and are attracted to plants infested by, e.g., *T. urticae*, *A. schlechtendali*, and thrips (Dicke et al., 1986; Dicke and Groeneveld, 1986). Similar data have been reported for other species of predatory mites (Dicke et al., 1989).

3. Experience. Satiated *P. persimilis* that have been feeding on *T. urticae* on Lima bean leaves [hereafter called *P. persimilis* (Li)] prefer the HIS of *T. urticae*-infested Lima bean leaves over the HIS of *T. urticae*-infested cucumber leaves in the Y-tube olfactometer (Dicke et al., 1990c) (Figure 5). When predators that have been reared on *T. urticae* on Lima bean leaves are transferred to *T. urticae*-infested cucumber leaves for seven days [hereafter called *P. persimilis* (Cu)], their preference is reversed; they prefer the HIS of *T. urticae*-infested cucumber leaves over the HIS of *T. urticae*-infested Lima bean leaves (Dicke et al., 1990c) (Figure 5). This indicates that the predatory mites distinguish between the two HIS through learning (for a definition of learning, see Papaj and Prokopy, 1989).

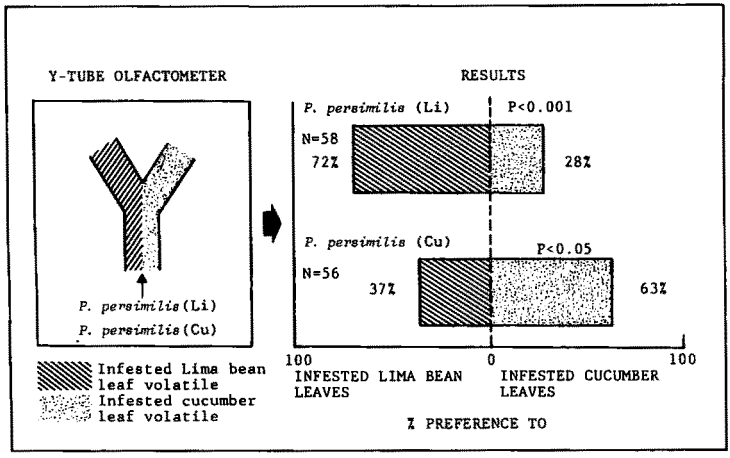

FIG. 5. Response in Y-tube olfactometer of two groups of satiated female *P. persimilis* [*P. persimilis* (Li) and *P. persimilis* (Cu)] towards *T. urticae*-infested Lima bean leaves versus *T. urticae*-infested cucumber leaves. The Y shape in the figure indicates the olfactometer.

When one of the two (Lima bean HIS and cucumber HIS) was offered versus clean air in the olfactometer, the two differently reared predatory mite groups responded differently. *P. persimilis* (Li) responded to the previously experienced HIS (Lima bean HIS) but not the the unexperienced HIS (cucumber HIS) (Figure 6). *P. persimilis* (Cu) showed an increase in response towards the newly experienced HIS (cucumber HIS), while its response to the previously experienced HIS (Lima bean HIS) remained unchanged for seven days (Dicke et al., 1990c) (Figure 6). These different responses of the two predatory mite groups explain the different preferences towards the two HIS in the olfactometer according to their predation experience as shown in Figure 5.

Next, the choice between *T. urticae*-infested Lima bean and *T. urticae*-infested cucumber leaves was compared for predators with different predation experience (Figure 7). The predatory mites had been reared on *T. urticae* on Lima bean plants and were subsequently transferred to *T. urticae*-infested cucumber leaves. Then, predatory mite groups with different exposure to *T. urticae*-infested cucumber leaves were prepared (see legend of Figure 7). The preference for cucumber HIS over Lima bean HIS clearly increased with increased exposure (Dicke et al., 1990c) (Figure 7). This indicates that the learning mechanism involved is sensitization. Further research is necessary to find out whether associative learning is involved in this learning mechanism.

The above discrimination of the predatory mites between the two infested

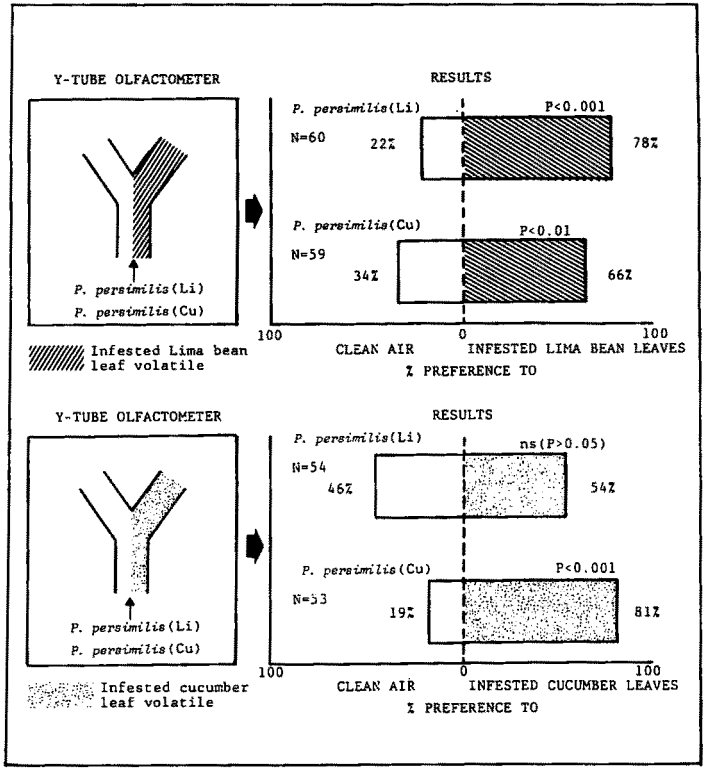

FIG. 6. Response in Y-tube olfactometer of two groups of satiated female *P. persimilis* [*P. persimilis* (Li) and *P. persimilis* (Cu)] towards *T. urticae*-infested leaves versus clean air. The Y shape in the figure indicates the olfactometer.

leaf volatiles can be made on the bases of the chemical differences in the two infested leaf volatiles (Dicke et al., 1990a,b; Takabayashi et al., 1994) (Table 4; and see section "Variation in HIS According to the Attacking Herbivore Species" above). Both infested Lima bean leaves and infested cucumber leaves emitted (3*E*)-4,8-dimethyl-1,3,7-nonatriene and (*E*)-β-ocimene as major herbivore-induced volatiles. However, infested Lima bean leaves emitted linalool and methyl salicylate, whereas infested cucumber leaves did not emit linalool and only trace amounts of methyl salicylate. The reverse was the case for (*E*, *E*)-α-farnesene, three oximes, and three nitriles (Table 4): these compounds were found in the infested cucumber leaf volatiles but not in the infested Lima bean leaf volatiles.

A similar effect of predation experience has been recorded with respect to

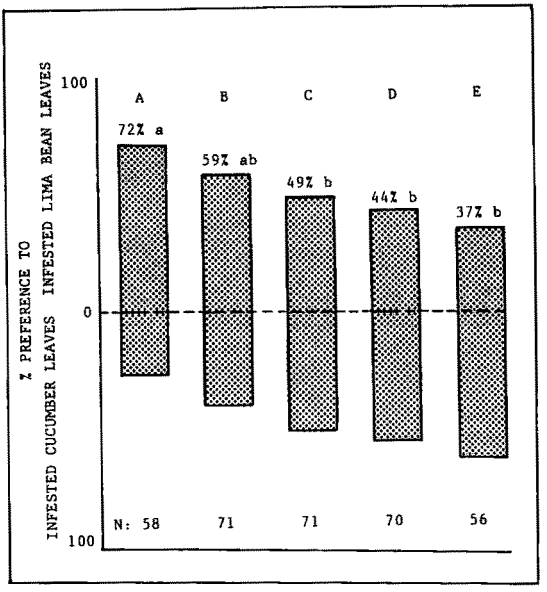

FIG. 7. Response in Y-tube olfactometer of five groups (A–E) of satiated female *P. persimilis* towards *T. urticae*-infested Lima bean leaves versus *T. urticae*-infested cucumber leaves. A: predators reared on infested Lima bean leaves for seven days, B: predators with 3 hr experience on previously infested cucumber leaves, C: predators with 3 hr predation experience on infested cucumber leaves, D: predators with 24 hr predation experience on infested cucumber leaves, E: predators reared on infested cucumber leaves in seven days. Volatiles of Lima bean leaves infested by 400 *T. urticae* versus volatiles of cucumber leaves infested by 4000 *T. urticae* were compared. Percentages above bars that are followed by the same letter are not significantly different (Kruskal-Wallis test, with multiple comparisons, $\alpha = 0.05$).

the response of *P. persimilis* to volatiles from uninfested cucumber and Lima bean plants (Takabayashi and Dicke, 1992). The host plant on which the predatory mites had consumed their prey affected the response of the predators to the volatiles of the leaves of uninfested plants: there was a tendency for the predator feeding on prey on a certain host plant to make itself more "tuned to" cues emitted by this plant species. The tuning into volatiles from uninfested plants is rather low (Takabayashi and Dicke, 1992) compared to the tuning into infested plants (Dicke et al., 1990c).

These data show that predatory mites feeding in a spider-mite patch on a certain plant species tend to specialize temporarily on the associated HIS. In doing so, they stay within the prey patch on the same plant species. As plants may affect predator fitness directly and indirectly (Dicke and Sabelis, 1988a),

this temporary specialization on a successfully experienced plant will improve predator fitness. For instance, if the prey contain plant secondary chemicals that are maladaptive to predators, the specialization will not continue. On the other hand, the temporary specialization of the predator on successfully experienced plants will ensure that they consume prey that does not vary in quality in terms of plant secondary chemicals.

From the plant viewpoint, if the predators prefer the preexperienced plant species with temporary specialization through learning, the plants may evolve a more specific HIS composition to attract the predators more effectively. Thus the specificity of HIS production and the ability to learn may have coevolved.

CONCLUSION

This paper has looked at tritrophic interactions between plants, spider mites, and predatory mites. In these systems, terpenoids as wells as the phenolic compound methyl salicylate were emitted from infested leaves, and they played an important role in the induced indirect defense mechanisms of plants against spider mites; predatory mites used these chemicals to locate infested leaves. However, this system is not as simple as it first appears. For instance, each plant species and cultivar produces its own characteristic combination of HIS. Thus, predators face a diversity of HIS according to the diversity of host plant species used by their prey. Predators may deal with this variation by innate recognition of the different HIS blends as wells as by temporary specialization to HIS through learning. HIS variation is also observed within an individual plant; young cucumber leaves and old cucumber leaves produce different blends of HIS. Predators prefer the volatiles of infested young cucumber leaves and are thus directed to the growing part, which is very important in the plant's competition for light with its neighboring plants.

Similar interactions over three trophic levels have also been found in systems consisting of plants, caterpillars, and parasitic wasps (Turlings et al., 1990a,b, 1993; Dicke, 1994). In such systems, terpenoids were also mediators of the induced indirect defense of plants against herbivores. However, there are striking differences between the predator system and the larval parasitoid system. Predators remove herbivores directly from plants, which is obviously beneficial to both predators and plants. Thus, the interaction between the two organisms is immediately symbiotic. In contrast, the beneficial effect of the larval parasitoid lags behind or is absent: the herbivorous host continues to feed until egression of the parasitoids and thus inflicts damage, sometimes at an even higher rate (e.g., Parker and Pinnel, 1992). Parasitoids that do not contribute to plant fitness do not affect the individual plant and are therefore irrelevant to the evolution of HIS (Sabelis and de Jong, 1988). Further comparative studies of predator sys-

tems and parasitoid systems are necessary to elucidate the evolution of HIS production.

Herbivore-induced plant volatiles may serve functions other than HIS, such as: plant pheromones (Bruin et al., 1991, 1992; Dicke et al., 1990c), spider-mite pheromones (Dicke, 1988), and spider-mite kairomones (Sabelis and van de Baan, 1983). Thus a plant infested by herbivores is considered to be a "chemically mediated hive of activity" involving multitrophic level interactions with direct and indirect effects. Such hives of activity are patchily distributed in the ecosystem. As seen in this paper, different combinations of herbivore-induced plant volatiles may create different types of hives. Chemical ecology has just started to shed light upon such systems. Further studies of these chemically mediated hives of activity will throw more light upon this new aspect of ecosystems.

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IN SEARCH OF ALLELOPATHY IN THE FLORIDA SCRUB: THE ROLE OF TERPENOIDS[1]

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Abstract—The hypothesis was tested that allelopathic agents released from fire-sensitive plants of the Florida scrub community deter the invasion of fire-prone sandhill grasses. The structures of the constituents of four endemic scrub species, *Conradina canescens*, *Calamintha ashei*, *Chrysoma pauciflosculosa*, and *Ceratiola ericiodes*, were established and their phytotoxic activity against two grasses of the sandhill was examined. Effects of the secondary metabolites from the above scrub species and their degradation products upon the germination and radicle growth of little bluestem (*Schizachyrium scoparium*) and green sprangletop (*Leptochloa dubia*), two native grasses of the Florida sandhill community, were determined. The studies included determination of the water solubility and release mechanism of terpenes and other allelopathic agents from the source plants and their aqueous transport to the target species. Some of the natural products were nontoxic until activated by light and/or oxidation after release from the source plant into the environment.

Key Words—*Calamintha ashei* (Labiatae), *Conradina canescens* (Labiatae), *Chrysoma pauciflosculosa* (Asteraceae), *Ceratiola ericiodes* (Empetraceae), terpenoids, allelopathy, mechanisms of release, water transport, activation.

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[1] Dedicated to one of the pioneers in allelopathy, Cornelius H. Muller.

INTRODUCTION

In the ongoing debate about the existence or nonexistence of allelopathy, this poorly understood area of chemical ecology is still considered an "illegitimate child" by opponents and a "stepchild" by proponents. Much of the criticism expressed in the past stems from the wealth of anecdotal information in the ecological and agricultural literature of alleged allelopathic actions and the paucity of experimental verification. The term "allelopathic action" in connection with the description of phytotoxic effects of a source plant upon ecologically unrelated target species has caused further consternation among ecologists. This has led to critical statements that "almost all species can, by appropriate digestion, extraction and concentration, be persuaded to yield a product that is toxic to one species or another" (J.L. Harper, 1975, 1977). Another cause for the extensive criticism of allelopathy resides in the fact that "chemistry [or the lack of it] has been the Achilles heel of allelopathy" (Putnam and Tang, 1986b), because most studies have been performed with little or no knowledge of the active principles, their chemical nature, their transport mechanism, quantities of released agent(s) in aqueous leachates, and concentrations in the soil. In recent years, in a collaborative effort between chemists and ecologists, we have studied the chemical interaction of higher plants in two adjacent plant communities in Florida, the scrub and the sandhill, in an attempt to provide a better understanding of the complex interactions among plant species. The goals of this project were to shed light on the multiple biological interactions in the two plant communities, to test the "influence of one plant upon another" (Molisch, 1937) and to determine the role of a biotic component in determining plant community composition.

The Florida scrub, in particular its early seral stage, represents an ecosystem with numerous advantages for the study of possible plant–plant interactions:

1. The sandy soil of the scrub contains little organic matter (Kalisz and Stone, 1984), thereby facilitating detection and quantification of allelotoxins in the soil below and near the source plant.
2. The quartz sand reduces uncontrolled absorptions of allelochemicals by clay soil particles (Muller, 1965a).
3. The source and target species are clearly defined. Early bioassays (Richardson and Williamson, 1988) provided evidence for allelopathic activity of several fire-sensitive scrub perennials against fire-prone sandhill species.
4. Last but not least, the Florida scrub hosts about 400 plant species including some 40 endemics, many of which are threatened or endangered (Menges and Salzman, 1992). This little studied community chemically represents a valuable resource for undiscovered natural products with potential biological activities (Eisner et al., 1990).

Following several years of ecological studies, four endemic shrubs common in immature white sand scrub communities were initially chosen for our combined chemical and biological studies. In this review, data will be presented from studies of two mints, *Calamintha ashei* (Weatherby) Shinners (Labiatae) and *Conradina canescens* (Torr. and Gray) (Labiatae), the Florida rosemary [*Ceratiola ericoides* (A. Gray)] of the family Empetraceae, and the woody goldenrod [*Chrysoma pauciflosculosa* (Michx.) Greene] of the Asteraceae family. Besides the new chemical data, which had to be established for all four species, their effects on the putative target species of the sandhill community were determined.

Our discussion will also address questions related to the production and storage of potentially allelopathic agents from the above scrub source plants. Furthermore, we will present evidence for possible mechanisms of release of potential phytotoxins and/or pretoxins and their transport from the leaf surface and/or roots into the soil and their inhibition of germination and growth of graminoid target plants of the sandhill community, which are used as test species to estimate the magnitude of allelopathic effects. Examples from *Chrysoma* and *Ceratiola* will be provided that demonstrate that after release into the environment photochemical and/or air-oxidative modifications can alter plant products to form derivatives of increased activity. Results of our studies on the qualitative and quantitative aspects of terpenoids and other plant products will hopefully contribute to a better understanding of the complex nature of the ecological roles of plant products in plant–plant interactions.

The allelopathic and phytotoxic properties of plant terpenoids have received attention in numerous publications and reviews. Sigmund (1924) was the first to demonstrate that essential oils as well as pure monoterpenes strongly inhibit seed germination and plant growth. Earlier treatments of phytotoxic and possible allelopathic actions of secondary plant metabolites including terpenoids were presented in books by Rice (1984), Thompson (1985), Putnam and Tang (1986a), Waller (1987), and Harborne (1988). Phytotoxic terpenoids and their possible involvement in allelopathy were covered in reviews on mono- and sesquiterpenes (Evanari, 1949; Fischer, 1986, 1991) and biological activities of sesquiterpene lactones were reviewed by Picman (1986) and Stevens and Merrill (1985). A summary of terpenoid allelopathy in desert ecosystems was presented by Friedman (1987), and a recent general review on allelopathic plant terpenoids was provided by Fischer (1991).

FLORIDA SCRUB AND SANDHILL COMMUNITIES

In the southeastern coastal plains of the United States, two plant communities, the sandhill and the scrub, are found on the sandy ridges of relict shorelines and coastal dunes. The two communities share a subtropical climate with

frequent heavy rains, particularly during the summer months of June to October. Although both plant communities are dominated by pines and oaks, their species composition is distinctly different. The sandhill is an open woodland with pines and oaks and a dense ground cover of grasses, mainly wiregrass (*Aristrida stricta* Michx.) and beard grasses (*Schizachyrium* and *Andropogon* spp.). Common fire-adapted pine species are slash pine (*Pinus elliottii* Engelm.) and longleaf pine (*P. palustris* Mill.). Oak species in the sandhill include sand live oak (*Quercus germinata* Small), turkey oak (*Q. laevis* Walt.) and bluejack oak (*Q. incana* Bartr.). With the exception of patches of saw palmetto [*Serenoa repens* (Bartr.) Small] shrubs are not common in the sandhill.

Throughout the sandhill vegetation are strands and islands of the much less common sand pine scrub community. The young scrub is mainly composed of endemic shrubs. A few such as *Ceratiola* are universally present, but most, such as *Chrysoma* and the mints, are restricted to local sites. In contrast, mature scrub has a closed canopy of sand pine (*P. clausa* Vasey ex Sarg.) and a dense understory of scrub oaks (*Q. chapmanii* Sarg., *Q. germinata*, and *Q. myrtifolia* Willd.), but like young scrub, there is almost no herbaceous ground cover. On sunny days, the well-drained white sandy soil has surface temperatures greater than 50°C (Richardson, 1985), and during the dry season as well as between summer rains moisture stress is significant in the upper horizons. In addition, the scrub and sandhill soils are considered nutrient poor with low levels of potassium and nitrogen (Christensen, 1988; Kalisz and Stone, 1984; Richardson, 1985), perhaps adding to the stress of scrub species.

Nearly 100 years ago, Nash (1895) noted that "The scrub flora is entirely different from that of the high pine land [sandhill], hardly a single plant being common to both; in fact these two floras are natural enemies and appear to be constantly fighting each other . . . . Later, Webber (1935) called the Florida scrub "a fire-fighting machine." Fire susceptibility and fire frequency of the two communities differ drastically. Grasses and pine needles are the fuel for surface fires that sweep through the sandhills every three to eight years (Williamson and Black, 1981). Surface fires move through the sandhills until they reach the ecotone, separating the sandhill from patches or strands of scrub. In most instances the fires are extinguished due to the lack of fuel caused by dramatic reduction of the surface vegetation in the ecotone. Postfire conditions in the sandhill stimulate plant reproduction and development without overall change of species composition.

Every 20–50 years crown fires devastate the slow-growing, fire-sensitive evergreen scrub stands. However, the community regenerates successfully by resprouting of oak species and fire-initiated release of sand pine seeds from serotinous cones (R.M. Harper, 1915; Richardson, 1977; Williamson et al., 1992b). The characteristic differences distinguishing the two plant communities are summarized in Table 1 (Williamson et al., 1992b).

TABLE 1. COMPARISON OF FLORIDA'S SCRUB AND SANDHILL COMMUNITIES AND CALIFORNIA'S CHAPARRAL AND GRASSLAND COMMUNITIES (Williamson et al., 1992b)

Community		
Florida	scrub	sandhill
California	chaparral	grassland
Physiognomy		
Ground cover	little	complete
Shrub cover	very dense	very sparse
Fuel traits		
Surface litter quantity	low	high
Surface litter quality	compressed	loose, aerated
Crown litter quantity	high	none
Foliage phenology	evergreen	deciduous
Fire traits		
Frequency	20–50 years	3–8 years
Type	crown	surface
Evidence of allelopathy	yes	no

Fire appears to be the major factor in preventing invasion of scrub plants into the sandhill. In fire-free intervals in the sandhill, scrub species will colonize and grow well, often faster than in the scrub, but then they are killed by the next natural surface fire sweeping through the graminoid ground cover (Laessle, 1958; Veno, 1976; Hebb, 1982). In mature scrub the closed canopy seems to prevent colonization of sandhill (or scrub) species, which generally require full sunlight. However, this does not explain the lack of sandhill species colonizing young scrub communities, which are open. Numerous studies of soil profiles of the two communities have produced meager differences in physical and chemical soil characteristics (R.M. Harper, 1914; Webber, 1935; Laessle, 1958, 1968). The most recent study concludes that any differences are attributable to the different plant communities and their fire regimes (Kalisz and Stone, 1984). The lack of significant differences in soil nutrients (Kalisz and Stone 1984; Richardson, 1985) and the fact that the addition of fertilizers in scrub plots did not increase germination or seedling growth relative to unfertilized control plots (Richardson, 1985) led to our studies in search for allelopathic actions in the scrub.

As summarized in Table 1, differences in the Florida scrub and the adjacent sandhill parallel the differences between California's chaparral and adjacent grasslands (Williamson, 1990). In addition, the sometimes sharp ecotone between scrub and sandhill resembles the bare zone between chaparral and grasslands. These similarities cannot be explained on the basis of soils, mainly clays in California versus sands in Florida, or on the basis of climate, dry in California

with precipitation concentrated in the winter versus wet in Florida with precipitation concentrated in the summer. However, parallels exist in fire regimes and in evidence of allelopathic suppression of grasses and herbs in both the California chaparral and the Florida scrub (Williamson, 1990).

ROLE OF MONOTERPENES IN FLORIDA SCRUB

We have tested the hypothesis (Richardson and Williamson, 1988) that allelopathy has evolved as a mechanism to prevent the invasion of fire-prone grasses and pines into the scrub, in particular, in immature or disturbed scrub communities (Williamson, 1990; Williamson et al., 1992b). Since graminoids provide fuel for frequent surface fires, the slow-growing, fire-sensitive scrub colonizers would be adversely affected by these fires. It was, therefore, proposed that allelopathic agents released from scrub species inhibit the growth of the fire-fueling grasses and create fuel breaks around individual plants and ecotones along dense scrub communities (Richardson and Williamson, 1988; Williamson et al., 1992b).

Our recent studies have provided evidence that biotic instead of abiotic factors are mainly responsible for the patterns of the scrub and sandhill communities (Richardson, 1977, 1988; Richardson and Williamson, 1988; Williamson and Black, 1981; Fischer et al., 1988; Fischer, 1991; Williamson et al., 1992b; Weidenhamer and Romeo, 1989). Earlier studies involved monthly collections of fresh leaves of *Calamintha ashei*, *Conradina canescens*, and *Ceratiola ericoides* from central Florida for bioassays. The aqueous soakings of fresh foliage of each of the three species, as well as decaying litter in the case of *Ceratiola*, were tested against three native sandhill grasses, *Schizachyrium scoparium* (Michx.) Nash, *Andropogon gyrans* Ashe, and *Leptochloa dubia* (HBK) Nees, for germination and radicle growth inhibitions (Richardson and Williamson, 1988). The effects of the leaf washes of all three scrub species upon the germination of target sandhill grasses was significant, with an overall average of inhibition of 20%. *Calamintha ashei*, *Conradina canescens*, and *Ceratiola ericoides* leaf washes gave 44%, 34%, and 20% inhibition of *Leptochloa*, respectively (Williamson et al., 1992b). Effects of the leaf washes on *Schizachyrium* were lower but decaying *Ceratiola* litter showed a 26% reduction of germination.

Monoterpenes of Conradina canescens *and their Biological Activities*

This member of the mint family is an evergreen of the sand pine scrub. It exists in two major disjunct populations, one in the Florida Central Ridge in Highland and Polk counties and the other in the Gulf Coastal region from Horn Island, Mississippi, to near Tallahassee, Florida. Within its range, it is relatively

common in areas where full sunlight occurs. Under the shady canopy of sand pine and the subcanopy of oaks of the mature scrub, it is less abundant. Recurved, undamaged leaves of *Conradina* emit no odor; however, even mild mechanical disturbance results in the emission of a strong terpene odor (Williamson et al., 1989). Scanning electron micrographs of the leaf surfaces of *Conradina* showed glandular trichomes encircled by the filamentous ones (Williamson et al., 1989). Cross-sections revealed secretory cells under a layer of the cuticle that harbored the secretion. Similar glandular and nonglandular trichomes are found in some, but not all, mint species (Metcalfe and Chalk, 1965). Dipping the leaves into organic solvents dissolved the fold of cuticle and the underlying secretions, whereas the filamentous trichomes remained intact. The resulting extract contained a mixture of monoterpenes and triterpenes.

Aqueous leaf soaks of fresh *Conradina* leaves were strongly inhibitory to sandhill grasses, and their chemical analysis indicated the presence of monoterpenes as well as the triterpenes, ursolic acid **(9)** and betulin **(10)**, which appear to be present in copious amounts in the cutical wax (Figure 1). Detailed chemical analysis of *Conradina* foliage provided a mixture of monoterpenes, typical of the mint family and also the active components of other allelopathic mints (Muller et al., 1964; Muller, 1965a,b; Muller and del Moral, 1966; Fischer, 1986, 1991). Besides several minor monoterpenes, the known entities, 1,8-cineole **(1)**, camphor **(5)**, borneol **(6)**, myrtenal **(7)**, myrtenol **(8)**, α-terpineol

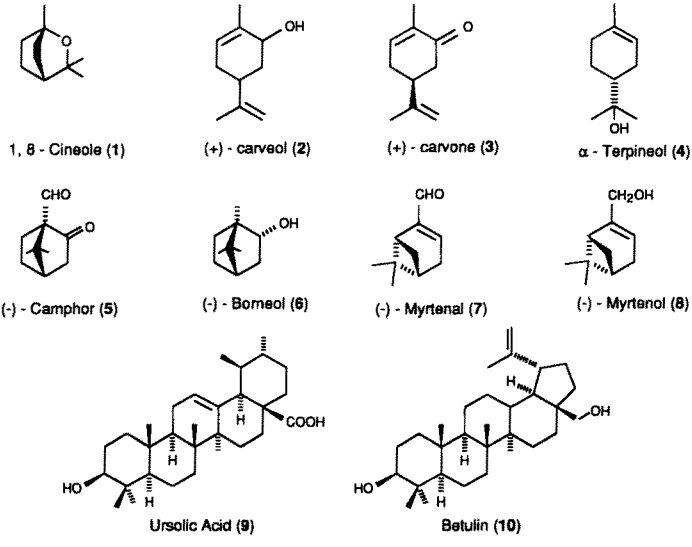


FIG. 1. Monoterpenes and triterpenes found in *Conradina canescens*.

(4), carveol **(2)**, and carvone **(3)** were the major constituents (Figure 1). Several of these monoterpenes (e.g., borneol, camphor, and 1,8-cineole) have been previously described as potent plant germination and growth inhibitors (Asplund, 1968, 1969; Muller, 1965a; Fischer, 1986, 1991).

Biological activities of saturated aqueous solutions of the major monoterpenes of *Conradina* on *Schizachyrium* and *Leptochloa* as well as lettuce were determined. Petri dish bioassays of the monoterpenes, applied to *Leptochloa* and *Schizachyrium*, revealed complete inhibition of germination by five compounds and statistically significant inhibition of germination in each case with the exception of 1,8-cineole, which had no effects on *Leptochloa* (Table 2). Thus, saturated aqueous solutions of most of the monoterpenes tested provided strong phytotoxicity. This result was highly unexpected since this group of natural products was assumed to have negligible water solubility due to the nonpolar character of the monoterpenes relative to other more polar plant metabolites such as organic acids and phenolics (Harborne, 1988). The above bioassay data strongly suggested that the monoterpenes in *Conradina* are not only potent phytotoxins but appear to have sufficiently high water solubilities to cause strong inhibitory effects in aqueous solution. In contrast, previous allelopathic effects of monoterpenes in the California chapparal had involved volatility as a mechanism whereby the volatile monoterpene reach the soil to be adsorbed on lipophilic clay particles and seed/seedling membranes (Muller and del Moral, 1966).

Storage, Water Solubility and Aqueous Transport of Monoterpenes

Secondary plant products are generally localized in specialized cells, tissues, organs, and organelles. Storage sites include glandular trichomes, resin ducts, secretory cavities, surface wax, vacuoles, and cell walls (Gershenzon, 1993). Defensive natural products could in principle have multiple functions, that is, be active against herbivores and also act as allelopathic agents (Langenheim, 1994). For instance, leaf surface waxes and resins (Gershenzon, 1993; Fischer et al., 1988) as well as constituents of glandular trichomes (Duke and Paul, 1993; Spring, 1991; Kelsey et al., 1984) may be active against herbivorous insects and also inhibit the germination and growth of competing plant species (Duke, 1991). Furthermore, lipophilic constituents in resin ducts of plant roots can be released into the surrounding soil at concentration levels sufficient to act as potent allelopathic agents (Tang, 1986; Kobayashi et al., 1980; Menelaou et al., 1992). Lipophilicity and the site of storage of secondary plant products appear to correlate since lipophilic plant metabolites, including most terpenoids, accumulate in secretory structures or in plant surface wax, whereas hydrophilic compounds are present in cell walls and/or vacuoles (Gershenzon, 1993).

The function of plant metabolites in allelopathic actions differs distinctly

TABLE 2. EFFECTS OF SATURATED AQUEOUS SOLUTIONS OF MONOTERPENES DETECTED IN LEAF SOAKS OF *Conradina canescens* ON GERMINATION (G) AND RADICLE LENGTHS (R) OF *Leptochloa dubia* AND *Schizachyrium scoparium*, AS PERCENT OF WATER CONTROLS[a]

	Leptochloa		Schizachyrium	
Compound	G	R	G	R
1,8-Cineole	100	97	20**	26**
(−)-Camphor	12**	34**	16**	61
(−)-Borneol	0**		8**	47*
(+)-α-Terpineol	0**		0**	
Myrtenal	2**	5**	8**	13**
Myrtenol	0**		0**	
(−)-Carvenol	0**		0**	
(+)-Carvone	0**		0**	

[a]Treatment was significantly different from water controls at $*P < 0.05$; and $**<0.01$.

from those of antiherbivore defense compounds in that the defense actions seem to be exclusively beneficial to the living plant tissue. In contrast, allelopathic activity may also be caused by phytotoxins released from dead tissue, such as plant leaf litter, or plant residues. Litter of evergreens in natural ecosystems, e.g., *Ceratiola ericoides*, as well as decomposing plant residues in agricultural monocultures are often highly phytotoxic (Rice, 1984; Williamson et al., 1992a). Sometimes, significantly higher allelopathic effects have been observed when decomposing plant material is involved (Rice, 1984; Fischer et al., 1988).

In general, aqueous transport may be essential to effective allelopathy where the aqueous runoff during frequent rains is transported to the soil solution and the target species. Alternative transport as volatiles was proposed in the California chaparral (Muller, 1965a,b; Muller and del Moral, 1966) and still may require aqueous dissolution in dew or soil solution (Muller et al., 1964). The "volatility" mechanism seemed less likely to operate within the immature scrub of the Florida coastal regions where frequent winds and high soil surface temperature would prevent condensation of monoterpenes on soils in the proximity of the plant.

Due to the relatively nonpolar character of monoterpenes, the group has been classified as volatile and assumed to have negligible water solubility in comparison to other more polar, organic natural products. For instance, Harborne (1984) states that "terpenoids are generally lipid-soluble" whereas "phenolic substances tend to be water-soluble." In spite of reports of significant solubilities (Rhode, 1922; Seidell, 1940–41; Smyrl and LeMaguer, 1980), cita-

tions in standard chemical references continued to represent monoterpenes as insoluble in water (Budavari, 1989; Weast, 1976, 1989).

Based on our preliminary data, allelopathic effects of the two scrub mints, *Calamintha ashei* and *Conradina canescens*, seemed to exist and are most probably due to monoterpenes as active constituents (Richardson and Williamson, 1988; Tanrisever et al., 1987, 1988; Macias et al., 1989; Williamson et al., 1989). Bioassays of saturated aqueous solutions of monoterpenes suggested that the most likely mechanism of allelochemical release was aqueous leaching of foliage and litter in apparent incongruity with the assumed aqueous insolubility of monoterpenes. Therefore, as part of our ongoing investigations of allelopathic mechanisms, we determined the water solubility of a structurally diverse group of monoterpenes, eight hydrocarbons and 23 oxygen-bearing monoterpenes (Weidenhamer et al., 1993). Their solubilities were also compared to known phenolic phytotoxins, juglone, ferulic acid, and hydrocinnamic acid (Davis, 1928; Blum and Dalton, 1985; Tanrisever et al., 1987; Harborne, 1988), which are presumed to be water soluble.

Solubility among the monoterpenes varied considerably, ranging from < 10 ppm to 6990 ppm (Table 3). The hydrocarbon monoterpenes had low solubilities, all under 35 ppm, but oxygenated monoterpenes such as aldehyde, ketones, alcohols, and ethers had solubilities 10–100 times greater than the hydrocarbons with a similar skeleton. Alcohols were somewhat more soluble than related ketones in the monocyclic skeletons. In bridged bicyclic monoterpenes, ring strain favors formation of the geminal diols from the ketones, making them more soluble than comparable alcohols, for instance, camphor (550 ppm) versus borneol (274 ppm) and verbenone (6990) versus myrtenol (1010 ppm). The monoterpenes exhibited a broad range of aqueous solubilities unexpectedly comparable to the common allelopathic phenolics and organic acids, juglone (52 ppm), ferulic acid (174 ppm), and hydrocinnamic acid (3490 ppm).

These results demonstrate that generalization about the biological activities and ecological functions of monoterpenes based on putative differences in aqueous solubilities need to be reevaluated. For example, Tukey (1969) in his classic foliar leaching studies concluded that "carbon dioxide, ethylene and terpenes" are released as volatiles, while rain and dew leach "mineral nutrients, carbohydrates, amino and organic acids, and growth regulators." Describing the role of secondary metabolites in litter decomposition, Horner et al. (1988) stated that "Leaching losses of fairly water soluble components (e.g., most simple phenolics, phenylpropanoids, flavonoids, and tannins) should exceed those of components that are only slightly or negligibly soluble in water (e.g., terpenes and lignin, respectively)." In regard to allelopathy, we and other authors have in the past differentiated the "volatile terpenes" from the "water-soluble" phenolics and aromatic acids (Whittaker, 1971; Harborne, 1988; Fischer et al., 1989; Williamson et al., 1992b). Apparently, the pioneering research of C.H.

TABLE 3. SOLUBILITIES OF MONOTERPENES IN WATER (from Weidenhamer et al., 1993)

Name of Compound	Water solubility (ppm)
[(1*S*)-*endo*]-(−)-Borneol	274
Bornyl acetate	23
Calaminthone	972
Calaminthone, desacetyl	1005
Camphene	23
(1*S*)-(−)-Camphor	550
(1*R*)-(+)-Camphor	531
(−)-Carveol	1115
(*S*)-(+)-Carvone	596
1,8-Cineole	332
p-Cymene	15
(*S*)-(+)-Dihydrocarveol	727
(*S*)-(+)-Dihydrocarvone	461
(+)-Evodone	409
Geraniol	404
Geranyl acetate	18
Limonene	13
Linolyl acetate	< 10
Menthol	183
Menthone	155
Myrcene	< 10
(1*R*)-(−)-Myrtenal	305
(1*R*)-(−)-Myrtenol	1010
Nerol	332
α-Pinene	22
β-Pinene	32
(1*R*)-(+)-Pulegone	385
(+)-Sabinene	< 10
α-Terpinene	14
4-Terpineol	1360
(1*S*)-(−)-Verbenone	6990

Muller and coworkers (Muller et al., 1964; Muller, 1965a,b, 1966, 1969; McPherson et al., 1971; Muller and Chou, 1972) might have unintentionally contributed to this perception, as they found several monoterpenes emitted as volatiles from *Salvia leucophylla* (Labiatae) and *Artemisia californica* (Asteraceae), whereas phenols and phenolic acids were washed from the leaves of *Adenostoma fasciculatum* (Roaseceae) and *Arctostaphylos glandulosa* (Ericaceae).

Phytotoxic effects of monoterpenes vary as much as their water solubilities, although in many cases compounds are active well below their aqueous solubility levels (Fischer, 1991). A comparison of bioactivity of the two monoterpenes borneol and (+)-camphor with the phenol juglone, the active constituent of *Juglans nigra* (Davis, 1928; Harborne, 1988), gave unexpected results (Figures 2 and 3). Although there is variation among these compounds and variation between target species, the biological activity of the two monoterpenes matched or exceeded in several cases that of juglone, the well known allelopathic agent of black walnut. Furthermore, the solubility of borneol and camphor exceeded that of juglone by 5–10×. Thus, the potential activity of the monoterpenes is much greater than juglone where toxicity is mediated through aqueous soil solutions. Reaching active concentrations requires solubilities and solubilization rates sufficiently high for activity. Some presumed insoluble compounds may dissolve

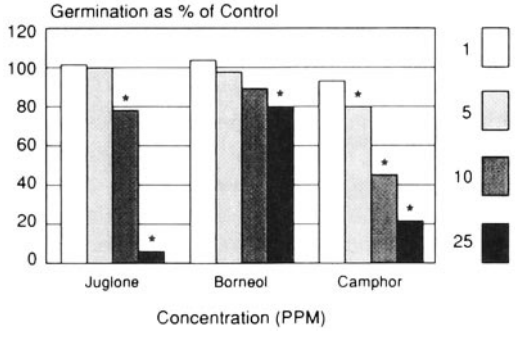


FIG. 2. Germination of *Lactuca sativa* in aqueous solutions of juglone, borneol, and camphor. Asterisk indicates significant difference from the control at $P \leq 0.05$.

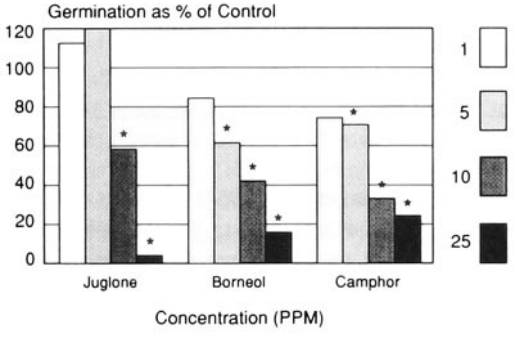


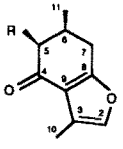
FIG. 3. Germination of *Rudbeckia hirta* in aqueous solutions of juglone, borneol, and camphor. Asterisk indicates significant difference from the control at $P \leq 0.05$.

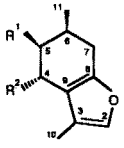
through formation of micelles with other natural products (Fischer et al., 1988; Fischer, 1991; Williamson et al., 1992a). The natural surfactant, ursolic acid **(9)**, is found in copious amounts on the leaf surface of all three scrub species, *Ceratiola*, *Conradina*, and *Calamintha*. It forms micelles in leaf washes, but does not seem to increase aqueous solubility of monoterpenes (Fischer et al., 1988; Weidenhamer et al., 1993). Even if solubilities were improved by micellization, activities may decrease if the compounds remain bound by the surfactant and thus, unable to penetrate target plant membranes. Therefore, the major ecological role of cuticular waxes in allelopathic processes and other biological functions may be due to their fixative property, which enhances long-term retention of active volatiles that would otherwise be lost to volatilization.

Monoterpenes of Calamintha ashei *and their Biological Activities*

Calamintha ashei, commonly referred to as Ashe's savory, is endemic to the central ridge of Florida and is locally common in hickory and sand pine scrub (Menges and Salzman, 1992; Van der Kloet, 1986). Extensive chemical analysis of this mint provided copious amounts of ursolic acid **(9)**, small amounts of the known sesquiterpene, caryophyllene epoxide, and a series of new menthofuran-type monoterpenes (Figure 4). In addition, six known highly methoxylated flavones were obtained (Tanrisever et al., 1988; Macias et al., 1989; Hernandez and Fischer, 1988; Menelaou, 1990).

When fresh aerial parts of *C. ashei* were soaked in water at ambient temperature followed by reextraction of the water soaks with dichloromethane, the organic extracts contained (+)-evodone **(11)**, (−)-calaminthone **(12)**, and desacetylcalaminthone **(13)** (Menelaou, 1990). Besides these three major monoterpenes, six highly unstable minor menthofurans **(15–20)** were isolated from the





		R
11	(+) - Evodone	H
12	Calaminthone	OAc
13	Desacetyl - calaminthone	OH
14	Menthofuran	H, 4-desoxy

	R^1	R^2
15	OAc	OAc
16	OAc	H
17	H	OAc
18	OH	OH
19	OAc	OH
20	OH	OAc

FIG. 4. Monoterpenes isolated from fresh leaves of *Calamintha ashei*.

water and dichloromethane extracts but could not be tested for biological activity due to lack of sufficient quantities.

The proposed mechanism for the release of allelochemicals from *Calamintha* and other scrub perennials is the leaching of monoterpenes from leaves and decaying litter by rainfall (Tanrisever et al., 1987, 1988; Fischer et al., 1988). In order to ascertain the role of menthofuran monoterpenes in the allelopathic potential of *Calamintha ashei*, we evaluated the phytotoxicity of the menthofuran monoterpenes in bioassays.

Earlier studies (Tanrisever et al., 1988) had shown that an aqueous solution of unknown concentration of a chromatographic fraction containing calaminthone **(12)**, (+)-evodone **(11)**, and caryophyllene oxide completely suppressed germination of *Schizachyrium* but had no effect on lettuce germination. Significant inhibitory effects were observed when (+)-evodone was administered as a volatile or in a saturated aqueous solution of ursolic acid **(9)**, the latter showing no significant effects when tested alone. Two facts from the earlier bioassay are of interest: (1) the highly specific activity of a mixture of calaminthone **(12)**, (+)-evodone **(11)**, and caryophyllene oxide on little bluestem contrasts with insignificant inhibition of lettuce; and (2) the strong inhibitory effect exhibited by the terpene mixture toward *Schizachyrium*. Dramatic synergistic phytotoxic effects by mixtures of camphor, pulegone, and borneol were previously observed by Asplund (1968, 1969), although we have failed to find such synergisms in other tests (see below).

More recent bioassays of (+)-evodone **(11)** at 0.05–1 mM concentration revealed strong inhibitory activity against four test species, *Schizachyrium*, *Leptochloa*, the sandhill herb *Rudbeckia hirta*, and *Lactuca* (Weidenhamer et al., 1994). In all cases, germination was inhibited more than radicle growth (Figure 5). All four target species were affected, with germination of *Rudbeckia* and *Lactuca* significantly inhibited even at 0.05 mM. In contrast, germination of the two grasses, *Schizachyrium* and *Leptochloa*, was inhibited only at the higher concentrations of 1.0 and 0.5 mM, respectively. Radical elongation was reduced in three of the four target species, but generally to a lesser degree than germination. Activity of the second major constituent of *Calamintha*, desacetylcalaminthone **(13)**, was limited to inhibition of germination and radicle elongation of *Leptochloa* at 1.0 mM, and germination of *Rudbeckia* was inhibited at concentrations as low as 0.25 mM.

The equimolar mixtures of (+)-evodone and desacetylcalaminthone showed inhibitory effects that were additive rather than synergistic or antagonistic. *Schizachyrium* germination was 53%, 67%, and 59% for 0.05 mM solutions of desacetylcalaminthone, (+)-evodone, and the equimolar mixture, respectively. *Lactuca* germination was 100%, 33%, and 66% for 0.25 mM solutions of desacetylcalaminthone, (+)-evodone, and their equimolar mixture, respectively. *Rudbeckia* germination was only slightly reduced (20%) by 0.25 mM desace-

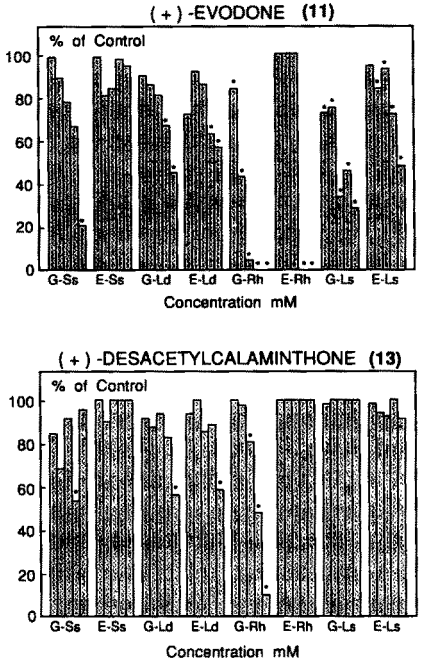


FIG. 5. Germination (G) and radicle elongation (E) of *Schizachyrium scoparium* (Ss), *Leptochloa dubia* (Ld), *Rudbeckia hirta* (Rh), and *Lactuca sativa* (Ls) at five concentrations: right to left 1.0, 0.5, 0.25, 0.10, and 0.05 mM for (+)-evodone and (+)-desacetylcalaminthone.

tylcalaminthone, but was drastically inhibited by 0.25 mM (+)-evodone (3% germination) and the 0.25 mM equimolar mixture (6% germination). Effects of the other three monoterpenes were considerably lower. Calaminthone exhibited strong inhibition of *Rudbeckia* at 0.8 mM but showed few effects at lower concentrations or on the other three species. Menthofuran stimulated 30–100% increases in radical elongation of *Leptochloa* at all five concentrations.

Water Solubility of Calamintha *Monoterpenes*

Determination of the aqueous solubility of the *Calamintha* monoterpenes was necessary to evaluate their potential phytotoxicity. Aqueous solubilities of the three major *Calamintha* monoterpenes were determined by gas chromatography, with values of 2.49 mM (409 ppm) for (+)-evodone **(11)**, 5.58 mM (1005 ppm) for desacetylcalaminthone **(13)**, and 4.36 mM (972 ppm) for calaminthone **(12)** (Weidenhamer et al., 1994). Menthofuran **(14)** slowly degraded,

giving an unreliable value of 1.69 mM (254 ppm). The aqueous solubilities of desacetylcalaminthone **(13)** and (+)-evodone **(11)**, the two major menthofuran constituents of *Calamintha ashei*, exceeded concentration levels necessary for inhibition of germination and radicle growth of the sandhill species tested. Calaminthone **(12)** and menthofuran **(14)**, which had low activity in the bioassays, also had relatively high solubilities (<1 mM).

Prior to determination of the aqueous solubilities (Fischer et al., 1988; Tanrisever et al., 1988), we proposed that the allelopathic activity of *Calamintha* must be mediated by the solubilization of the *Calamintha* menthofurans with naturally occurring surfactants such as the triterpene ursolic acid **(9)**, which occurs in large quantities in *Calamintha* foliage (Fischer et al., 1988; Tanrisever et al., 1988). We had previously demonstrated that formation of micelles in leaf soaks of these plants occurs (Tanrisever et al., 1988), but no measurements of water solubilities or solubilization rates of pure compounds were made. The solubility data presented above clearly showed that the *Calamintha* menthofurans were soluble in excess of 100 ppm, sufficient for these compounds to be potent phytotoxins. The new quantitative data showed clearly that oxygenated monoterpenes are sufficiently water soluble to exert biological activity, and ursolic acid does not increase monoterpene solubility (Weidenhamer et al., 1993). Therefore, our previous hypothesis of the importance of micelle formation by biological detergents such as ursolic acid needs revision.

The ecological significance of the formation of micelles in leaf soaks of *Calamintha* and other allelopathic shrubs of the Florida scrub may have been overestimated based on the presumed insolubilities of the menthofuran monoterpenes. However, triterpenes and other lipids may play a significant role in the fixation of leaf volatiles, in general, and volatile monoterpenes, in particular, to prevent their otherwise rapid release into the atmosphere. Furthermore, effects of triterpenes and other micelle-forming plant products present in the leaf waxes undoubtedly have a significant detergent effect on the rate of water solubilization in a natural setting.

LABORATORY VERSUS FIELD EXPERIMENTS

The proposed mechanism for the release of *Conradina* and *Calamintha* allelochemicals appears to proceed via the leaching of allelopathic compounds from leaves and decaying litter. Frequent heavy rains, which begin in June and extend through October, define the growing season in the scrub and may play a major role in transport of allelotoxins. The impact of large raindrops may cause glandular trichomes to rupture, thereby releasing large quantities of monoterpenes from the leaves. Whether leaves or litter are more important will require more detailed quantitative studies in the field.

Another concern in all studies of allelopathy is the question of realistic bioassays (Hollis et al., 1982). Single applications of a higher concentration of an allelochemical are less realistic than pulses of lower concentration. Inhibition of growth may depend on regular exposure to an allelopathic agent (Blum and Rebbeck, 1989). A small but constant flux of allelochemicals into the soil, if continually taken up by target species, may have significant inhibitory effects on growth, even if high concentrations in the soil solution are never achieved (Williamson and Weidenhamer, 1990). While data are accumulating in the allelopathy literature on the concentrations of toxins in soil at a single point in time, the more important question is how much of an allelochemical is available over a period of time. Answering this question in the case of *Calamintha* and *Conradina* will require measurements of the total amounts of monoterpenes entering the soil during rainstorms through the growing season. This may require the development of new analytical methods, such as traps placed in the soil for an extended period of time followed by quantitative analysis. Environmental parameters such as temperature, nutrient limitation and plant density can significantly modify plant response to an allelochemical (Einhellig and Eckrich, 1984; Einhellig, 1987; Williamson et al., 1992a; Weidenhamer et al., 1989). These environmental factors, which may have no effect at ambient laboratory conditions, may strongly enhance or reduce inhibitory actions in the field.

Nutrient limitation is known to increase inhibition of growth by phenolic compounds (Glass, 1973; 1974; J.R. Harper and Balke, 1981). The effect of hydrocinnamic acid, a photochemical breakdown product of ceratiolin from the scrub endemic *Ceratiola ericoides*, is intensified by reduced levels of nitrogen and potassium (Williamson et al., 1992b). Since macronutrient levels in the Florida scrub are generally very low (Kalisz and Stone, 1984; Richardson, 1985), nutrient limitation may be an important factor in the phytotoxicity of scrub allelochemicals.

High temperature and low soil moisture increase phytotoxic effects of some phenolic allelochemicals (Einhellig and Eckrich, 1984; Einhellig, 1987). On sunny days, surface temperatures often exceed 50°C on the well-drained sands of the Florida scrub (Richardson, 1985), and hydric stress can occur during the dry season and during breaks in summer rains. Plant densities in the Florida scrub are low, and this may also contribute to enhanced phytotoxicity of scrub allelochemicals. At low densities of seeds and germinating seedlings, phytotoxic effects are increased (Weidenhamer et al., 1987, 1989). It is not known whether the effects of the monoterpenes from *Conradina* and *Calamintha* will also be intensified in the harsh scrub environment.

It is perhaps significant that the major monoterpene constituents of *Conradina* and *Calamintha* are also the most active in the bioassays conducted. The question of whether other compounds might be involved in the allelopathic effects must be considered. *Calamintha* contains a rich mixture of flavonoids in

addition to the menthofuran monoterpenes (Hernandez, 1988; Hernandez and Fischer, 1988; Menelaou, 1990). As a result of the low biological activity of the flavonoids in laboratory experiments (Menelaou, 1990), their concentrations in the leaf soaks and mists were not determined. Furthermore, the biological activity of other unidentified polar constituents in the leaf washes of *Calamintha* is not known and requires further study.

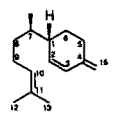
ALLELOPATHIC ACTIVATION IN FLORIDA SCRUB

The complexity of allelopathic actions in natural ecosystems is due to a multitude of events in a dynamic process of simultaneously occurring chemical transformations. After release of a secondary metabolite from leaves, litter, and/or the roots of a source plant into the environment, a cascade of spontaneous chemical, photochemical, and/or microbial events may follow. These often result in postrelease activations of nontoxic plant products into active derivatives with specific toxic effects for the target species, which can be enhanced by synergistic effects (Asplund, 1969; Fischer et al., 1988) and simultaneous reduction of autotoxicity (Fischer et al., 1988; Williamson, 1990). Ultimately, active compounds will be deactivated by microbial degradations. Two examples are given below.

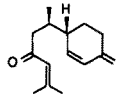
Oxidative Activation of Diterpenes in Woody Goldenrod (Chrysoma pauciflosculosa)

The woody goldenrod (*Chrysoma pauciflosculosa*) of the family Asteraceae is a dominant shrub on open scrub sites in the Florida panhandle. In an earlier study, leaf washes of *Chrysoma* had significantly inhibited the germination of *Lactuca* in laboratory tests (Eleuterius, 1979). Therefore, this member of the immature scrub in the dunes of the Florida panhandle was included in our chemical and biological studies.

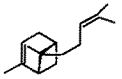
Aqueous extracts of fresh leaves of *C. pauciflosculosa* contain ca. 500 ppm of organic extractables (Menelaou et al., 1993). 17-Hydroxygrindelic acid **(24)** was a major constituent with the diterpene aldehyde **(25)** and the diacid **(26)** as well as the sesquiterpenes (+)-β-turmerone **(22)** [(+)-curlone], (+)-sesquiphellandrene **(21)**, and (−)-α-*trans*-bergamotene **(23)** being minor components (Figure 6). Once released into the environment, the moderately active allylic alcohol moiety in 17-hydroxygrindelic acid (HGA, **24**) is subject to light-mediated air oxidation to give first the more active aldehyde (OGA, **25**) and ultimately the diacid **(26)** (Figure 7), processes which seem to involve free radical intermediates (Menelaou et al., 1993). At a range of 36–144 μM (12–48 ppm) 17-oxogrindelic acid **(25)** reduced the germination and radicle growth of the two sandhill grasses, *Schizachyrium* and *Leptochloa*, but had no



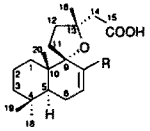
(+) - Sesquipheliandrene (21)



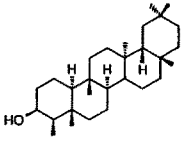
(+) - ent - β - Turmerone (22)
[(+) - Curlone]



α - trans - Bergamotene (23)



	R
17- Hydroxygrindelic acid (24)	- CH_2OH
17- Oxogrindelic acid (25)	- CHO
Diacid (26)	- COOH



Epifriedelinol (27)

FIG. 6. Sesqui-, di-, and triterpenes from *Chrysoma pauciflosculosa*.

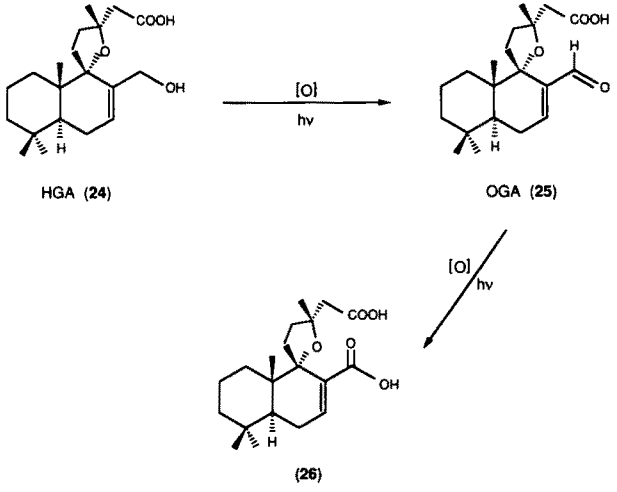


FIG. 7. Photochemical allelopathic activation of diterpenes of *Chrysoma pauciflosculosa*.

significant effects on the germination and slight stimulatory effects on radicle growth of *Rudbeckia hirta*. At a concentration range of 60–357 μM (20–120 ppm) the diterpene alcohol **(24)** was less active in reducing germination and radicle growth of the same target species and the diterpene diacid **(26)** gave no significant activity at comparable concentration.

The *C. pauciflosculosa* sesquiterpenes had only minor effects on the germination and radicle growth of the four target species (Menelaou, 1990). *Schizachyrium* radicle growth was significantly stimulated by 10^{-4} M solutions of (+)-ent-β-turmerone **(22)** and (−)-α-*trans*-bergamotene **(23)**, but *Leptochloa* was not affected by any of the three sesquiterpenes. *Rudbeckia* radicle growth was reduced to about 80% of the control by 10^{-4} M solutions of (+)-ent-β-turmerone and (−)-α-*trans*-bergamotene. Lettuce germination was inhibited by aqueous saturated (65% of control) as well as 10^{-4} and 10^{-5} M solutions (49% and 77% of control) of (+)-ent-β-turmerone and was stimulated significantly by a 10^{-4} M solution of (−)-α-*trans*-bergamotene.

In the assays of mixtures of oxogrindelic acid **(25)** with the three sesquiterpenes, (+)-ent-β-turmerone, (+)-sesquiphellandrene and (−)-α-*trans*-bergamotene, there was no indication of synergistic or antagonistic effects of a diterpene aldehyde–sesquiterpene mixture. This suggested that the three sesquiterpenes do not seem to be significantly involved in allelopathic effects of *C. pauciflosculosa*.

Ceratiolin Cascade in Florida Rosemary (Ceratiola ericoides)

Although unrelated to the theme of allelopathic terpenes, the following example demonstrates the complexity of chemical events in allelopathic actions. The biologically inactive dihydrochalcone, ceratiolin, is leached from fresh leaves and litter of *Ceratiola* and degraded to hydrocinnamic acid (HCA), a selective toxin for native Florida grasses and pines (Tanrisever et al., 1987; Williamson

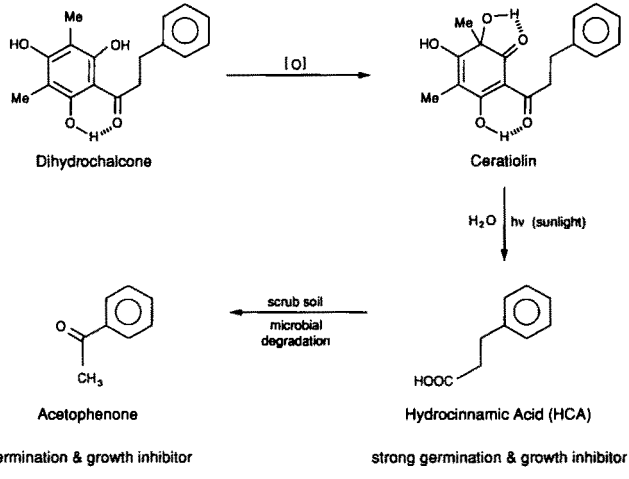


FIG. 8. Photochemical allelopathic activation in the Florida scrub: the ceratiolin cascade.

et al., 1992a). As shown in Figure 8, decomposition of ceratiolin in aqueous solution is a light-dependent process, suggesting that in the scrub, formation of the active HCA represents a novel photochemical process of allelopathic activation (Tak and Fischer, unpublished results). As soil microbes metabolize and degrade organic plant products (Blum and Shafer, 1988) and as HCA is released from living and decaying foliage of *Ceratiola* and transported in aqueous soil solution at toxic levels (Jordan, 1990), further degradation was anticipated. HCA spiked in nonsterile scrub soil at ambient temperature disappeared after about 60 hr. After 12 hr, acetophenone, which is also inhibitory toward target grasses, was detected in soil extracts, reaching a maximum after 60 hours and decreasing to nondetectable levels after 144 hr (Jordan, 1990). In sterile soil after application of HCA, acetophenone was not detected. Therefore, the degradation process must be a microbial, rather than an air-oxidative decomposition of HCA (Jordan, 1990). In contrast to the photochemical activation–microbial degradation process of the ceratiolin cascade, allelopathic activation of the dihydrochalcone phloretin from apple roots (*Pirus malus* L.) involves formation of the active *p*-hydroxydihydrocinnamic acid exclusively by microbial action (Boerner, 1960).

CONCLUSIONS AND FUTURE STUDIES

Our studies in the Florida scrub have provided insight into new mechanisms of release and action of allelopathic agents that might be used as models for studies of other natural ecosystems. Chemical, microbial, or spontaneous decomposition of nontoxic plant products into potent phytotoxic derivatives provides possible mechanisms for specific, direct toxic effects toward a target species. In *Ceratiola ericiodes*, the nontoxic plant product ceratiolin is only the precursor, and its photochemical decomposition product HCA, which is mainly formed in the litter, acts as the specific allelotoxin for sandhill target grasses.

As emphasized earlier in this issue (Langenheim, 1994), synergistic allelopathic effects, exemplified by the dramatic increase in inhibition of germination caused by mixtures of monoterpenes can be expected. Bioactivity enhancements due to synergism of terpene mixtures could dramatically increase biological activities at concentrations one to two orders of magnitude lower than single component activities (Asplund, 1969; Fischer et al., 1988). Furthermore, observation of highly selective phytotoxicity of *Calamintha* terpenes with selective germination inhibition of different target species represents another useful guide for future studies. Nonpolar cutical waxes and resins can possibly function as fixatives to prevent the loss of allelopathic volatiles by rapid evaporation. This property as a slow-release solvent for organic volatiles could be facilitated by a wide host of constituents in plant leaf waxes: triterpenes, long-chain fatty

acids, hydrocarbons, alcohols, esters, and fats. Enhanced long-term retention of active components undoubtedly plays a significant role in the retention of allelopathic plant volatiles.

Our collaborative study of the Florida scrub has made substantial progress in the understanding of the intricacies of plant–plant interactions in this highly endangered natural ecosystem. We have provided evidence that terpenes, in general, and monoterpenes, in particular, play a major role in the allelopathic action in the endemic scrub mints *Calamintha ashei* and *Conradina canescens*. Furthermore, our findings on the water solubility of a series of potent phytotoxic monoterpenes has opened new doors for future ecological studies of this group of compounds. We hope that this new knowledge forces change not only in relation to their allelopathic actions in natural ecosystems but also changes our thinking of this major group of natural products in general ecological terms.

Many significant questions remain to be answered before the original hypothesis of allelopathic effects by the four scrub endemics *Conradina*, *Calamintha*, *Chrysoma*, and *Ceratiola* can be confirmed or refuted. Crucial to this assessment will be long-term bioassays and field studies that must address the effects of terpenes and other compounds on the growth of native sandhill species under conditions comparable to the harsh environment of the Florida scrub. Application regimes that mimic the periodic release of these compounds during the rainy season in the Florida scrub will ultimately be required to ascertain the allelopathic properties of these shrubs.

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MONOTERPENES: THEIR EFFECTS ON ECOSYSTEM NUTRIENT CYCLING

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Abstract—This article explores the evidence for monoterpenes to alter rates of nutrient cycling, with particular emphasis on the nitrogen (N) cycle, from an ecosystem perspective. The general N cycle is reviewed and particular processes are noted where monoterpenes could exert control. The theoretical and conceptual basis for a proposed mode of action by which monoterpenes effect the processes of N mineralization and nitrification is presented, along with recent developments. It is hypothesized that monoterpenes retained in litter enhance the frequency of fire, which in turn changes many N-cycling processes. Experimental support for these roles is presented that includes effects at the cellular level and progresses through populations and communities (microbial and invertebrate) involved in N mineralization and immobilization processes. Since many inhibitors of ammonium oxidation also inhibit methane oxidation, monoterpenes also may alter processes within the carbon cycle. Finally, areas for future research that appear most promising are suggested.

Key Words—Nitrogen cycle, nutrient cycling, N mineralization, immobilization, inhibition of nitrification, ammonium monooxygenase, methane monooxygenase, ponderosa pine, fire cycle, monoterpenes.

INTRODUCTION

The potential for monoterpenes to alter rates of nutrient cycling, and nitrogen (N) cycling in particular, from an ecosystem-level perspective is the focus of this article. Although research in this area is limited, the majority of studies are on the effects of monoterpenes on processes within the N cycle. The N cycle is perhaps the most widely studied element cycle because of its perceived importance as a limiting factor for primary production in a wide variety of systems

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(Vitousek and Howarth, 1991). Nitrogen is an essential element and a large fraction of N within primary producers is directly involved in photosynthesis (Evans and Seemann, 1989). A rate-controlling factor for N availability is the mineralization process, which is nearly inseparable from the decomposition process and the carbon (C) cycle in general. In addition, there are strong links between the N and C cycles with some compounds capable of acting as alternative substrates for specific enzymes involved in the other element's cycle (specifically NH_3 and CH_4 are alternate substrates for the other's respective monooxygenases) (Ward, 1987; Carlson et al., 1991). Thus, I will begin the assessment of monoterpenes on nutrient cycling with the N cycle.

Since ecosystem-level processes are not the usual topic for this journal, this article first reviews the N cycle and notes particular processes where monoterpenes could exert control. The theoretical and conceptual basis for the proposed mode of action on particular processes within the N cycle is presented, along with recent developments. Although terrestrial and aquatic ecosystems share similar processes (with some distinct differences), this review will be limited to terrestrial ecosystems. Ponderosa pine (*Pinus ponderosa*) ecosystems will receive the greatest attention, the ecosystem in which the majority of my work was done. Interactions of monoterpenes with fire and its frequency in ponderosa pine forests are discussed. Next, evidence for the potential role of monoterpenes in the carbon cycle, specifically in the reduction in methane oxidation, is presented. This paper concludes by suggesting three areas for future research.

THE NITROGEN CYCLE

The supply of N often limits production in terrestrial ecosystems (Vitousek and Howarth, 1991). Inputs of N to terrestrial ecosystems are from precipitation, airborne particles trapped by the vegetation and soil (dry deposition and impaction), and through gaseous fixation (N fixation) (Figure 1). N fixation occurs through free-living organisms (i.e., cyanobacteria and lichens) and symbiotically with plants and their root-associated microorganisms. On an annual basis, inputs of N are thought to be small relative to the amount obtained from the soil.

To a large extent, the supply of N in terrestrial ecosystems is determined by the rate at which organic-bound soil N is released in inorganic form; a process termed N mineralization (Figure 1). The first inorganic form of N is ammonia (NH_3), which readily converts to ammonium (NH_4^+) in the soil solution. Ammonium (or ammonia) can be converted to nitrate (NO_3^-) through the two-step process of nitrification. Through the nitrification process, a portion of the ammonium nitrogen can be lost to the atmosphere as nitrous oxide. Both ammonium and nitrate are assimilated by the soil microbial community and by higher plants. Unlike ammonium, nitrate is negatively charged (an anion) and is mobile within

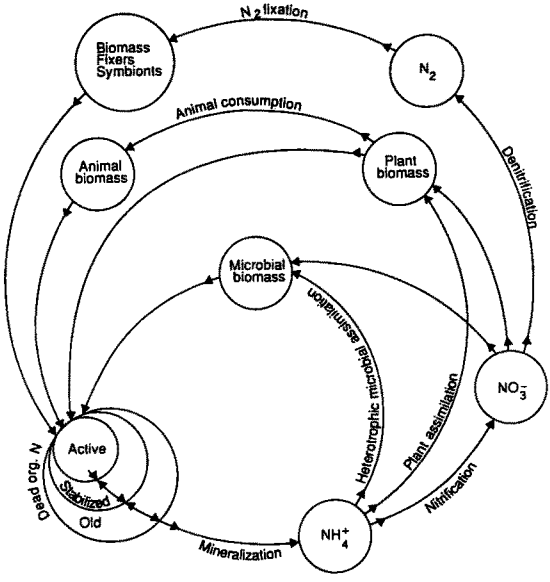

FIG. 1. The universal nitrogen cycle indicating pools (circles) and processes (arrows). Inputs (wet and dry deposition) and losses (leaching, erosion) are not indicated. The soil nitrogen pool (Dead org. N) is divided into three conceptual compartments (from Jansson, 1981).

the soil, which can lead to loss of nitrate from the terrestrial system to streams or groundwater. Nitrate can be utilized by other microorganisms as a terminal electron acceptor, which results in the production of nitrous oxide or dinitrogen gas, through the process of denitrification. Dinitrogen gas can return to the atmosphere, completing the cycle. Nitrous oxide also can escape to the atmosphere, where it can undergo reactions with other chemicals.

Both nitrate and ammonium are assimilated into plant and microbial biomass through growth. Nitrogen assimilated by a portion of the microbial community contributes to plant growth through mycorrhyzal associations (not shown in Figure 1). A portion of the microbial and plant biomass is consumed by animals. Eventually, microorganisms, higher plants, animals, and their associated wastes become part of the soil organic matter pool, a portion of which rapidly decomposes and undergoes the process of mineralization to continue the cycle.

Processes Potentially Controlled by Monoterpenes

The most widely studied process controlled at least in part by monoterpenes is herbivory. Gershenzon and Croteau (1991) and the proceedings edited by Harborne and Tomas-Barberan (1991) provide recent reviews on this aspect,

which will receive little attention in this article. The role of monoterpenes (and synergistic interactions with other secondary plant products) in the resistance of live plants to herbivores and pathogens was recently reviewed by Fritz and Simms (1992). Direct contribution by monoterpenes in ponderosa pine to resistance of herbivores and pathogens was demonstrated by Snyder (1992) and Himejima et al. (1992), respectively. Langenheim (this issue) provides additional references to the effects of individual monoterpenes, and quantitative variations in their composition contribute to defense properties for ponderosa pine and plants in general.

Although individual and total monoterpene concentrations may change during senescence (Wilt et al., 1993), monoterpene concentrations in litter and other plant detritus can nearly equal concentrations in some live materials (White, 1991a). The resistance properties of monoterpenes should be retained in litter or detritus; thus, monoterpenes might be expected to exert some control over decomposer organisms. If activity of microbial and/or invertebrate decomposers is inhibited by either individual monoterpenes or combinations of various monoterpenes, then decomposition and the release of inorganic ammonium (the process of N mineralization) would slow. Once ammonium is released to the soil environment, monoterpenes can exert control on two processes: (1) microbial assimilation by providing a C-rich substrate and promoting immobilization of ammonium by heterotrophic microorganisms (Bremner and McCarty, 1988); and (2) nitrification by inhibiting the oxidation of ammonium (White, 1986b, 1988, 1991a). Inhibition of nitrification would result in concurrent reductions in the process of denitrification and its resulting gaseous products.

Monoterpenes are emitted to the atmosphere and comprise the major portion of the class of compounds called "nonmethane hydrocarbons" (Lamb et al., 1985; Zimmerman et al., 1988), which are involved in numerous reactions in the atmosphere. Monoterpenes react in the atmosphere to enhance the conversion of oxides of N and sulfur (S) to NO_3 and SO_4, respectively, which may increase dry deposition of these compounds to vegetative surfaces. Inputs of N and S may act to "fertilize" the ecosystem; an aspect that has received much attention associated with acid rain effects. Other important reactions of monoterpenes and other nonmethane hydrocarbons within the atmosphere were reviewed by Logan et al. (1981).

Finally, monoterpenes may alter inputs to ecosystems by either stimulating or inhibiting N fixation, based on their known potential to favor or inhibit different microorganisms. Monoterpenes could alter the activity of epiphytic or soil lichens and free-living cyanobacteria.

In summary, monoterpenes could potentially interact with the N cycle by altering rates of: (1) herbivory, (2) decomposition, (3) N mineralization, (4) immobilization, (5) nitrification, (6) denitrification, and (7) inputs via wet and dry deposition and N fixation—nearly every process within the N cycle. The

conceptual basis of, and experimental support for, the interaction of monoterpenes with the processes of decomposition, mineralization–immobilization, and nitrification are expanded in the subsequent section.

N Mineralization and Nitrification: Patterns of Processes

Conceptual Patterns and Experimental Results. Mechanisms that control the processes of decomposition, N mineralization–immobilization, and nitrification are the focus of considerable research, particularly in terrestrial ecosystems, because of the general assumption that productivity is N-limited. The process of nitrification is of particular importance because loss of N from terrestrial ecosystems primarily occurs by leaching of nitrate and gaseous loss during or following nitrification.

Factors that regulate rates of N mineralization and nitrification for 17 forests located throughout the United States were studied by Vitousek et al. (1979, 1982). Their focus was to determine the potential for loss of nitrate from forest ecosystems following disturbance. Their specific disturbance of interest was clear-cutting, but "disturbance" should be interpreted liberally and for the purposes of this discussion could include the collection and removal of a soil sample. From a process perspective, clear-cutting limits the uptake of nutrients by plants (pathways illustrated in Figure 1), which allows the inorganic forms of N to accumulate in the soil. In contrast with gross mineralization (or gross nitrification; Davidson et al., 1991), the accumulation of inorganic N with this treatment represents net mineralization, which is the difference between gross mineralization and concurrent immobilization.

Based upon their conceptual response, Vitousek et al. (1979) predicted a pattern for the production of ammonium and nitrate following trenching in the field (Figure 2). After disturbance, a time lapse may occur prior to the accumulation of ammonium, which they termed the lag phase. Microbial activity may be high during the lag phase, but gross mineralization is balanced by immobilization. Once net mineralization occurs, various patterns are possible. The mineralized ammonium may be converted to nitrate, which may cause a concurrent decrease in ammonium (the substrate for nitrification). There may be a lag in nitrification, which allows ammonium to accumulate. Nitrification may occur so rapidly that ammonium production is not apparent, resulting in an immediate rise in nitrate rather than ammonium. Nitrate may be transported to lower soil horizons, which can be detected in soil lysimeters, or to surface streams if sufficient moisture is available.

The time for the entire response sequence to occur in field experiments (lag phase, net mineralization, and nitrification) differed substantially between forests. Although responses differed, they seemed to fall into three general categories (Figure 3). A very slow response (Figure 3C) includes only the lag phase

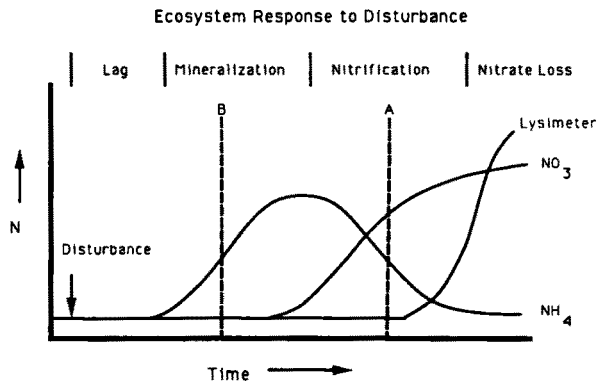

FIG. 2. Responses of soil ammonium, soil nitrate, and lysimeter nitrate concentrations to disturbance in a hypothetical ecosystem. The lines represent increases in a disturbed system relative to a control or to the levels prior to treatment. The phases of the response are given above that portion of the graph. Refer to the text for the meaning of lines A and B. (Redrawn from Vitousek et al., 1979, with permission).

with a slight rise in ammonium at the end of the measurement period (corresponding to the pattern up to line B in Figure 2). An intermediate response (Figure 3B) includes an increase in ammonium (a lag may or may not precede net mineralization) followed by an increase in nitrification (corresponding to the pattern from the disturbance to line A in Figure 2). A very rapid response (Figure 3A, corresponding to the portion of the predicted pattern beginning at line A in Figure 2) has the entire pattern preceding the rise in nitrate condensed within the first sampling period. These three characteristic responses represent stages along a continuum of potential responses, with the rapid and slow responses near opposite ends of this gradient. Different forest types show responses typical of these three patterns in both field experiments where plant uptake is limited and in laboratory incubations of soil where favorable moisture/temperature regimes are maintained (termed "mineralization potentials").

The three Indiana forests included in the studies by Vitousek et al. (1979, 1982) show responses that are typical of sites with rapid, intermediate, and slow responses. Furthermore, the response seen in laboratory mineralization potentials (Figure 4) mirrored the field response at all sites, only the response was faster in the laboratory. The Indiana maple site (Figure 4) is typical of a rapid response (corresponding to Figure 3A) with an immediate increase in nitrate and a gradual decline in ammonium. The Indiana oak site (Figure 4) is typical of an intermediate response (corresponding to Figure 3B) with an increase in ammonium followed by an increase in nitrate after a short lag. The Indiana pine

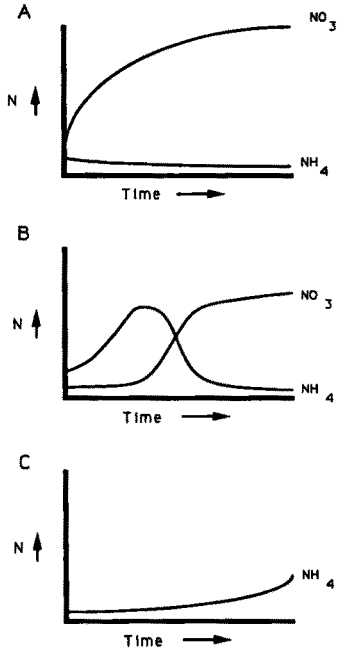

FIG. 3. Potential patterns for ammonium and nitrate concentrations is a soil following disturbance or during laboratory incubations for determination of mineralization and nitrification potentials. Patterns range from rapid (A), through intermediate (B), to slow (C).

site is typical of an extremely slow response (corresponding to Figure 3C) with a protracted lag phase and a slight rise in ammonium at the end of the incubation.

Four forests in New Mexico and three Pacific Northwest forests also were included in the studies of Vitousek et al. (1979, 1982). The New Mexico aspen site showed an intermediate-to-fast response in laboratory mineralization potentials (similar to Indiana oak site; Figure 4), but both the ponderosa pine and spruce–fir sites in New Mexico showed a slow response (slightly higher absolute values but similar to the pattern in the Indiana pine site; Figure 4). The similarity in patterns for these conifer sites in contrast to deciduous sites suggests the potential for an overall pattern based on gymnosperm–angiosperm differences; however, the entire range of potential responses was shown by the three conifer sites in the Pacific Northwest. Thus, mineralization and nitrification appear to be limited in only certain forests.

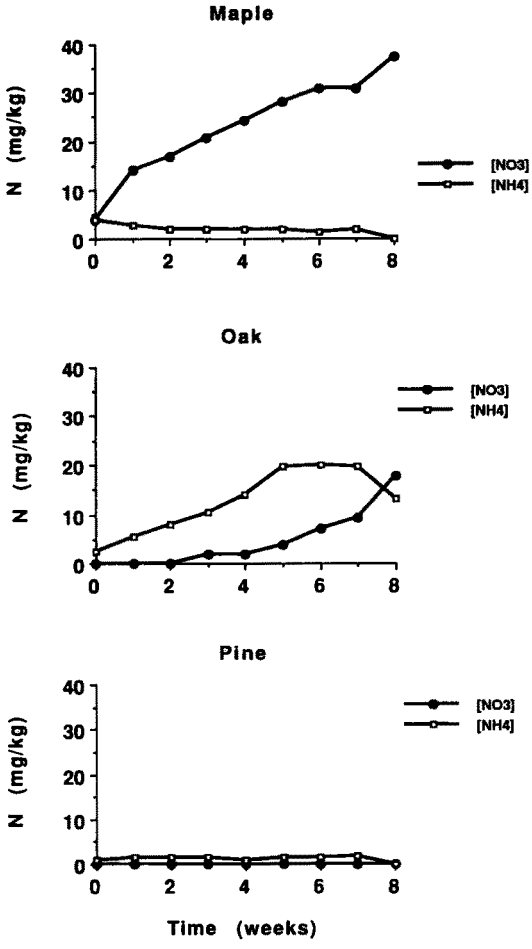

FIG. 4. Changes in inorganic N during laboratory incubation of mineral soil samples from the Indiana maple, oak, and pine forests reported in Vitousek et al. (1982; redrawn with permission).

N Mineralization and Nitrification: Monoterpenes as Controlling Factors?

Factors controlling N mineralization and nitrification in the New Mexico forest sites reported in Vitousek et al. (1979, 1982) were the focus of a separate study by White and Gosz (1987). In laboratory incubations, potentially limiting nutrients (N, P, and/or micronutrients) were added to forest floor and mineral soil from the ponderosa pine site in attempts to alter the mineralization pattern.

A positive response to nutrient additions would shorten the lag in mineralization or nitrification and increase production of inorganic N. The mineralization pattern was very resistant to change. White and Gosz concluded that N mineralization in the New Mexico ponderosa pine site was limited by (1) organic quality factors (which includes inhibition by an organic inhibitor) or by (2) the availability of a combination of limiting nutrients (including N, P, and micronutrients).

Based upon the resistance to increase rates of net mineralization and nitrification in laboratory studies, high concentrations of ammonium and nitrate were not expected in field collections. However, high concentrations of ammonium and nitrate were reported in a single field collection from both the trenched and control plots in the New Mexico ponderosa pine site of Vitousek et al. In September of 1977, concentrations of ammonium and nitrate in the forest floor (organic soil horizons) of both trenched and control plots were approximately 20 and 0 mg N/kg, respectively. Both ammonium and nitrate concentrations increased to approximately 80 mg N/kg soil in the October 1977 collection, but returned to the September 1977 levels by the next collection (February 1978).

The obvious question is how did such dramatic and rapid changes in soil inorganic N occur? Between the collection with low inorganic N concentrations (September 1977) and the next collection with high concentrations of ammonium and nitrate (October 1977), exceptionally hot, dry conditions forced complete closure of the forest to the public because of extreme fire danger. The high ammonium and nitrate concentrations (indicating rapid mineralization and nitrification) were measured in forest floor collections taken after rains had moistened the forest floor and reduced the fire danger. This suggested that the hot, dry conditions altered some critical controlling factor, which allowed rapid mineralization and nitrification to occur in the forest floor.

Higher than normal precipitation and an early freeze after the October 1977 collection prevented further collection at the New Mexico ponderosa pine site until later that winter. Low concentrations of ammonium and nitrate in February 1978 indicate limited mineralization and nitrification. Since senescence occurred during the interim period, it is likely that the controlling factor was contributed in litterfall and/or throughfall. If the controlling factor was an organic inhibitor, the inhibitor had to: (1) be denatured and/or removed (perhaps volatilized) during heating and drying of the forest litter, (2) contributed through litterfall and/or throughfall, and (3) persist within frozen soils.

I collected all samples of forest floor and soils from the ponderosa pine site. Before closure, the turpentine aroma of a pine forest was very strong. I remember this clearly because I have an allergy to turpentine in oil-based paints, and I recall suffering from allergic symptoms during the September 1977 collection. Following reopening of the forest, I noted the lack of turpentine aroma and had no allergic symptoms. It was as if the turpentines were volatilized from

the needles on the forest floor during the hot, dry conditions. Turpentine is a mixture of various monoterpenes, which are highly volatile, with smaller amounts of sesquiterpenes. Since monoterpenes have physical characteristics that are consistent with those of a potential organic inhibitor (highly volatile, in relatively high concentration in litterfall and throughfall, and freezing is a means of preserving monoterpenes in living tissues), this observation suggested the potential role of monoterpenes in the processes of N mineralization and nitrification.

INHIBITION OF NITROGEN CYCLING PROCESSES BY MONOTERPENES: MODE OF ACTION

It is important to recognize that the evidence for the action of monoterpenes spans multiple levels of biotic organization. Although the discussion has focused on ecosystem-level experiments, monoterpenes are suspected to act at the subcellular level by inhibiting specific intracellular enzymes. The following discussion starts with effects at the cellular level, which also is the organism level for single-cell bacteria, and progresses through populations and communities (microbial and invertebrate) involved in N mineralization–immobilization processes.

Inhibition of N Mineralization

The most likely mode of action for inhibition of N mineralization by monoterpenes is by stimulation of heterotrophic microorganisms and promotion of N immobilization (a reduction in net mineralization). Monoterpenes are C-rich compounds. Given the diverse microbial community within a soil, it is inevitable that these C-rich compounds will be utilized by some portion of the heterotrophic community. Because monoterpenes do not contain N, heterotrophic organisms would require a source of N, thus promoting immobilization of inorganic-N in new microbial biomass. In an analogous manner, a common method to promote microbial immobilization in coniferous forests is through the application of glucose in combination with sawdust (White et al., 1988; Gower et al., 1992).

Experimental support for stimulation of heterotrophic immobilization of N by the addition of monoterpenes was provided by Bremner and McCarty (1988) and White (1991a). Following additions of monoterpenes at levels ranging from 10 to 5000 μg/g to three Iowa soils used extensively for corn or soybean production, Bremner and McCarty (1988) reported a net reduction in the total amount of inorganic N (immobilization), a response that was similar to the addition of glucose to the same soils. Using soil from a ponderosa pine stand or a desert grassland in New Mexico, White (1991a) demonstrated that additions of various monoterpenes at levels of approximately 1250 μg/g of soil stimulated immobilization of available ammonium (Figure 5).

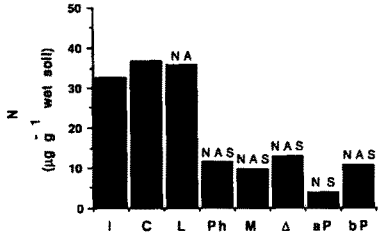

FIG. 5. Inorganic N content (solid is ammonium; shaded is nitrate) of mineral soil from a ponderosa pine stand (I) and after 28-day laboratory incubation with the addition of distilled water (C), or 1250 μg/g of limonene (L), phellandrene (Ph), myrcene (M), d-3-carene (Δ), α-pinene (aP), or β-pinene (bP). Bars with letters indicate that nitrate (N), ammonium (A), or their sum (S) was significantly different from the control (C).

Inhibition of Nitrification

In contrast to the mineralization process, nitrification is primarily an autotrophic process (technically, a chemolithotrophic process), although heterotrophic nitrification also can be significant in some soils (Schimel et al., 1984). Autotrophic nitrification is a two-step process; the first is carried out by organisms capable of oxidizing ammonium (or ammonia) to nitrite (NO_2^-) via hydroxylamine, and the second carried out by organisms capable of further oxidizing nitrite to nitrate (NO_3^-). Although many compounds are known to inhibit nitrification, no precise mode of action or biochemical mechanism is known for most inhibitory compounds (Powell, 1986). The majority of inhibitors are believed to act on the enzyme ammonia monooxygenase (AMO), which catalyzes the oxidation of ammonia to hydroxylamine (Hynes and Knowles, 1982; Hyman and Wood, 1985; Powell and Prosser, 1985). Recent work indicates that specific compounds act as either competitive or noncompetitive enzymatic inhibitors of AMO (Keener and Arp, 1993). This enzyme is yet to be isolated and purified, although its amino acid sequence has been determined in part (McTavish et al., 1993). Many of AMO's characteristics have been ascertained experimentally.

In general, efficiency of known nitrification inhibitors increases in compounds with an increasing degree of unsaturation in carbon–carbon (C—C) bonds. Thus, alkynes (acetylene) have greater inhibitory activity than the corresponding alkene (ethylene). For compounds with terminal triple bonds (alkynes with general formula R—C≡C—H), inhibitory efficiency decreases with increasing aliphatic chain length (i.e., acetylene > propylyne > butyne), and with shifting of the unsaturated bond away from the terminal position (1-butyne > 2-butyne) (McCarty and Bremner, 1986). However, when a six-member unsaturated ring compound (either benzene or pyridine) is substituted to a ter-

minal triple bond, nitrification inhibitory activity apparently is nearly as high as any compound that is a solid at room temperature (McCarty and Bremner, 1986). A commercial nitrification inhibitor, nitrapyrin [2-chloro-6-(trichloromethyl)pyridine], contains a pyridine ring with a chloro substitution in the 2 position (next to the N on the pyridine ring) and a trichloromethyl substitution in the 6 position (also next to the N on the ring). Thus, the unsaturated six-membered ring appears to be one important characteristic of AMO inhibitors, along with the degree of unsaturation at a terminal position.

A potential mode of action for monoterpenes is based upon structural characteristics they have in common with known inhibitory compounds (White, 1988). The monoterpenes can be separated into four types based on their skeletal (or molecular) structures (Figure 6): (1) acyclic (e.g., myrcene), (2) monocyclic (e.g., limonene and alpha-phellandrene), (3) bicyclic (e.g., α- and β-pinene), and (4) tricyclic (e.g., tricyclene, not pictured). Based upon the occurrence of a ring structure and the degree of unsaturation of terminal C—C bonds, White (1988) predicted that inhibitory activity should be greatest for monoterpenes with: (1) 6-C rings; and (2) terminally located unsaturated C—C bonds. Furthermore, based on the results of McCarty and Bremner (1986), White (1988) predicted that inhibitory activity would decrease with placement of unsaturated bonds further from the terminal position. White (1990) predicted that inhibition was competitive, since high concentrations of ammonium could apparently overcome inhibition by monoterpenes. Even if the structures proposed by White (1988) were proven to be unrelated to inhibitory activity, the proposed mode of action predicts that monoterpenes should vary in the degree of inhibition due to differences in molecular structures and differential affinity with the enzyme

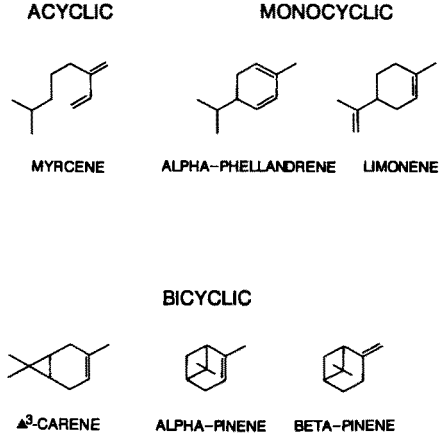

FIG. 6. Structures of various monoterpenes.

(AMO). Thus, this mode of inhibition allows for temporal and spatial variations in nitrification inhibition due to factors within the environment that change the types and amounts of monoterpenes within the soil. Allowance for variation in nitrification is necessary to explain the observed variation in these patterns.

Inhibition of Nitrification: Evidence for Mode of Action. Tests of nitrification inhibitors utilize three types of experimental designs: cell-free solutions with either soluble or membrane-bound monooxygenase, whole-cell solutions with live cultures (usually monocultures), or soil slurries containing mixed communities of active microorganisms. Cell-free experiments test the effects of a compound on the enzyme (or on multiple enzymes). Whole-cell experiments test the effects on intracellular enzymes, but also incorporate the effects on other functions of the cell, including direct toxic effects on other biochemical processes and integrity of the cell wall. Whole-soil experiments integrate whole-cell effects with the concurrent effects (either synergistic or antagonistic) of the physical, chemical, and biological components of the soil environment. Thus, the results from experiments with one design may not be the same as in experiments with another design.

To my knowledge, the effects of various monoterpenes on AMO through cell-free experiments have not been tested. The potential of inhibition of nitrification in stands of coastal redwood (a producer of monoterpenes) was first suggested by the lack of nitrate in soils from various stands of coastal redwood (Bollen and Wright, 1961). In whole-cell experiments, Courtney et al. (1991) reported that relatively low concentrations of monoterpenes that are abundant in coastal redwood inhibited the production of nitrite in log-growth-phase cells of *Nitrosomonas europaea*. In other whole-cell experiments with a species of *Nitrobacter*, redwood monoterpenes did not inhibit the conversion of nitrite to nitrate (J.H. Kim, K.J. Courtney, and J.H. Langenheim, unpublished manuscript, May 1993). Although inhibition of AMO by monoterpenes was not directly tested in these studies, monoterpenes appear to inhibit the production of nitrite (the first step in the process of nitrification) and do not inhibit the conversion of nitrite to nitrate, which is consistent with the proposed mode of action.

Recent work by Keener and Arp (1993) suggests that monoterpenes should act as noncompetitive rather than competitive inhibitors. In kinetic studies with whole-cell cultures, all nonpolar organic compounds tested by Keener and Arp showed patterns consistent with noncompetitive inhibition of AMO. Keener and Arp hypothesized that nonpolar compounds would bind with AMO at a hydrophobic region, which is not the active site with respect to ammonia. Similarly, Hyman et al. (1985) demonstrated that benzene also exhibited a noncompetitive inhibition pattern. Some monoterpenes have ring structures similar to benzene, and all are nonpolar. Thus, it is more likely that monoterpenes are noncompetitive inhibitors of AMO in whole-cell conditions, rather than competitive inhib-

itors are suggested by White (1990). Other potential mechanisms for inhibition of AMO include interfering with the reductant supply to AMO, or oxidation of monoterpenes could yield products that covalently bind with and inactivate AMO (Keener and Arp, 1993).

Indirect support for inhibition of AMO by monoterpenes comes from Howard and Howard (1991), although the intent of their study was to test the proposed inhibition of nitrification by polyphenols. In whole-cell experiments, water extracts of litter from various deciduous and coniferous trees were tested for their inhibitory activity towards *Nitrosomonas europaea* and towards *Nitrobacter agilis* in separate experiments. Inhibition of *Nitrosomonas europaea* without inhibition of *Nitrobacter agilis* would be consistent with inhibition of AMO, although other mechanisms are not excluded. In both experiments, aqueous extracts of each litter were added to pure cultures of each species. Furthermore, each extract was tested following removal of polyphenols by PVP (polyvinylpyrrolidone). Activity of *Nitrosomonas europaea* was decreased by over 50% by the addition of fresh extracts of all but two species (elm, *Ulmus glabra*; and ash, *Fraxinus excelsior*). After removal of polyphenols, inhibitory activity of all conifer extracts was unchanged or actually increased, except Norway spruce (*Picea abies*), which still inhibited but not as much as the original extract. This test indicates that compounds in water extracts of conifer litter inhibit conversion of ammonium to nitrite, but that compounds other than polyphenols are responsible for the inhibition (see Fischer et al., this issue, for discussion on water solubility of monoterpenoids). In addition, many of the water extracts of conifer species did not inhibit the conversion of nitrite to nitrate (Howard and Howard, 1991), again consistent with inhibition of AMO.

Although the specific mechanism cannot be determined from studies with mixed microbial communities within a soil, it is essential to demonstrate that monoterpenes inhibit nitrification at the process level, in addition to the cellular level. In soils from a ponderosa pine stand in New Mexico, net nitrification in mineralization potential experiments with soils before a prescribed burn was very low (White, 1986a). Within a few months following the prescribed burn, net nitrification was significantly increased compared to soils from unburned areas. Although fire directly combusts the highly flammable monoterpenes, fire also alters many other factors within the forest floor or soil. To test for the effects of monoterpenes and/or other volatile compounds, White (1986b) used "trapped vapor" experiments to assay the effects of vapors on nitrification in soils from burned plots. Portions of unburned forest floor, which were placed in beakers within sealed jars containing soil from burned plots (no direct contact between the forest floor and soils) significantly inhibited nitrification. Nearly complete inhibition of nitrification occurred when postburn soils were exposed to vapors of a mixture of monoterpenes in ponderosa pine (White, 1986b). These experiments show that apparent inhibition of nitrification was released or dimin-

ished following fire, and inhibition was again demonstrated following contact with vapors from the forest litter or with vapors from a mixture of monoterpenes.

In further studies, White (1991a) demonstrated that monoterpenes varied in their inhibition of net nitrification and that inhibition was a function of monoterpene concentration, which is consistent with the variable effects of specific monoterpenes on whole-cell cultures of *Nitrosomonas europaea* in studies by Courtney et al. (1991). With low to intermediate additions of monoterpenes (10–125 μg/g), nitrification was inhibited with little change in net mineralization. High monoterpene additions (1250 μg/g) stimulated net immobilization of ammonium. Bremner and McCarty (1988) also reported that addition of monoterpenes at about 2000 μg/g to soils currently in production of corn and soybean resulted in net immobilization of ammonium. Since ammonium is the substrate for nitrification, a reduction in substrate concentration may produce the same apparent result as inhibition of nitrification, which prompted Bremner and McCarty to conclude that monoterpenes were not nitrification inhibitors.

In a comment on the work of Bremner and McCarty (1988), White (1990) stated that tests with soils in agricultural production did not constitute proof that monoterpenes were not nitrification inhibitors. Rather, White felt that the real challenge was to find a reasonable explanation for the apparent differences in the results of Bremner and McCarty (1988) and White (1986b, 1991a). One such explanation is offered by the work of DeLuca and Keeney (1993), who compared cultivated soils in Iowa (including soils from the same soil series used by Bremner and McCarty 1988) to soils of the same series that had remained in prairie. Patterns of N cycling in prairie soils were inherently different than patterns in soils under agricultural production. DeLuca and Keeney (1993) determined that cultivated soils are low in available organic substrate (C limited) and high in inorganic N, particularly nitrate, relative to prairie soils of the same soil series. Furthermore, prairie soils had ammonium concentrations higher than nitrate, whereas soils under crop production had nitrate in excess of ammonium. In contrast, prairie soils had sufficient available C to immobilize nitrate (soils were N limited), resulting in higher concentrations of ammonium relative to nitrate in prairie soils (DeLuca and Keeney, 1993). These results support the hypothesis of Schimel (1986), who suggested that cultivated soils lack available-C substrate, which results in net N mineralization and an accumulation of nitrate. Thus, the differences between the results of Bremner and McCarty (1988) and those of White (1986b, 1991a) could be as simple but fundamental as differential effects of monoterpenes when added to C-limited versus N-limited soils, respectively.

When added to available-C-limited soils (such as those under cultivation used by Bremner and McCarty), monoterpenes act as an available-C source (as determined by Bremner and McCarty), which stimulates immobilization of available N until the substrate (monoterpenes) is consumed. After the available-C

substrate added as monoterpenes was consumed by heterotrophic microorganisms (or lost by volatilization during "aeration" of the flasks as in the experiments of Bremner and McCarty, 1988), the remaining ammonium can undergo nitrification, which is about the only process that can yield further energy. In available-C-rich or N-limited soils (like those in ponderosa pine ecosystems), the addition of relatively small amounts of available C has little effect on the heterotrophic community. Thus, monoterpenes can persist in N-limited soils, enabling their effects on nitrifying organisms to be expressed.

Inhibition of Nitrification by Monoterpenes: Conceptual Pattern. White (1991a) proposed a descriptive model to illustrate the effects of monoterpene additions to a mixed soil microbial community (Figure 7). In light of the work by Bremner and McCarty (1988) and subsequent work by DeLuca and Keeney (1993), it should be noted that monoterpenes are expected to exert control over the process of nitrification only in N-limited soils. When monoterpenes are not present in such soils, mineralization and nitrification are controlled by other factors and nitrate production may be high. As monoterpene concentrations increase, the proportion of nitrate relative to ammonium starts to decline. At still higher monoterpene concentrations, nitrification is inhibited and ammonium is the only inorganic form. At yet higher concentrations, net immobilization of N would be expected under field or laboratory conditions.

Although extracts of various litters may inhibit nitrification and net mineralization in laboratory conditions, whether or not these processes are inhibited under field conditions will depend upon two primary factors: (1) the rate of contribution to the soil system via throughfall, litterfall, root exudation, etc.; and (2) their persistence in the field, which may be a function of multiple factors. Other papers within this symposium have identified mechanisms for dispersal

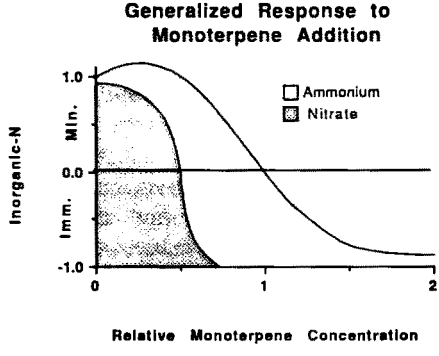

FIG. 7. Conceptual pattern in the inorganic N content of soils as a function of monoterpene content. (Redrawn from White, 1991a).

within the environment (Fischer et al., this issue), so I will concentrate on factors that affect persistence.

The persistence of monoterpenes in litter is not well known. I know of no published study that has determined the persistence of monoterpenes in plant litter. Measurement of monoterpene concentrations within the organic horizons and upper mineral soil may provide an indirect estimate of persistence. In the ponderosa pine stand used in the studies of White (1986a,b, 1991a,b), there was about a 10-fold decline in monoterpene concentrations between adjacent horizons. Total monoterpene concentrations were about 1400 μg/g in the fresh litter, about 150 μg/g in year-old litter, about 30 μg/g in the F-H organic horizon, and finally about 0.6 μg/g in the 0- to 10-cm-depth mineral soil horizon (Figure 8). In a stand of *Pinus monophylla* along the extreme western edge of the Great Basin, Wilt et al. (1993) reported total monoterpene content declined from a mean of 3.6 mg/g dry weight in senescent needles to 0.12 mg/g within a "dark decomposing layer" of litter material.

On two separate occasions, the L and F-H horizons in three forest types in the Kananaskis Valley of southwestern Alberta, Canada were analyzed for monoterpenes in my laboratory (only three samples of each horizon from each forest). The three sites, which varied in their tree species composition, were

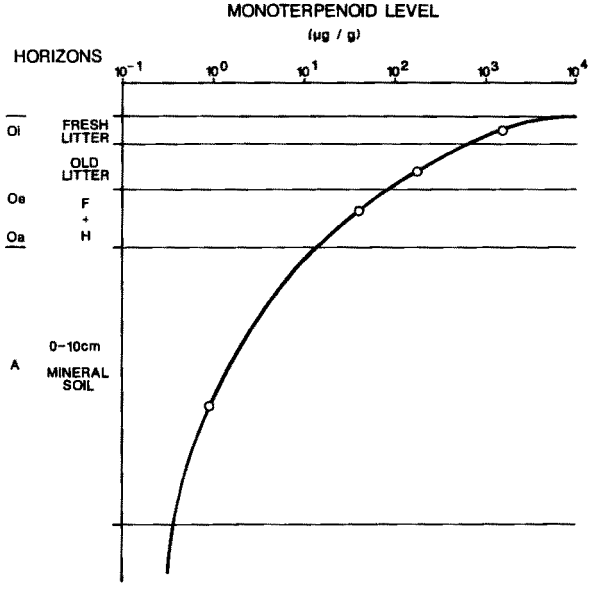

FIG. 8. Total monoterpene concentration of indicated horizons for a typical ponderosa pine forest in New Mexico (in the absence of recent fire).

named for the tree in the greatest abundance at each site: fir (*Abies lasiocarpa*); spruce (*Picea glauca*); and pine (*Pinus contorta*) (see Prescott et al., 1989a,b, 1992 for site descriptions and other characteristics of these forests). Total monoterpenes in fresh litter collected in January 1989 differed by at least an order of magnitude between the different forests (Figure 9) (C.S. White, unpublished results). However, the forest type with the lowest monoterpene concentration in the litter (pine) had the highest concentrations in the F-H horizon (Figure 9). Thus, even though monoterpene concentrations were much higher in litter from the spruce and fir forest, greater persistence of monoterpenes in the lodgepole pine forest lead to higher monoterpene concentrations in the lower organic horizons. Mineralization potentials also were measured on the F-H and 0- to 10-cm-depth mineral soil horizons from these forests. The lodgepole pine forest had relatively low net N mineralization and did not produce nitrate in the three samples of either horizon (Figure 10). Net nitrification occurred in the mineral soils from the fir forest and in one of three samples of mineral soil from the spruce forest (Figure 10). Thus, preliminary data suggest that higher concentrations of monoterpenes in the F-H horizon are negatively correlated with

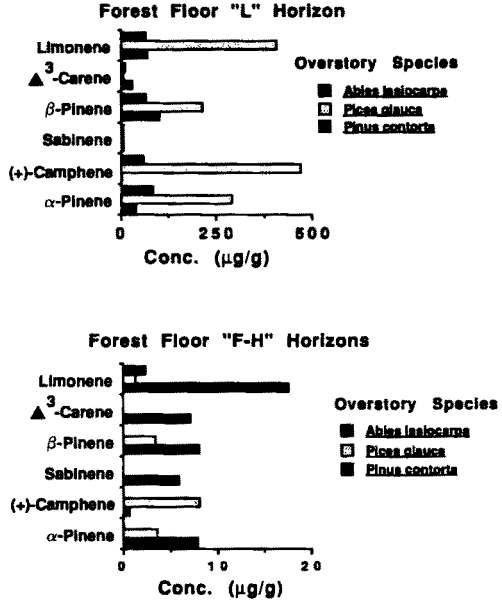

FIG. 9. Concentrations of indicated monoterpenes in the L and F-H horizons of three forest-types in Alberta, Canada collected in June 1990 (N = 3). (See Prescott et al., 1989a,b, 1992 for details about the forest types.)

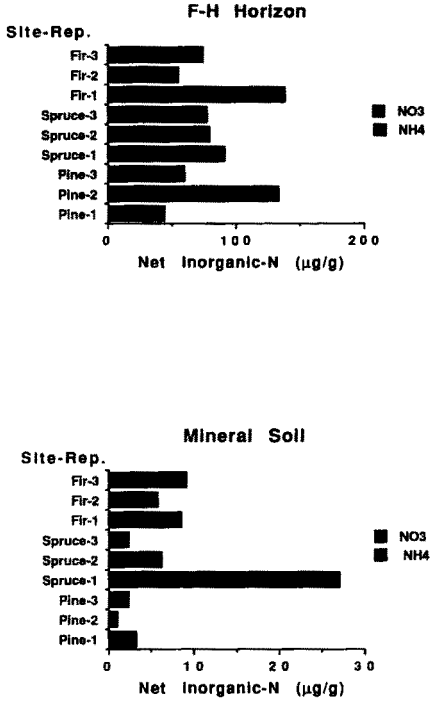

FIG. 10. Net N mineralization during 26-day aerobic incubation of individual samples of F-H and mineral soil samples from three forest-types in Alberta, Canada, collected in June 1990. (See Prescott et al., 1989a,b, 1992 for details about the forest types.)

nitrification rates in the F-H and mineral soil horizons (consistent with inhibition by monoterpenes).

It may be pure coincidence that lodgepole pine and ponderosa pine are both fire-prone or fire-adapted species, but this suggests that monoterpenes in litter of fire-adapted pines may persist longer than in litter of nonadapted species. Thus, fire-prone species may produce litter that has greater retention of volatiles, resulting in higher concentrations of volatiles within the forest floor, which also increases the probability of fire within those systems. This aspect will be developed further in the next section.

FIRE, MONOTERPENES, AND PONDEROSA PINE

Mutch (1970) hypothesized that fire-adapted tree species produced compounds that enhanced the probability of fire at more frequent intervals. His hypothesis was based upon comparisons of the amount of extractable plant

material in species with differing fire frequencies. Ponderosa pine had the highest amount of extractable compounds and had a high fire frequency (or short fire interval). Since 1970, improvements in analytical techniques and availability of instruments (principally in gas chromatographs and associated techniques) have led to the understanding that monoterpenes are a major portion of the extractable plant material referred to by Mutch.

Monoterpenes are highly flammable compounds and are present in high concentrations in ponderosa pine litter and in conifer litter in general. Monoterpenes are degassed early in the combustion process, and heat from their combustion provides the energy for direct pyrolysis of the solids to occur (Chandler et al., 1983). Thus, higher monoterpene concentrations would increase the probability of ignition and increase the ability to carry a fire, which could lead to shorter fire intervals in these systems. This is also compatible with observed high concentrations of monoterpenes within the forest floor of lodgepole pine and ponderosa pine forests, both of which are fire-adapted species.

White (1991a) proposed a conceptual model for the effects of increasing monoterpene concentrations on the inorganic N content of a soil. The model also may mimic the effects of fire on soil inorganic N because natural dynamics of monoterpenes in soils should follow the fire cycle. After fire, monoterpenes are in low concentration and net mineralization and nitrification are high (White, 1991b). With time since fire, monoterpenes increase, lowering net mineralization and inhibiting nitrification. Eventually, monoterpenes accumulate to the point that N cycling processes are greatly reduced and their presence concurrently increases the probability of another fire. Low net N mineralization and net nitrification might indicate when a fire could be beneficial from a nutrient cycling perspective, regardless of the apparent fuel loads.

The apparent concurrence of low rates of N cycling with high monoterpene concentrations, which would promote a fire, suggests that the development of low mineralization rates might be related to the historic fire interval at that site (fire intervals prior to controls by man). Some experimental evidence from Bandelier National Monument, New Mexico (C.S. White, unpublished results), appears to support this hypothesis. The historic fire interval for a ponderosa pine stand was approximately eight years. Samples of soils taken from that stand eight years after the last fire event showed low net N mineralization and nitrification in laboratory experiments (Figure 11). In contrast, another ponderosa pine stand on the same bedrock that was managed by prescribed fire in the previous year had high rates of net N mineralization and nitrification. Other samples taken from sites along a fire chronosequence within Bandelier National Monument were disturbed by heavy elk and showed intermediate rates of N mineralization and nitrification. The higher rates of N mineralization and nitrification at the sites used by elk are a response to the additional supply of N from elk feces and urine.

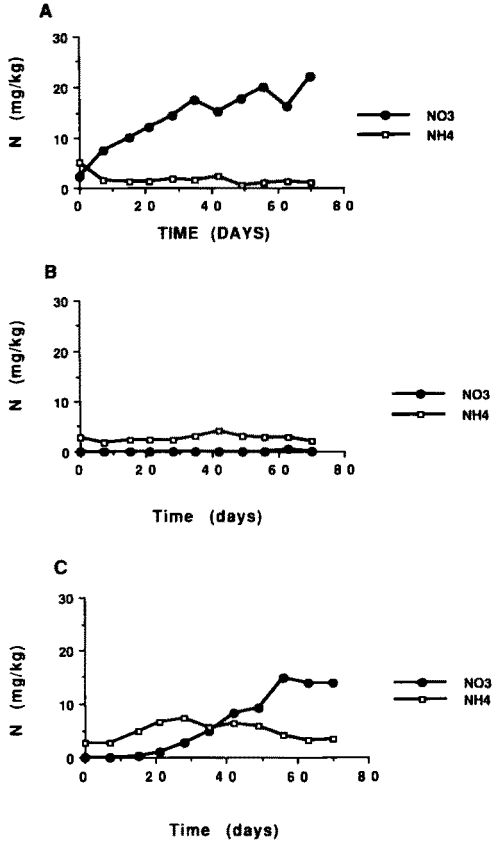

FIG. 11. Nitrogen mineralization patterns for mineral soil from three ponderosa pine stands in Bandelier National Monument in New Mexico. Interval since last fire was one year (A), eight years (B), and 15 years (C). Elk use at site C probably accounts for the higher mineralization–nitrification patterns.

INTERACTION OF MONOTERPENES WITH THE CARBON CYCLE: SIMILARITIES BETWEEN AMMONIA AND METHANE OXIDATION

Methane and ammonium are very similar structurally and, perhaps not coincidentally, both are oxidized by monooxygenases: methane monooxygenase and AMO, respectively. Many compounds that inhibit AMO also inhibit methane oxidation. In fact, either compound may substitute for the other as a substrate for their respective enzymes, although affinity is lower for the alternate substrate (Ward, 1987). If monoterpene's mode of action is by inhibition of AMO, then

monoterpenes also may inhibit methane oxidation. In experiments conducted in my laboratory (C.S. White, unpublished results), oxidation of methane in moistened soils was significantly inhibited in the presence of monoterpenes (Figure 12). Thus, monoterpenes may inhibit the oxidation of methane in environments where both compounds occur.

In studies of the global concentrations of methane, the predicted pattern of highest concentrations of methane in regions of flooded agriculture (i.e., rice paddies), which occur at near-equatorial latitudes, did not occur. Rather, atmospheric methane concentrations were highest in northern latitudes (Steele et al., 1987; Blake and Rowland, 1988), which have extensive coniferous forest that have high concentrations of monoterpenes. Methane is produced in anoxic conditions when all alternative electron acceptors (oxygen, nitrate, manganese, iron, and sulfate, in order of their disappearance) are depleted. An example is by anaerobic organisms in the sediments of rice paddies. Northern latitudes contain extensive regions with environments conducive to methane generation (Blake and Rowland, 1988). However, oxic conditions often occur in the waters above anoxic sediments, where methane oxidation could occur. Inhibition of methane oxidation in the overlying water column could contribute to the increase in atmospheric methane concentrations in northern latitudes. Increased atmospheric methane concentrations and monoterpenes may not be related; however, the correlative patterns are suggestive and may deserve further attention.

SUMMARY

From a nutrient cycling perspective, monoterpenes are recognized as agents to reduce herbivory and protect against pathogens, which retain material in plant biomass and reduce or delay return of plant nutrients through litterfall and/or mortality. Monoterpenes in the atmosphere are involved with the production and consumption of ozone and hydroxyl radicals, which increase the rate of oxidation of N and S compounds and their net deposition rate, at least in the immediate area. In the soil, monoterpenes can act as substrate for a portion of the heterotrophic microbial community, which contributes to net C mineralization, but promotes net N immobilization in microbial biomass. In systems with high C-to-N ratios, reduction of available N through immobilization may feedback to further reduce net decomposition rates and net N mineralization. By inhibiting specific monooxygenases, monoterpenes may inhibit nitrification and methane oxidation. Inhibition of nitrification would reduce the production of nitrous oxide during the nitrification process and via denitrification. By promoting fire, monoterpenes further interact with N and C cycles directly through the combustion process and indirectly through changes in rates of nutrient cycling after fire. In conclusion, monoterpenes as a group may be an example of sec-

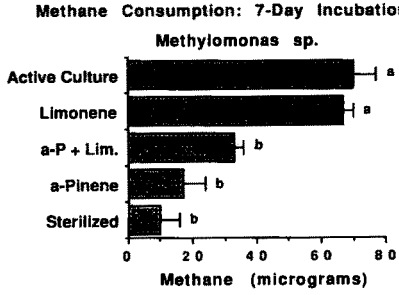

FIG. 12. The amount of methane consumed during seven-day incubation of solutions containing methanotrophic bacteria (*Methylomonas* sp. in 50 ml nutrient broth contained in 125-ml serum bottles) with equal concentrations of methane in the headspace (standard error indicated to right of bar). Treatments were control (Active Culture), 1.5 μl limonene added to solution (Limonene), α-pinene and limonene (0.75 μl each) added to solution (a-P + Lim.), 1.5 μl α-pinene added to solution (a-pinene), and sterilized control (Sterilized). Bars with different letters are significantly different ($P < 0.05$).

ondary plant metabolites that occur in parts per million concentrations in the soil yet influence ecosystem-level properties.

FUTURE RESEARCH: HYPOTHESES AND DIRECTIONS

1. To date, our interpretation of response to monoterpenes in the field is limited to our analysis that measures "total" monoterpenes, but surely only a fraction of this amount is active at any given time. Microorganisms are surrounded by a film of water; thus, a compound must occur in this film of water to alter the function of the microorganism. Monoterpenes are thought to be only slightly water soluble, but as reported by Fischer et al. (this issue), this perception probably underestimates the amount in soil solutions that contain a variety of other organic and inorganic compounds that alter the solubility of monoterpenes. Actual analysis of this active phase that surrounds the microorganisms may be nearly impossible; however, monoterpenes within the soil atmosphere would represent the amount in the transfer phase. Dynamics of the vapor phase may be measured by purging the soil atmosphere and analyzing the volatiles. Recent work in atmospheric sampling techniques may prove valuable in this effort.

2. Monoterpenes exhibit differential persistence in litters from different conifers. "Free" monoterpenes appear to undergo rapid transformations within the environment. Morphological characteristics may contribute to the persistence of monoterpenes in plant litter. I propose that retention of monoterpenes within

plant litter is related to the types of structures in which they are sequestered. Monoterpenes sequestered in glands and easily broken surficial structures should be less persistent than those sequestered in lignified structures, such as resin ducts. Thus, the rate of turnover or residence time of monoterpenes within litter or soils may differ substantially between vegetation types and be related to the persistence of the structure in which they are sequestered: short residence times in easily disrupted structures, longer residence time in resistant structures.

3. In addition to morphological features, other factors should enhance or limit the persistence of monoterpenes in the soil environment. Factors associated with poor litter quality (such as high C/N or lignin/N ratios), which usually slow decomposition, may enhance persistence of monoterpenes. Favorable moisture/temperature regimes may enhance microbial breakdown of monoterpenes. In contrast, extremely dry or hot conditions may enhance volatile loss of monoterpenes, which also enhances the probability of fire since these compounds are highly flammable. Thus, decomposition via combustion may be a mechanism to enhance rates of nutrient cycling in systems with high monoterpene content in litter and environmental conditions unfavorable for heterotrophic decomposers.

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IDENTIFICATION OF OLFACTORY CUES USED IN HOST-PLANT FINDING BY DIAMONDBACK MOTH, *Plutella xylostella* (LEPIDOPTERA: PLUTELLIDAE)

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Abstract—Olfactory attraction of female diamondback moths (*Plutella xylostella*) to odors of intact and homogenized host plants, as well as individual compounds characteristic of host plants, were investigated by behavioral and electrophysiological methods. Moths were attracted to odors of *Brassica juncea* and *B. napus* seedlings in a Y-tube bioassay. Solvent fractions of homogenized *B. juncea* leaves were attractive to moths whether or not isothiocyanates (IC) were present. Moths were attracted in Y-tube bioassays and to field traps baited with individual ICs. Volatiles from *B. juncea* and *B. napus* elicited an electroantennogram (EAG) response and were attractive in the Y-tube bioassay. Allyl IC was shown to be the attractive component in homogenized plant volatiles but was found to be virtually absent from intact plant volatiles. Gas chromatographic fractionation of intact plant volatiles revealed a terpene-containing fraction to be most attractive to the moths. We were unable to isolate individual attractive compounds from this fraction. Our results suggest that certain elements of this fraction, possibly in combination, are important olfactory cues for host-plant finding by the diamondback moth with mustard oils playing an important and possibly synergistic role, particularly when plants are damaged.

Key Words—*Plutella xylostella*, Lepidoptera, Plutellidae, *Brassica*, host plant attraction, EAG, bioassay, host plant location, plant volatiles, mustard oils, isothiocyanates.

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INTRODUCTION

The diamondback moth, *Plutella xylostella* (L.), is a highly mobile insect, of worldwide distribution, whose major host plants include most species of the Brassicaceae. In Canada, it is not capable of surviving the winters, but reinvades from the southern United States every spring (Smith and Sears, 1982). The adult must be capable of rapidly locating its host plants following migration or following local movement, for example, when the local host plants are unsuitable. As it is primarily a nocturnal insect, it is likely that odor cues are important in host-plant finding. This suggestion is supported by experimental results, which indicate that the diamondback moth responds to certain host-plant odors by moving upwind in their presence (Palaniswamy et al., 1986; Pivnick et al., 1990). Insects from at least five different orders are known to be attracted to volatiles of cruciferous plants (Feeny et al., 1970; Read et al., 1970; Wallbank and Wheatley, 1979; Shimizu and Usui, 1986; Pivnick et al., 1991). Where a primary attractant compound has been identified in crucifer-feeding specialists or their parasites, this has always been allyl isothiocyanate (allyl IC) (Feeny et al., 1970; Read et al., 1970; Free and Williams, 1978; Wallbank and Wheatley, 1979) and other isothiocyanates as well (Matsumoto, 1970). Isothiocyanates are hydrolytic breakdown products of glucosinolates, characteristic constituents of the Brassicaceae (Kjaer, 1960). In this paper, we investigate the response of diamondback moths to host-plant odors with the aid of a behavioral Y-tube bioassay, electroantennogram recordings, and field trapping.

METHODS AND MATERIALS

Insect Rearing

Insects were reared as previously described (Pivnick et al., 1990). Potted Oriental mustard *Brassica juncea* (L.) Czerniak (cv. Domo), grown in the greenhouse, was used for rearing and in all experiments.

Bioassays

Four different bioassays were used to assess or screen intact plants, plant extracts, and individual compounds, as follows:

Y-Tube Behavioral Bioassay. This bioassay has been described previously (Pivnick et al., 1990). Insects were tested on day 8 having been deprived of mates and plant odors for the previous 24 hr (where day 1 is 0–24 hr post-emergence). The bioassay lasted for the first 6 hr of the 8-hr scotophase. Tests were carried out with approximately 30 females per bioassay. Four bioassays were carried out per test for the initial concentration (500 mg plant equivalents) of fractions obtained in the solvent extraction and fractionation procedure

(described below). For lower concentrations, combined fractions, and all subsequent tests, five bioassays were carried out.

Electroanntennogram (EAG) Response. The signal detection and recording equipment and insect antennal preparation used for the EAG has been described previously (Chisholm et al., 1975). Odor samples were delivered to the insect antenna in 1-ml puffs. Test materials were administered through cartridges consisting of a glass pipet containing a small filter paper disk impregnated with the sample, with the exception of gas chromatograph (GC) fractions, which were tested directly from the GC collection tubes. The wide end of each cartridge was capped with a rubber septum. The cartridge was inserted into a glass delivery tube situated approximately 2 cm from the insect antennal preparation. Air was delivered through a needle tip inserted through the septum. Young females were found to respond best to plant compounds or extracts. Therefore, all EAGs were carried out on day 2 females (24–48 hr old), which had access to 10% sucrose solution until testing. The 1-ml air puffs were repeatedly administered in the following order: blank, standard, blank, test material, blank, standard, blank, and so on. Each moth was tested once with each test material, and a moth was used until a series was completed or until the moth's response to the standard diminished substantially. The standard was 1 μg phenylethyl IC in 10 μl of dichloromethane. Antennal responses to plant fractions are reported as a percentage of the mean response to the standard before and after the response to the test material. Mean response to the blank before and after was first subtracted from both the response to the standard and to the test material. Antennal responses to individual plant compounds are reported in millivolts.

Field Trapping. Baits used in field trapping were prepared so that release rates were relatively constant over the trapping period. Baits were placed in glass tubing, sealed at one end, with dimensions similar to those used by Pivnick et al. (1992). Tubing dimensions were identical for *n*-propyl IC and allyl IC. The release rate was directly proportional to the square of the diameter of the tubing and inversely proportional to the length of the air column above the test material. Release rates were measured during the experiments by measuring the length of the air column at the beginning and end of each trapping period. All traps were replicated four times.

Vertical cylinders of white cardboard (15 cm diameter and 30 cm tall) with Stickem Special (Michel & Pelton Company, Emeryville, California) on the outside served as traps. The traps, with baits placed in the center of the inside of the cylinder, were placed 10 m apart alongside plots of canola in Saskatoon for two periods of two weeks each, with baits replaced and bait loss measured after each two week period. The middles of the traps were placed on stakes at the height of the crop.

Traps were baited with compounds used in the EAG and Y-tube tests at release rates of approximately 4 and 0.4 mg/day. Allyl IC was also tested at 40

mg/day. Due to the small quantity of 3-methylthiopropyl IC available, it was not used in the field tests.

Oviposition and Larval Development. Ten pairs of newly emerged adult diamondback moths were placed in each of five cages containing one pot of one plant of 2-week-old *B. juncea* cv. Domo and one of a similar aged *S. alba* cv. Ochre plant plus a container of 10% sucrose solution. The moths were allowed to mate and oviposit for three days, at which time the adults were removed and the number of eggs on each plant counted. The plants were then maintained in separate cages while eggs hatched and larvae commenced feeding. When the majority of larvae had reached the second instar, 50 larvae from each cage were transferred to fresh, similar aged plants of the same species (10 larvae per plant) to complete development. Emergence of mature diamondback moths was then monitored.

Materials Tested

Intact Seedlings Tested in the Y-Tube. Oriental mustard, *Brassica juncea*; canola *B. napus* L. cv. Westar; white mustard, *Sinapis alba* L. cv. Ochre; and faba bean, *Vicia faba* L. cv. Outlook, were planted in 50 ml of sterile soil in 250-ml Erlenmeyer flasks. Twenty-five seeds of the first three species were planted and were tested seven days later when their total fresh weight (determined after the bioassay) was approximately 2 g. For *V. faba*, 10 seeds were planted and were tested after 10 days when the total weight was approximately 6 g.

Solvent Extraction and Fractionation. Leaves of 4-week-old *B. juncea* plants (25 g) grown in sterile growing media (Turface, Applied Industrial Material Corp., Deerfield, Illinois) in 15-cm-diameter pots, three plants per pot, were extracted with boiling methanol (100 ml) and 80% (v/v) methanol in water (Chisholm and Wetter, 1967). The methanol and some water were removed under reduced pressure at 50°C and the aqueous residue filtered and made up to 50 ml (fraction i). The aqueous solution of glucosinolates was sequentially extracted for 48 hr with pentane (fraction ii) and dichloromethane (fraction iii) to leave an aqueous extract (fraction iv). The latter was treated with myrosinase (EC .3.2.3.1) prepared from seeds of *S. alba* (Harris, 1970) and volatiles (fraction v) collected using a closed loop stripping apparatus as described previously (Pivnick et al., 1990). The myrosinase-treated solution was then extracted with dichloromethane as before (fraction vi) to leave an aqueous residue (fraction vii). The solvent extracts were concentrated to 0.5 ml by slow distillation through a 10-cm Vigreaux column at 50°C. Thus 10 μl solvent extract and 1 ml aqueous extract contained 500 mg plant equivalent (fresh weight). Samples were stored in the refrigerator until required. For Y-tube bioassays, 1 ml of aqueous extract was presented on a cotton wick or 10 μl of solvent extract was spotted on a 7-mm-diameter filter paper disk.

Individual Volatile Plant Compounds. Compounds were obtained from Aldrich Chemical Company, Milwaukee, Wisconsin (allyl IC = allyl isothiocyanate, 2-phenylethyl IC, *n*-propyl IC, 4-allylanisole, (1*S*)-(−)-verbenone, and α-terpineol), and the British Drug Houses, London, England (borneol). 3-Methylthiopropyl IC was synthesized according to published methods (Kjaer et al., 1955).

For both Y-tube and EAG bioassays, compounds were dissolved in dichloromethane at the appropriate concentration so that 10 μl spotted on 7-mm-diameter filter paper disks would contain the desired amount of compound. For EAGs, the disks were kept in the rubber septum-capped glass pipets and stored in a freezer when not in use. Each disk was used three times for EAG bioassays before discarding, during which time no change in EAG activity was noted. For the Y-tube bioassays, disks were used once only.

In EAG tests, all compounds were tested on five insects at three different doses: 0.1, 1, and 10 μg/disk. Compounds that elicited a significant response at the lowest dose were tested at two lower doses (0.01 and 0.001 μg/disk). Compounds were tested with the Y-tube bioassay with five replicates at five doses.

Collection of Volatiles and Gas Chromatograph Fractionation. Volatiles from 25 g of homogenized leaves of Oriental mustard were collected as described previously (Pivnick et al., 1990). After collection and elution, the resulting sample contained 500 mg fresh weight plant equivalents in 10 μl dichloromethane.

For collection of volatiles from intact seedlings, an open loop collection apparatus was constructed from a glass cylinder (21 cm OD × 45 cm high) fitted with an acrylic lid (25 cm OD × 2.5 cm thick) carrying three Swagelok fittings to accommodate inlet and outlet tubing (6 mm polyethylene) and a manometer. An O-ring was countersunk in the lid to make an air-tight seal with the rim of the cylinder. The lid was held in place by eye-bolts attached to a metal strip passed around the bottom of the apparatus. Traps were made by fusing two pieces of glass tubing 4 mm ID × 4 cm and 2 mm ID × 6 cm and placing 10 mg coconut charcoal (60 mesh, Fischer Scientific) between two plugs of polypropylene wool (Aldrich Chemical Company) in the wider section. Compressed air was passed through a Supelpure HC trap (Supelco Canada, Oakville, Ontario, Canada), and pressure was controlled using a pressure regulator and a relief valve made from the base of a Bunsen burner. Airflow through the apparatus was as follows: from the laboratory source air flowed through the Supelpure trap and pressure regulator into the cylinder via the inlet fitting, then out of the cylinder via the outlet fitting and through the charcoal trap. The apparatus was vented into the lab. With the design, airflows of 20–60 ml/min could be maintained while keeping the pressure increase inside the chamber to 100 Pa, or lower. Approximately 100 seedlings of *B. juncea* or *B. napus* cv. Westar were

grown in Turface in a 15-cm pot, which was placed in the chamber the day after seedling emergence (usually five to six days after planting) and volatiles were collected for 48 hr. Traps were kept at 50°C to minimize condensation of water and were eluted with 2 × 100 μl of dichloromethane. Of each 200 μl sample, 50 μl was taken and reduced to 10 μl under a nitrogen gas stream at 0°C.

Volatiles were separated with a Hewlett Packard model 5890A gas chromatograph equipped with a thermal conductivity detector (TCD) on a DB-5 column (30 m × 0.53 mm, d_f 1.0 μm, J & W Scientific Inc., Rancho Cordova, California) in a Hewlett Packard 5890A GC. The injector was kept at 200°C and the detector at 250°C. Helium (10 ml/min at 100°C) was used as carrier gas. A 5-μl splitless injection (250 mg plant equivalents) was made at 40°C and the temperature held for 4 min. The column was then heated to 225°C at 4°C/min, and held for 15 min. Timed fractions (Figure 1) were collected on 350-mm × 2-mm-OD glass tubes bent so as to fit into 400-ml Dewar flasks packed with crushed Dry Ice. The collection tubes were connected to the outlet of the TCD by short Teflon sleeves into which short glass plugs were inserted to seal the tubes after each fraction was collected.

For EAG bioassays, the glass collection tubes were fitted with short steel tubes by the Teflon sleeves. Air puffs were routed directly through the collection tubes at 0°C. Fractions were tested on one or two insects, and subfractions were tested on one insect only.

For Y-tube behavioral bioassays, the tubes were eluted with 40 μl of dichloromethane, and the eluate was transferred directly from the tube to a 7-mm filter paper disk. After the solvent evaporated, the test material was placed in one arm of the Y-tube for testing.

Compound Identification. Gas chromatograph–mass spectral analyses (GC-MS) were obtained with a Finnigan 4000 instrument carrying a DB-5 column (60 m × 0.32 mm., d_f 0.25 μm, J & W Scientific). The injector was kept at 200°C and the source at 300°C. Helium flowing at 50 cm/sec and 100°C was used as carrier gas. Compound identity was confirmed by comparison with retention times and mass spectra of authentic samples.

RESULTS

Moths responded positively to intact seedlings of *B. juncea* and *B. napus*, but not to *S. alba* or *V. faba*, (Table 1). Because of the lack of attraction to *S. alba*, it was decided to test its suitability as a host plant in comparison with *B. juncea*. The number of eggs laid on *B. juncea* (227 ± 37, $N = 7$) and *S. alba* (302 ± 40, $N = 7$) did not differ significantly ($t = 2.21$). The number surviving to adult emergence out of 50 second-instar larvae also did not differ significantly

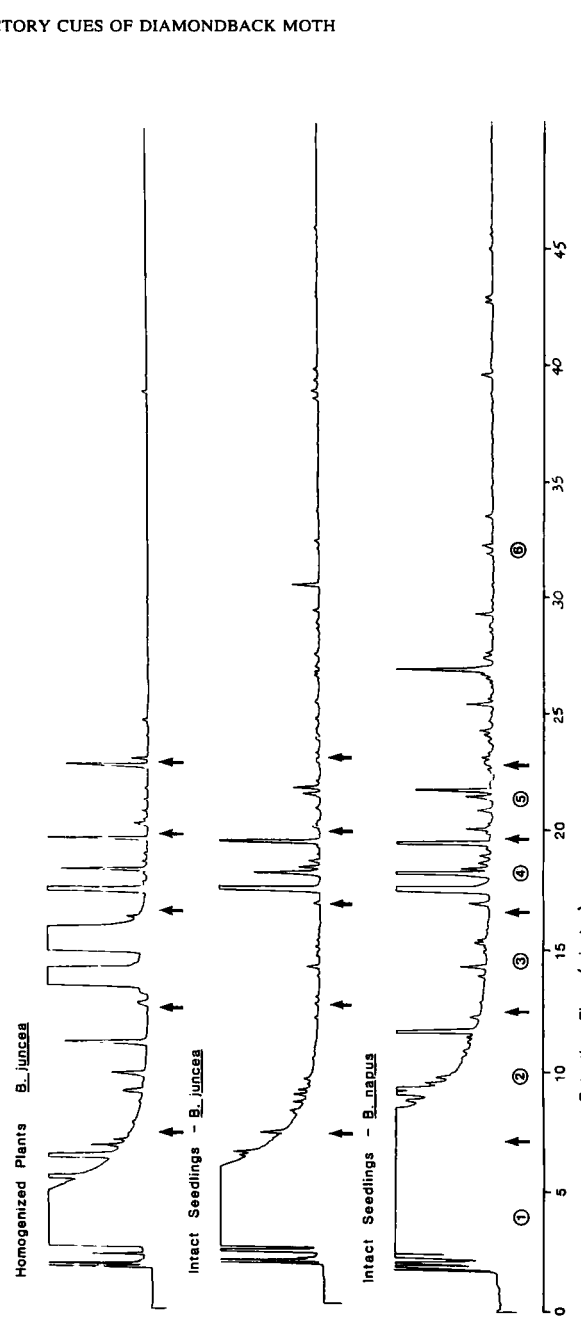

FIG. 1. Gas chromatograph (GC) traces of volatile collections from homogenized leaves of *Brassica juncea*; and intact seedlings of *B. juncea*, and *B. napus*. The arrows indicate the retention times at which cold-trapping collection tubes were changed in the first GC fractionation procedure to give fractions 1–6.

TABLE 1. Y-TUBE BIOASSAY RESPONSE OF *Plutella xylostella* FEMALES TO ODOR OF INTACT SEEDLINGS[a]

Species	Number of insects trapped		t^b	P^c
	Odor	Blank		
Brassica juncea	55	11	8.63	0.001
Brassica napus	60	14	4.89	0.01
Sinapis alba	39	25	2.12	NS
Vicia faba	19	26	3.09	NS

[a] Five replicates per test.
[b] Paired two-tailed t tests.
[c] NS, $P > 0.05$.

($t = 0.20$) between those reared on *B. juncea* (37.0 ± 5.5, $N = 5$) and those on *S. alba* (35.8 ± 4.4, $N = 5$).

Of the seven solvent fractions tested, all but fraction vi elicited a positive Y-tube response from the moths (Table 2). The crude aqueous fraction i remained attractive over a range of concentrations spanning four orders of magnitude. Fractions ii–iv, which ranged from nonpolar to polar and contained no isothiocyanates (verified by GC-MS), were all attractive. Fraction v, which contained volatile isothiocyanates (also verified), was attractive at only one dose (50 mg plant equivalents). Fraction vi, which was unattractive (at the one dose tested) also contained isothiocyanates (also verified). When fraction v at a subattractive dose was combined with fraction i at different doses, the response was consistently greater than that for fraction i alone at the same dose. However, the increase in attraction was small. When these two fractions were combined both at subattractive doses (when tested individually), the moths were attracted significantly, suggesting a weak additive or synergistic effect of two or more compounds in the mixture.

The lack of success in isolating attractive components with the solvent fractionation procedure prompted us to use GC fractionation of whole plant volatiles with EAG responses serving as an initial screening technique. These more rapid and finer fractionation and bioassay procedures were warranted as it was clear that a number of different attractive components were present. EAG tests on head space volatiles from homogenized *B. juncea* leaves showed that 250 mg fresh weight of plant equivalents was adequate for testing as female antennae responded strongly and consistently (Table 3). This material was even more stimulatory after collection from the gas chromatograph. The volatiles were separated into six fractions; the first five were approximately 5 min in

TABLE 2. Y-TUBE BIOASSAY RESPONSE OF *Plutella xylostella* FEMALES TO ODOR OF SOLVENT FRACTIONS OF *Brassica juncea* PLANTS AT FLOWER BUD STAGE

	Fraction	Concentration (mg plant equivalents)	Total number of insects trapped		t^a	P^b
			Odor	Blank		
(i)	Crude aqueous	500[c]	52	9	6.67	0.01
		50[d]	60	19	3.46	0.05
		5[d]	71	6	5.60	0.05
		0.5[d]	44	6	3.78	0.05
		0.05[d]	16	16	0	NS
(ii)	Pentane[c]	500	54	11	4.85	0.05
(iii)	Dichloromethane[c]	500	85	14	4.55	0.05
(iv)	Aqueous[c]	500	66	11	5.06	0.05
(v)	Volatiles after myrosinase treatment	500[c]	43	41	0.19	NS
		50[d]	75	4	6.96	0.01
		5[d]	35	20	0.73	NS
(vi)	Dichloromethane after myrosinase treatment[c]	500	69	30	1.19	NS
(vii)	Aqueous residue[c]	500	74	23	3.51	0.05
	Fraction (i) + (v)[d]	500 + 50	68	5	12.24	0.01
		50 + 5	86	6	11.35	0.01
		5 + 5	80	9	7.36	0.01
		0.05 + 5	22	9	2.99	0.05

[a] Paired two-tailed t tests.
[b] NS, $P > 0.05$.
[c] Four replicates per test.
[d] Five replicates per test.

duration, and the last, approximately 30 min (Figure 1; for exact times see Table 3). EAG activity was greatest for fractions 3 and 6, less so for fractions 4 and 5 and even less for fractions 1 and 2. Of all fractions, fraction 3 contained the greatest quantity of plant compounds and fraction 6 the least. Upon subdividing fractions 3 and 6, only subfraction 3.5 remained highly active (Table 3). This subfraction was the largest peak in the entire GC trace (other than the early solvent peaks) (Figure 1). Analysis by GC-MS and comparison with authentic samples established this component as allyl IC. The preceding large peak was similarly shown to comprise allyl thiocyanate and *trans*-2-hexenal, a "green plant volatile" (Visser and Avé, 1978). The allyl thiocyanate is likely to be an artifact of the GC procedure and not actually released by the plant (Slater, 1992).

TABLE 3. ELECTROANTENNOGRAM RESPONSE OF *Plutella xylostella* FEMALES TO HEADSPACE VOLATILES OF HOMOGENIZED *Brassica juncea* AND TO COLD-TRAPPED GAS CHROMATOGRAPH (GC) FRACTIONS OF VOLATILES

Test material	EAG response ($\bar{X} \pm$ SE)[a]	N	P[b]
Before GC injection			
250 mg plant equivalents	115 ± 17	3	0.05
50 mg plant equivalents	91 ± 31	3	NS
GC fractions (250 mg plant equivalents)			
Total GC effluent	277 ± 21	6	0.0001
Fraction 1 (8.00)[c]	32 ± 22	13	NS
Fraction 2 (13.00)	35 ± 13	9	0.05
Fraction 3 (17.80)	184 ± 30	9	0.001
Fraction 4 (21.30)	88 ± 20	9	0.01
Fraction 5 (24.75)	56 ± 10	9	0.001
Fraction 6 (50.25)	136 ± 21	9	0.001
Subfraction 3.1 (14.00)	41 ± 15	11	0.05
Subfraction 3.2 (14.50)	−1 ± 13	11	NS
Subfraction 3.3 (15.00)	57 ± 13	11	0.01
Subfraction 3.4 (15.85)	28 ± 10	11	0.02
Subfraction 3.5 (16.75)	173 ± 24	11	0.001
Subfraction 3.6 (17.80)	29 ± 10	11	0.02
Subfraction 6.1 (29.75)	56 ± 16	6	0.02
Subfraction 6.2 (34.75)	72 ± 30	6	NS
Subfraction 6.3 (40.00)	54 ± 16	6	0.05
Subfraction 6.4 (42.00)	56 ± 11	6	0.01
Subfraction 6.5 (50.25)	49 ± 15	6	0.05

[a] Responses reported are expressed as percent standard (1 μg phenylethyl isothiocyanate) administered before and after fraction.

[b] P is the probability that the mean EAG response is different from zero as determined by the t statistic; NS indicates that $P > 0.05$.

[c] Numbers in parentheses refer to the retention time (minutes) at the end of the fraction collection period.

All subfractions from fraction 6 elicited moderate EAG stimulation but no subfraction contained much activity, compared with fraction 6 in its entirety.

Y-tube bioassays (Table 4) of the headspace volatiles collected from homogenized *B. juncea* demonstrate that the moths readily discriminate between a solvent blank and the plant odor mixture, at higher and lower concentrations than that which elicited a strong EAG response (250 mg) (Table 3). The mixture was also attractive after 250 mg plant equivalents were injected and cold-trapped from the GC. Fraction 3 was attractive but all the other fractions combined were not. Subfraction 3.5 was attractive at 250 mg and at 25 mg but not at 2.5 mg plant equivalents. The remainder of fraction 3 was not attractive. A blank GC

TABLE 4. Y-TUBE BIOASSAY RESPONSE OF *Plutella xylostella* FEMALES TO HOMOGENIZED *Brassica juncea* HEAD SPACE VOLATILES AND TO COLD-TRAPPED GAS CHROMATOGRAPH (GC) FRACTIONS OF VOLATILES

Test material	Concentration (mg plant equivalents)	Number of insects trapped		t^a	P^b
		Odor	Blank		
Before GC injection	500	48	14	3.72	0.05
	50	49	4	9.00	0.001
GC fractions					
Fractions 1-6	250	63	10	3.01	0.05
Fraction 3	250	32	13	3.30	0.05
Fractions 1, 2, 4, 5, 6	250	21	8	0.22	NS
Subfraction 3.5	250	41	6	3.85	0.05
	25	34	4	4.47	0.05
	2.5	32	22	1.18	NS
Subfractions 3.1-3.4, 3.6	250	19	6	2.41	NS
Blank	0	20	17	0.19	NS

[a] Five replicates per test. Paired two-tailed *t* tests.
[b] NS, $P > 0.05$.

collection gave no response. We conclude that allyl IC is the main attractant in homogenized *B. juncea* volatiles.

Four isothiocyanates, which are known to occur in the Brassicaceae, were tested with the EAG. Of these, three elicited EAG responses at the two highest doses, while two compounds elicited significant EAG responses at the lowest level tested (0.001 μg) (Table 5). In Y-tube bioassay trials, allyl IC, 3-methylthiopropyl IC, and 2-phenylethyl IC were neutral at low doses, attractive at intermediate doses, and the latter two were repellent at the highest doses (Table 6). Traps containing the most attractive bait caught only twice the number of moths as did the blank traps during field trapping experiments (Table 7). For each compound, the higher the bait release rate, the higher the trap catch. With all compounds, significantly more moths were attracted at the highest release rate than with the blanks.

Allyl IC is primarily released when cells are ruptured, yet we have shown intact-plant odors also to be attractive to diamondback moths. Hence, we collected volatiles from intact seedlings, and repeated our GC fractionation procedure to isolate other attractive compounds. Volatiles from intact seedlings of *B. juncea* produced a strong EAG response when cold-trapped from the GC (Table 8). When the same timed fractions (as in Table 3; Figure 1) were assayed, the responses were similar to those elicited by the homogenized plant volatiles

TABLE 5. ELECTOANTENNOGRAM (EAG) RESPONSE OF *Plutella xylostella* TO FOUR INDIVIDUAL VOLATILE PLANT COMPOUNDS KNOWN TO OCCUR OR SIMILAR TO THOSE THAT OCCUR IN BRASSICACEAE

Compound[a]	Boiling point (°C)	EAG response, mV ($X \pm$ SE, $N = 5$), at different concentrations[b]				
		0.001 μg	0.01 μg	0.1 μg	1 μg	10 μg
Allyl IC	152			0.08 ± 0.07	0.42 ± 0.15*	0.48 ± 0.08**
n-Propyl IC	153			−0.13 ± 0.09	−0.09 ± 0.08	0.03 ± 0.14
3-Methylthiopropyl IC	240	0.32 ± 0.08*	0.57 ± 0.13*	0.68 ± 0.08*	1.03 ± 0.08***	1.36 ± 0.11***
2-Phenylethyl IC	280	0.18 ± 0.05*	0.27 ± 0.09*	0.74 ± 0.13*	0.84 ± 0.13**	1.16 ± 0.11***

[a]IC, isothiocyanate.
[b]$*P < 0.05$; $**P < 0.01$; $***P < 0.001$; probability that the mean EAG is different from zero as determined by the t statistic.

TABLE 6. Y-TUBE BIOASSAY RESPONSE OF *Plutella xylostella* TO INDIVIDUAL PLANT COMPOUNDS KNOWN TO OCCUR IN BRASSICACEAE

Compound (μg)[a]	Number of insects trapped		t^b	P^c
	Odor	Blank		
Allyl IC				
0.1	10	9	0.14	NS
1	26	8	3.88	0.05
10	59	9	18.26	0.001
100	57	10	23.50	0.001
1000	22	23	0.06	NS
3-Methylthiopropyl IC				
0.01	19	15	0.24	NS
0.1	54	10	3.96	0.05
1	84	7	14.95	0.001
10	20	30	2.25	NS
100	13	52	4.41	0.05
2-Phenylethyl IC				
0.1	25	18	0.50	NS
1	37	9	4.99	0.01
10	20	19	0.13	NS
100	8	51	2.66	NS
1000	0	28	9.30	0.001

[a] IC, isothiocyanate.
[b] Five replicates per test. Paired two-tailed t tests.
[c] NS, $P > 0.05$.

with one important exception. There was no significant response to fraction 3 (Table 8). All the other fractions elicited stronger EAG responses than in the previous fractionation procedure, although the relative strength of the responses was similar.

Fraction 6 (Fig. 1) was subdivided as before and all five subfractions elicited strong EAG responses (Table 8). The three most active subfractions (6.1, 6.2, and 6.3) were subdivided further and tested. Most of these subsubfractions were only moderately active but two (6.2.1, 6.3.2; Table 8) were very active. GC-MS analysis of subsubfraction 6.2.1 indicated at least 16 components (Figure 1, the middle GC trace) of which the following have been identified by comparing retention times (in seconds in parentheses) and mass spectra of available authentic compounds: the terpenes, borneol/isoborneol (1476), 4-terpinenol (1504), α-terpineol (1538), verbenone (1584), and *cis*-carveol (1606), as well as naphthalene (1516), estragole (4-allylanisole) (1554), and benzothiazole (1617). Peaks at retention times 1450, 1634, and 1665 sec appear to be silyl

TABLE 7. CAPTURES OF *Plutella xylostella* IN STICKY CYLINDRICAL TRAPS BAITED WITH PLANT COMPOUNDS KNOWN TO OCCUR IN BRASSICACEAE NEAR SASKATOON, JULY 20–AUGUST 17, 1989

Compound[a]	Release rate (mg/day)			Total number of moths[b]
	Estimated	Actual ($\bar{X} \pm$ SE)		
Allyl IC	40	55 ± 8	$N = 8$	328 a
2-Phenylethyl IC	4	9.6 ± 5.4	$N = 5$	313 ab
n-Propyl IC	4	2.8 ± 0.5	$N = 8$	256 abc
2-Phenylethyl IC	0.4	0.3 ± 0.1	$N = 8$	240 bcd
Allyl IC	4	1.2 ± 0.7	$N = 6$	225 cd
Allyl IC	0.4	0.3 ± 0.1	$N = 8$	225 cd
n-Propyl IC	0.4	0.3 ± 0.03	$N = 8$	210 cd
Control				157 d

[a]IC, isothiocyanate.
[b]Totals (of four replicates per bait) followed by different letters are significantly different at $P < 0.05$ as determined by two-way ANOVA and a protected LSD test.

compounds from pretreatment of the glass cylinder, while those at 1463 and 1497 seconds appear to be isomers with mass spectra similar to that of pinanone.

Volatiles from intact seedlings of *B. juncea* (Figure 1) were assayed in the Y-tube at three different doses. The intermediate dose (10% of a volatile collection) was most attractive and was, therefore, the main dosage used in the rest of the bioassays (Table 9). Cold-trapped volatiles collected from the GC were also active. Fraction 6 was attractive, but at a reduced dose (1%). Subsubfractions 6.2.1 and 6.3.2, which were highly active in the EAG (Table 8), were also attractive in the Y-tube bioassay, although in the latter case, the dosage of the material had to be increased to 100% of the GC collection for it to be attractive (Table 9). The four most abundant of the eight compounds identified by GC-MS analysis of subsubfraction 6.2.1 were tested in the Y-tube bioassay. The most abundant compound, α-terpineol, was tested at eight concentrations (0.001–10,000 μg), none of which were attractive to the moths. Verbenone, estragole, and borneol were also not attractive to the moths when tested at four concentrations (0.1–100 μg) in the Y-tube.

GC fractionation and EAG testing was repeated with volatiles collected from intact seedlings of *B. napus*. The EAG responses to these volatiles, fractions, and subfractions were very similar to those elicited by the corresponding volatiles, fractions, and subfractions of intact *B. juncea* seedlings (Table 8).

TABLE 8. ELECTROANTENNOGRAM (EAG) RESPONSE OF *Plutella xylostella* FEMALES TO VOLATILES FROM INTACT *Brassica juncea* AND *B. napus* SEEDLINGS FRACTIONATED BY COLD-TRAPPING ON GAS CHROMATOGRAPH (GC)

	B. juncea			*B. napus*		
GC fraction[a]	EAG response ($\bar{X} \pm$ SE)[b]	*N*	*P*[c]	EAG response ($\bar{X} \pm$ SE)[b]	*N*	*P*[c]
Fractions 1-6	341 ± 54	9	0.001	246 ± 35	10	0.001
Fraction 1 (8.00)[d]	61 ± 33	12	NS	−68 ± 13	10	0.001
Fraction 2 (13.00)	58 ± 15	12	0.01	9 ± 22	10	NS
Fraction 3 (17.80)	51 ± 24	12	NS	36 ± 26	10	NS
Fraction 4 (21.30)	103 ± 12	12	0.0001	126 ± 24	10	0.001
Fraction 5 (24.75)	130 ± 16	12	0.0001	139 ± 45	10	0.02
Fraction 6 (50.25)	454 ± 68	12	0.001	375 ± 47	10	0.001
Subfraction 6.1 (28.00)	271 ± 49	5	0.01	177 ± 37	5	0.02
Subfraction 6.2 (33.20)	334 ± 44	5	0.01	170 ± 41	5	0.02
Subfraction 6.3 (38.45)	255 ± 27	5	0.001	166 ± 31	5	0.01
Subfraction 6.4 (42.00)	120 ± 35	5	0.05	75 ± 22	5	0.05
Subfraction 6.5 (50.25)	152 ± 51	5	0.05	116 ± 9	5	0.0001
Subsubfraction 6.1.1 (23.30)	9 ± 15	5	NS			
Subsubfraction 6.1.2 (24.20)	7 ± 21	5	NS			
Subsubfraction 6.1.3 (24.75)	42 ± 18	5	NS			
Subsubfraction 6.1.4 (25.75)	39 ± 16	5	NS			
Subsubfraction 6.1.5 (26.85)	33 ± 17	5	NS			
Subsubfraction 6.1.6 (28.00)	61 ± 6	5	0.001			
Subsubfraction 6.2.1 (29.50)	149 ± 23	5	0.01			
Subsubfraction 6.2.2 (31.60)	47 ± 21	5	NS			
Subsubfraction 6.2.3 (33.20)	61 ± 30	5	NS			
Subsubfraction 6.3.1 (33.45)	4 ± 14	5	NS			
Subsubfraction 6.3.2 (37.90)	170 ± 34	5	0.01			
Subsubfraction 6.3.3 (38.45)	49 ± 20	5	NS			

[a] 25% of volatile collection was injected on the gas chromatograph.
[b] Responses reported are expressed as percent standard (1 μg phenylethyl isothiocyanate) administered before and after the fraction.
[c] *P* is the probability that the mean EAG response is different from zero as determined by the *t* statistic; NS indicates that $P > 0.05$.
[d] Numbers in parentheses refer to the retention time at the end of the fraction collection period.

DISCUSSION

It is not clear how diamondback moths actually respond to odor cues in nature. Based on what we do know about insect host-plant perception (see Metcalf, 1987; Visser, 1986) and location (see Finch and Skinner, 1982; Visser,

TABLE 9. Y-TUBE BIOASSAY RESPONSE OF *Plutella xylostella* FEMALES TO VOLATILES FROM INTACT *Brassica juncea* SEEDLINGS AND COLD-TRAPPED GAS CHROMATOGRAPH (GC) FRACTIONS OF VOLATILES

Test material	Percentage of collection used[a]	Number of insects trapped		N	t^b	P^c
		Odor	Blank			
Before GC injection	50	35	7	5	1.61	NS
	10	41	4	5	4.54	0.05
	1	38	10	5	2.39	NS
Fractions 1-6	10	69	18	5	7.97	0.01
	1	35	14	5	1.32	NS
Fraction 6	10	50	42	5	0.41	NS
	1	67	29	5	3.25	0.05
Fraction 6.2.1	10	79	17	5	3.71	0.05
	1	17	12	3	1.25	NS
Fraction 6.3.2	100	46	22	5	8.20	0.01
	10	12	18	3	1.00	NS

[a] Entire volatile collection injected on GC, cold-trapped and eluted. This is the percentage of eluate used in bioassays.
[b] Five replicates per test. Paired two-tailed t tests.
[c] NS, $P > 0.05$.

1988), diamondback moths probably fly low over vegetation in fields or weedy patches, and when favorable odors are detected from a short distance, they may move toward them, probably employing some combination of odor-conditioned anemotaxis, orthokinesis, and klinokinesis (see Kennedy, 1977), until they land. From this point, detection may be primarily due to detection of nonvolatile constituents including glucosinolates (Reed et al., 1989). In an agricultural setting, with vast monocultures of host plants, as exist in canola-growing areas of the Canadian prairies, host-plant location is likely to be simpler.

Diamondback moths respond positively to many different plant volatiles. Yet, results of our Y-tube bioassays with intact seedlings indicate specificity of response. The two *Brassica* spp. were strongly attractive. *S. alba*, a species that we demonstrate here to be an acceptable host, was not attractive. This same species contains oviposition-deterrent compounds, although these are overridden by oviposition stimulants present in significant amounts (Reed et al., 1989). Faba bean seedlings also were not attractive. In contrast, Palaniswamy et al. (1986) reported faba bean and *S. alba* extracts to be attractive to both sexes of diamondback moths. Our results are not strictly comparable to those of Palaniswamy et al. (1986) as we used intact seedlings, not homogenized leaf extracts, in our bioassay. Further, Palaniswamy et al. (1986) assumed independent assort-

ment of individual insects in their statistical analysis. We have found this assumption to be invalid and liable to lead to unfounded conclusions even when using insects of only one sex (unpublished data).

The results of our solvent fractionation of *B. juncea* suggest that there are other compounds as well as the mustard oils, both polar (soluble in water and dicholoromethane) and nonpolar (soluble in pentane), which attract diamondback moths. Moreover, allyl IC, present in large quantities when *B. juncea* is homogenized, was repellent to the moths at high concentrations. There was also some evidence of possible synergism between allyl IC and other *B. juncea* volatiles (see Table 2) as has been found with certain combinations of plant volatiles for other phytophagous insects (see Visser, 1986).

In our bioassays of GC-fractionated volatiles of homogenized *B. juncea*, which may be likened to volatiles produced by insect-damaged plants, the major stimulatory and attractive component was allyl IC. However, fraction 6 was also highly stimulatory in the EAG in spite of the small amount of volatile compounds indicated by GC (Figure 1). Since the Y-tube bioassays lasted 6 hr, it is perhaps not surprising that the small quantity of volatile material in fraction 6 was not attractive. While fraction 6 was found to contain minor amounts of 2-phenylethyl IC by GC-MS, the EAG activity was dispersed, indicating that several compounds are responsible for the collective EAG stimulation.

We were able to confirm our findings (regarding the importance of allyl IC) with EAG and Y-tube bioassays by obtaining pure compounds and testing them similarly. Allyl IC was found to be stimulatory with the EAG and attractive in the Y-tube bioassay over a definable range of concentrations. In field trapping experiments, allyl IC was attractive at the highest dose tested.

In addition to allyl IC, we tested *n*-propyl IC, 2-phenylethyl IC, and 3-methylthiopropyl IC. In the EAG, the latter two compounds elicited responses at a much lower dose than allyl IC, while *n*-propyl IC was not at all stimulatory. Clearly, the moths do perceive *n*-propyl IC as they were attracted to it in the field trapping experiment. While there is obviously some difference in response to allyl IC and *n*-propyl IC, the main difference between these two and the other two compounds in EAG response may result from their relative volatility (as reflected by their boiling points), since release rates from disks used in the EAG will depend on volatility. Extensive EAG testing of diamondback moths with other compounds, not reported here, leads us to conclude that the volatilities of allyl IC and *n*-propyl IC are close to the minimum boiling point that will elicit an EAG response at the maximum dose used (10 μg). Light et al. (1988) were able to obtain EAG responses to more volatile compounds such as ethanol. However, they used a larger filter paper disk for odor release and 10 times more compound, an amount identical to that used by Averill et al. (1988). In other studies of EAG responses to plant volatiles of which we are aware, methodology has been similar to ours (e.g., Ma and Visser, 1978), and volatiles tested with

EAG have generally been of molecular weight ≥ 99 (the molecular weight of allyl IC) (Metcalf, 1987). This inherent problem of sensitivity of the EAG to the combination of dose and volatility also indicates that some potentially active compounds will be missed when screening GC fractions with an EAG test.

Differences in volatility likely explain why allyl IC was not repellent at the highest dose tested in the Y-tube as were the other two compounds. Higher doses of allyl IC are likely to be repellent, as suggested when the allyl IC-dominated plant fractions were tested in the Y-tube. In field trapping, where volatility was not an issue because release rates were controlled, no important differences between compounds were noted. However, all compounds were weakly attractive at best. Even at a very high release rate, allyl IC attracted only twice as many moths as did the blank traps. This may indicate a short distance of response but odor competition undoubtedly affected these traps, which were on the edge of a canola field in late summer. Further attempts to field test other compounds including other isothiocyanates and terpenes proved fruitless due to low diamondback moth densities.

Isothiocyanates are probably the prime olfactory attractants to large patches of damaged plants. We have previously shown that *B. juncea* releases huge quantities of allyl IC over short time periods when damaged in a manner that simulates insect feeding (Pivnick and Jarvis, 1991). Pivnick et al. (1992) found that several isothiocyanates, including allyl IC and 3-methylthiopropyl IC, attract the flea beetles *Phyllotreta cruciferae* (Goeze) and *P. striolata* (F.), with allyl IC being the most attractive compound of those tested. In tests of volatiles of several homogenized crucifer species, the cabbage root fly, *Delia brassicae* (Wiedemann) is also attracted strongly to allyl IC (Wallbank and Wheatley, 1979). Allyl IC and *n*-propyl IC have also been shown to attract the northern false chinch bug, *Nysius niger* Baker, to field traps (Pivnick et al., 1991). However, in this case a synthetic mustard oil, ethyl-4-isothiocyanatobutyrate, proved to be more attractive than allyl IC. Based on these findings it would be easy to overestimate the role of allyl IC in attracting crucifer-feeding insects to their host plants. We have estimated that a group of 27,000 intact *B. juncea* at the flower bud stage would be required to produce enough allyl IC to attract nearby flea beetles or northern false chinch bugs (Pivnick and Jarvis, 1991). Thus, attraction of insects to isolated plants or small patches of plants in natural ecosystems almost certainly involves stimuli other than isothiocyanates alone, or is only effective from a few centimeters distant. For this reason, we investigated the response of diamondback moths to volatiles collected from intact host plants, where isothiocyanate concentration is much lower.

Tollsten and Bergstrom (1988) demonstrated that homogenized leaves of *Brassica* spp. release primarily five- and six-carbon alcohols, aldehydes, and acetates (the "green leaf volatiles" of Visser and Avé, 1978), as well as glucosinolate breakdown products (isothiocyanates, nitriles, and sulfides). On the

other hand, intact *Brassica* spp. release a diverse group of terpenes and some benzenoids, primarily benzaldehyde and phenylacetaldehyde, with very small amounts of the volatiles typical of homogenized leaves (Tollsten and Bergstrom, 1988). In light of this, our results indicate that diamondback moths are attracted to isothiocyanates when plants are damaged. While they may detect many other volatiles released from damaged plants, the quantities are less substantial and probably less important in attraction.

Tollsten and Bergstrom (1988) suggest that different plant stages may differ widely in the composition of volatiles released. The majority of the compounds present, however, should be the same. In our experiments, the decision to collect intact volatiles from seedlings rather than flower bud-stage plants, as we had done for homogenized plant volatiles, was based on the difficulty involved in collecting volatiles from large plants as compared to seedlings. We also felt that the plant stage difference would likely be dwarfed by the difference between intact and macerated plants.

Our EAG results indicate that the moths appear to be particularly sensitive to some combinations of plant volatiles, probably including terpenes, released from intact plants. These are the likely candidates for plant volatiles used as primary cues in host-planting finding when no damage is present. The presence of small amounts of isothiocyanates may increase responsiveness.

Our attempts to identify a primary attractant in intact crucifer volatiles have been unsuccessful to this point. It is interesting to note that one of the compounds identified in fraction 6, estragole, strongly attracts the western corn rootworm, *Diabrotica virgifera* Leconte (Lampman et al., 1987). It may be that the diamondback moth will not respond to a single compound in isolation (other than isothiocyanates), but rather responds to a mixture of odors more representative of the whole plant. This is suggested by the observation that when we subdivided fraction 6, the sub- and subsubfractions became less stimulatory in the EAG and correspondingly less attractive in the Y-tube bioassay. If there are two groups of primary cues dependent on damage level (e.g., terpenes or "green leaf volatiles" from undamaged plants and isothiocyanates from damaged plants), the level of damage necessary for a shift from predominance of one group to the other and the time course of the shift is not known.

In summary, diamondback moths detect and respond to a wide variety of compounds released by both intact and damaged plants. However, their high sensitivity to an as yet uncharacterized mixture, possibly dominated by terpenes in the volatiles of intact *Brassica* spp., would suggest that certain compounds from intact plants are of importance in host-plant finding. The strong response of diamondback moths to high levels of isothiocyanates typical of damaged plants indicates that these are likely also used in host-plant finding in certain situations. As well, low levels of isothiocyanates may strengthen response to mixtures of other plant volatiles.

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REDUCED OFFSPRING PRODUCTION IN BARK BEETLE *Tomicus piniperda* IN PINE BOLTS BAITED WITH ETHANOL AND α-PINENE, WHICH ATTRACT ANTAGONISTIC INSECTS

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Abstract—Bolts of Scots pine, *Pinus sylvestris* L., attacked by the bark beetle *Tomicus piniperda* (L.) were baited with ethanol and α-pinene to attract antagonistic insects and thereby enhance their detrimental effects on the production of bark beetle progeny. Unbaited and caged bolts were included in the experiments as controls. Attraction of beetles to the bolts and subsequent emergence were estimated using traps. Six phloem-feeding species (potential competitors of *T. piniperda*) and four predatory species were caught in significantly higher numbers at the baited bolts than at the unbaited ones. The number of offspring and the productivity of *T. piniperda* were four to seven times higher in unbaited bolts than in baited bolts. Exclusion of other insects, by using cages, resulted in a nine-fold increase in the number of *T. piniperda* offspring per square meter and productivity (offspring per egg gallery) compared with unbaited, exposed bolts. *Hylurgops palliatus* (Gyll.) (Scolytidae) and *Rhagium inquisitor* (L.) (Cerambycidae) attacked both the baited and unbaited bolts, whereas *Acanthocinus aedilis* (L.) (Cerambycidae) and *Pytho depressus* (L.) (Pythidae) reproduced almost exclusively in the baited ones. Large numbers of larvae of *Thanasimus* (Cleridae) and *Rhizophagus* (Rhizophagidae) emerged from both the baited and unbaited bolts. Adults of *Plegaderus vulneratus* (Panzer) and *Cylister linearis* (Er.) (Histeridae) emerged almost exclusively from the baited bolts. The low progeny production of *T. piniperda* in the baited bolts was attributed largely to the influence of adults of *Rhizophagus* and *Epuraea* (Nitidulidae), and larvae of *Thanasimus* and *A. aedilis*.

Key Words—*Tomicus piniperda*, *Thanasimus formicarius*, *Rhizophagus depressus*, *Rhizophagus ferrugineus*, *Epuraea* spp., *Acanthocinus aedilis*,

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predators, competitors, attractants, α-pinene, ethanol, Scolytidae, Cleridae, Rhizophagidae, Cerambycidae, Coleoptera.

INTRODUCTION

Many bark beetle species use olfactory orientation when searching for suitable breeding material. They can be attracted by volatiles released from host trees or by pheromones or both (e.g., Borden, 1982). Entomophagous insects also use plant volatiles and prey insect pheromones as stimuli in long-range attraction (Vet and Dicke, 1992). Many natural enemies of bark beetles are attracted by host tree volatiles and bark beetle pheromones (Bakke and Kvamme, 1981; Borden, 1982; Chénier and Philogène, 1989; Schroeder and Lindelöw, 1989).

In recent years, tactics to decrease the progeny production of tree-killing bark beetles by manipulating entomophagous or competitive insects with pheromones and host tree volatiles have been tested. These tactics include induction of attack by a secondary bark beetle species, which then competitively excludes a tree-killing bark beetle species, or augmentation of entomophagous insects on bark beetle-attacked trees. Kohnle (1985) tried to induce attacks of secondary bark beetles in windthrown Norway spruce, *Picea abies* L. (Karst.), with pheromones and ethanol in order to decrease the reproductive success of the bark beetles *Ips typographus* (L.) and *Pityogenes chalcographus* L. The experiment did not succeed since no attacks of the secondary species were induced. In contrast, pheromone-induced attacks by the bark beetle *Ips pini* (Say) on lodgepole pines, *Pinus contorta* var. *latifolia* Engelm., newly attacked by the tree-killing bark beetle *Dendroctonus ponderosae* Hopk., resulted in a 72.5% reduction in *D. ponderosae* progeny production compared with that in *D. ponderosae*-attacked control trees (Rankin and Borden, 1991). Chatelain and Schenk (1984) tried to enhance the adverse effect of entomophagous insects on the bark beetle *D. ponderosae* by baiting bark beetle-infested trees with the pheromone frontalin. Although the clerid *Thanasimus undulatus* (Say) showed a significant response to frontalin, no significant reduction in the progeny production of *D. ponderosae* was achieved. Nor did the overall insect community beneath the bark vary substantially between baited and unbaited trees.

Degradation processes in dead or dying tree tissue result in the formation of ethanol, whereas this substance is absent or present in only small amounts in healthy trees (Moeck, 1970; Cade et al., 1970; Ikeda et al., 1980; Sjödin et al., 1989). α-Pinene is one of the dominant monoterpenes in Scots pine. Large quantities of α-pinene are released from resin exuding from newly damaged parts of conifers (Ikeda et al., 1980; Strömvall and Pettersson, 1991). However, as a result of resin crystallization and a decrease in resin flow, the release rate of α-pinene decreases substantially within a few days. Thus, by increasing the release rates of host-tree volatiles to levels above those occurring naturally and

by maintaining elevated release rates throughout the developmental period of the bark beetle brood, the long-range attraction of associated insects should be facilitated.

T. piniperda is one of the economically important forest insects of Scots pine in northern Europe. At high population levels, beetles feeding in shoots of healthy trees can result in considerable growth reduction (Långström and Hellqvist, 1990). The flight period of *T. piniperda* occurs in early spring. The beetles reproduce in logs, stumps, and pines that are windbroken, windthrown, or otherwise weakened. *T. piniperda* lacks an aggregation pheromone (Perttunen et al., 1970; Byers et al., 1985; Lanne et al., 1987). The beetles are strongly attracted by the Scots pine monoterpenes α-pinene, terpinolene, and 3-carene, and weakly attracted by ethanol (Byers et al., 1985; Klimetzek et al., 1986; Schroeder, 1988; Schroeder and Lindelöw, 1989). The predators *Thanasimus formicarius* (L.) (Cleridae), *Rhizophagus depressus* (F.), *R. ferrugineus* (Payk.) (Rhizophagidae), and *Epuraea* spp. (Nitidulidae) as well as the potential competitor *Hylurgops palliatus* (Gyll.) (Scolytidae) are attracted by α-pinene and ethanol (Schroeder, 1988; Schroeder and Lindelöw, 1989). No experimental studies of the influence of predation on *T. piniperda* offspring production have been conducted.

The objective of this study was to find out if it is possible to reduce *T. piniperda* progeny production by baiting pine stems with attractants for natural enemies and competitors.

METHODS AND MATERIALS

Experiments were conducted in a 40-year-old Scots pine stand near Uppsala in central Sweden in 1992. The stand covered 20 ha and had been thinned during late autumn of 1990. In 1991 *T. piniperda* (and other insects) reproduced in the stumps and slash.

Four to six bolts (length 45–50 cm, diam. 13–17 cm) were cut from the lower part of each of 10 Scots pines in the spring of 1992. The bolts were baited with α-pinene to further increase the numbers of *T. piniperda* attracted to the bolts and the number initiating egg galleries (Byers et al., 1985; Schroeder and Eidmann, 1987). The main flight of *T. piniperda* occurred in April 26, and all bolts were heavily attacked. On April 29, the α-pinene dispensers were removed. On May 1, experiments 1 and 2 were started. On May 5, the ends of all bolts were sealed with paraffin and experiment 3 was started. A summary of the experiments conducted is presented in Figure 1.

Experiment 1. Attraction of Beetles to Unbaited and Baited Bolts during Developmental Period of T. piniperda *Brood (Figure 2A).* Insects were caught in a modified version of the flight barrier trap described by Chapman and King-

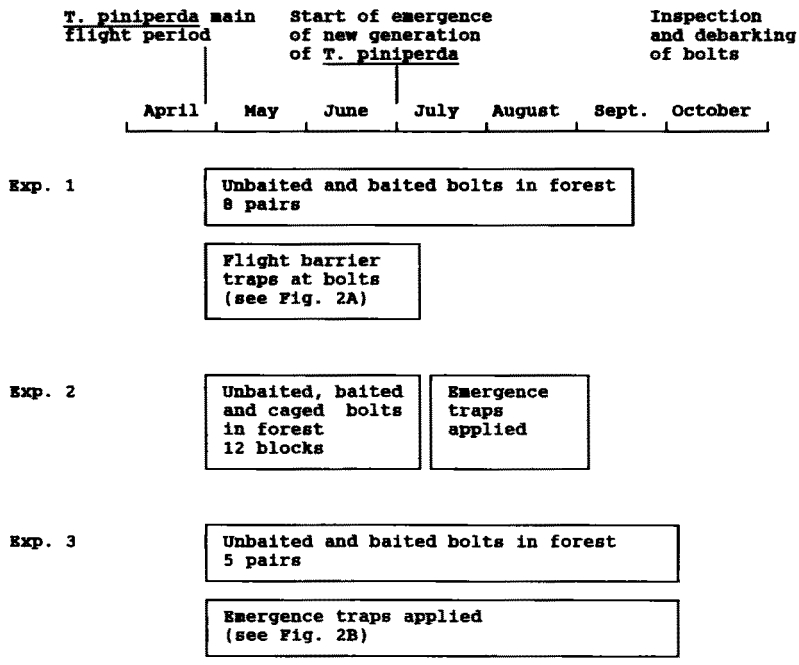

FIG. 1. Summary of experiments. The bolts in each pair or block originated from the same tree and were placed 25–50 m apart. The minimum distance between pairs or blocks was 50 m.

horn (1955). The barrier consisted of a 15-cm-high, transparent plastic sheet held over a water-filled trough. The traps were emptied at least every second week.

Experiment 2. Emergence of Insect Larvae and Adults from Unbaited, Baited, or Caged (Unbaited, 0.5-mm Screening) Bolts during Developmental Period of T. piniperda *Brood.* The Experimental setup was as in experiment 1 but with no flight barrier traps. On July 2, just after the new generation of *T. piniperda* started to emerge, each bolt was hung in the shade in an emergence trap consisting of a white cotton bag (diam. 40 cm, length 110 cm) with a collecting funnel forming the bottom. The traps were emptied once or twice weekly.

Experiment 3. Emergence of Beetle Larvae from Unbaited and Baited Bolts (Figure 2B). This experiment was conducted to estimate the proportion of larvae that had left the bolts by July 2, when the emergence traps of experiment 2 were applied. Only larvae of *Thanasimus*, *Rhizophagus*, and *Epuraea* emerged in considerable numbers before July 2. Thus, only data for these species were

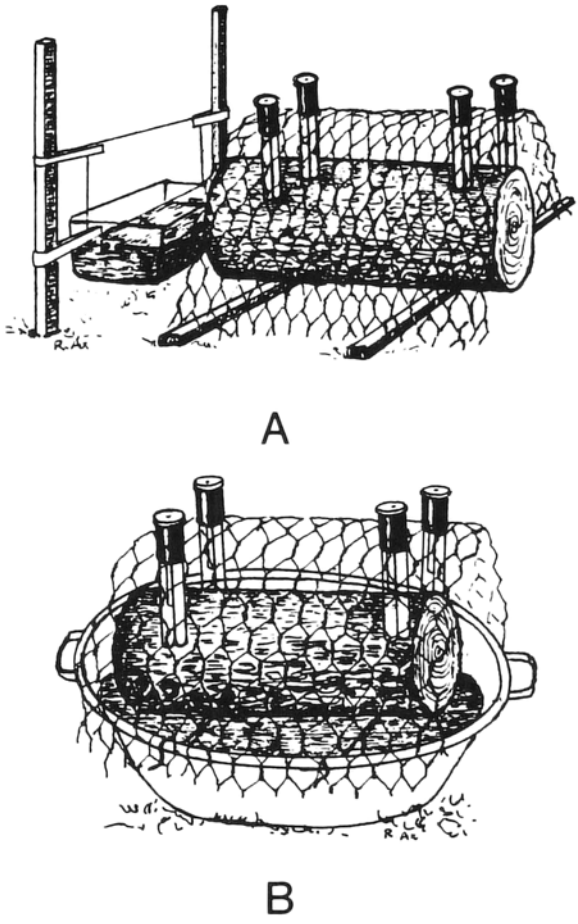

FIG. 2. Arrangement of experiments 1 (A) and 3 (B). All bolts were covered with 5-cm-mesh chicken net to protect them from woodpeckers. The four dispensers on each bolt were fitted into the screening.

analyzed. The bolts were placed above water-filled tubs, which acted as traps and which were emptied at least every second week.

In the autumn, *T. piniperda*, *H. palliatus*, and *Acanthocinus aedilis* (L.) emergence holes were counted on the bolts from all three experiments. The bark was peeled off and the numbers of bark beetle egg galleries as well as larger living insects were recorded. The length of each *T. piniperda* egg gallery was measured.

The new-generation adults of *T. piniperda*, collected in the emergence traps of experiment 2, were easily recognized by their light-brown color. Of 574 sampled specimens of *Epuraea* spp. caught in experiment 1, 528 (92%) were *E. marseuli* (Reitt.). In a previous study, 95% of the *Epuraea* caught in traps baited with ethanol and α-pinene were *E. marseuli* (*E. bickhardti*) (Schroeder, 1993). Adult Staphylinidae and larval *Thanasimus* were not determined to species. *Thanasimus formicarius* and *T. femoralis* (Zett.) occur in Sweden. Since only *T. formicarius* were caught in the flight barrier traps, it is probable that most of the larvae were of this species. In experiment 3, the length of *Thanasimus* larvae was recorded. The larvae of *Rhizophagus* were identified as *R. depressus* and *R. ferrugineus*.

On May 12 and 19, adult *T. formicarius*, *A. aedilis*, *Rhagium inquisitor* (L.), and *Pytho depressus* (L.) on the experimental bolts were counted. Each bolt was observed for 5 min.

The chemicals used were (−)-α-pinene (Fluka 97%, $[\alpha]_D^{20}$ −42 ± 3°) and 95% ethanol (5% water). The dispensers (four per bolt) (Figure 2) consisted of three test tubes, two of which (depth 150 mm, inner diam. 14 mm) were filled with ethanol and one (depth 100 mm, inner diam. 9.5 mm) with α-pinene. Each tube was supplied with a strip of filter paper (Munktell No. 3; ethanol: 145 × 13.5 mm, α-pinene: 96 × 9 mm) reaching from the bottom to about 1 mm from the rim. A plastic 35-mm film canister was fitted over the open ends of the tubes. All unbaited bolts (except caged ones) were supplied with dispensers without chemicals. During warm weather, the ethanol dispensers were refilled at least once a week. The α-pinene dispensers were refilled approximately once every month.

The dispensers were designed to release the two substances in higher than natural amounts known to strongly attract some beetle species associated with *T. piniperda* (Ikeda et al., 1980; Byers et al., 1985; Schroeder, 1988; Schroeder and Lindelöw, 1989). Field release rates were estimated by measuring the amounts of chemicals left before refilling (Table 1). The initial liquid surface levels differed among the three measurement periods and may have influenced the release rates. More than 50 mg of α-pinene and more than 2000 mg of ethanol were released per day per bolt.

Thermistors were inserted into bored holes under the bark of one caged and one uncaged bolt. Temperature readings were taken every hour from May 13 to June 30. On each bolt two thermistors were placed on the upper side and one on the underside. One of the upper thermistors on the uncaged bolt only worked occasionally and was excluded.

Data from experiments 1 and 3 were analyzed by two-tailed paired-sample *t* test and data from experiment 2 by two-way analysis of variance followed by the Tukey multiple comparison test (Zar, 1984). To increase the homogeneity

TABLE 1. AMOUNTS OF α-PINENE AND ETHANOL RELEASED PER DISPENSER DURING FIELD EXPERIMENTS (15 REPLICATES)

Time period	Amount released/h (mg, $\bar{X} \pm$ SE)		Mean air temp. during period (°C)
	α-Pinene	Ethanol	
May 12–19 (7 days)	4.6 ± 0.2	80.1 ± 1.7	12.9
June 3–11 (8 days)	2.0 ± 0.1		16.9
June 3 (4 hr)		322.0 ± 20.0	21.3

of variances, data were $\log(x + 1)$-transformed. It was assumed that the log-transformed values came from normally distributed populations.

RESULTS

The application in experiment 1 of dispensers releasing ethanol and α-pinene on pine bolts attacked by *T. piniperda* resulted in increased catches of at least 19 species of beetles in the barrier traps (Table 2). The difference between numbers of beetles caught at bolts with and without dispensers was statistically significant ($P < 0.05$) for six phloem-feeding species, four predatory species, and one species with unclear feeding habits.

Higher numbers of associated insects were present on the baited bolts than on the unbaited bolts. On May 12, 86 *T. formicarius* and 10 *A. aedilis* were counted on the baited bolts, whereas only 15 *T. formicarius* and no *A. aedilis* were found on the unbaited bolts. On May 19, 14 *T. formicarius*, 2 *A. aedilis*, 10 *R. inquisitor*, and 8 *P. depressus* were observed on the baited bolts, whereas only 1 *T. formicarius* and 1 *R. inquisitor* were observed on the unbaited bolts.

The number of offspring per square meter and productivity (offspring per egg gallery) of *T. piniperda* was four to seven times higher in unbaited than in baited bolts in the three experiments (Table 3). In experiment 2, exclusion of other insects resulted in a ninefold increase, compared with unbaited exposed bolts, in number of *T. piniperda* offspring and productivity.

The breeding density of *T. piniperda* was about 10% lower in baited bolts than in unbaited ones in experiments 1 and 3, and about 20% lower in the baited treatment than in the unbaited treatment in experiment 2 (Table 3). The *T. piniperda* egg galleries were about 6 mm longer in the uncaged unbaited bolts than in the baited bolts in all three experiments (Table 3). Mean gallery length

TABLE 2. BEETLES CAUGHT IN FLIGHT BARRIER TRAPS AT BAITED AND UNBAITED SCOTS PINE BOLTS ATTACKED BY *T. piniperda* IN EXPERIMENT 1

Species	Catch ($\bar{X}$ ± SE)[a]		Feeding habit[b]	Occurrence (+) of reproduction in bolt
	Baited	Unbaited		
Scolytidae				
Tomicus piniperda (L.)	15 ± 4	5 ± 1	phloem-feeding L	+
Hylurgops palliatus (Gyll.)	58 ± 8*	26 ± 7	phloem-feeding L	+
Dryocoetes autographus (L.)	26 ± 7*	2 ± 1	phloem-feeding L	
Hylastes brunneus Er.	8 ± 1*	2 ± 1	phloem-feeding L	
Hylastes cunicularius Er.	2 ± 1	0.3 ± 0.2	phloem-feeding L	
Hylastes opacus Er.	11 ± 2	3 ± 1	phloem-feeding L	
Trypodendron lineatum (Oliv.)	74 ± 42	1 ± 1	mycetophageous L	+
Curculionidae				
Hylobius abietis (L.)	17 ± 4*	3 ± 1	phloem-feeding L	
Cerambycidae				
Rhagium inquisitor (L.)	8 ± 1*	0.4 ± 0.2	phloem-feeding L	+
Acanthocinus aedilis (L.)	0.8 ± 0.4	0	phloem-feeding L	+
Cleridae				
Thanasimus formicarius (L.)	27 ± 6*	5 ± 1	predator A, L	+
Rhizophagidae				
Rhizophagus depressus (F.)	47 ± 5*	6 ± 2	predator A, L	+
Rhizophagus ferrugineus (Payk.)	30 ± 6*	1 ± 1	predator A, L	+
Nitidulidae				
Glischrochilus quadripunctatus (L.)	27 ± 5*	2 ± 1	unclear	
Pityophagus ferrugineus (F.)	6 ± 1*	0.3 ± 0.2	predator A, L	
Epuraea spp. (dominantly *E. marseuli* (Reitt.)	654 ± 79*	63 ± 23	unclear	+
Pythidae				
Pytho depressus (L.)	7 ± 2*	0	phloem-feeding L	+
Histeridae				
Plegaderus vulneratus (Panzer)	1 ± 0.4	0	predator A, L	+
Cylister linearis (Er.)	0.5 ± 0.3	0.1 ± 0.1	predator L	
Staphylinidae spp.	80 ± 7	17 ± 4	unclear	+

[a] Within each row, a significant difference between paired means is indicated with an asterisk, two-tailed paired-sample t test of log $(x + 1)$-transformed values, $P < 0.05$.
[b] Feeding habits obtained from literature cited; L = larva, A = adult.

in caged bolts was 7 mm longer than that in uncaged unbaited bolts. Corresponding differences in egg gallery length were statistically significant in experiments 2 and 3.

Larvae of *Thanasimus*, *Rhizophagus*, and *Epuraea* started to emerge from the bolts of experiment 3 during the first half of June. By July 3, 66% of the

TABLE 3. OFFSPRING PER SQUARE METER, BREEDING DENSITY, PRODUCTIVITY, AND MEAN LENGTH OF EGG GALLERIES OF *T. piniperda* IN BAITED, UNBAITED, AND CAGED SCOTS PINE BOLTS

	Uncaged ($\bar{X}$ ± SE)[a]		Caged ($\bar{X}$ ± SE)[a]
	Baited	Unbaited	Unbaited
Experiment 1			
Offspring/m^2	55 ± 8*	365 ± 100	
Egg galleries/m^2	191 ± 19	213 ± 17	
Offspring/egg gallery	0.28 ± 0.03*	1.88 ± 0.57	
Egg gallery length (mm)	45.4 ± 1.8	51.8 ± 2.3	
Experiment 2			
Offspring/m^2	63 ± 17*	247 ± 71	2491 ± 316*
Egg galleries/m^2	281 ± 27*	362 ± 34	347 ± 29
Offspring/egg gallery	0.24 ± 0.05*	0.86 ± 0.31	7.64 ± 1.03*
Egg gallery length (mm)	43.9 ± 1.4*	49.9 ± 1.6	56.9 ± 1.9*
Experiment 3			
Offspring/m^2	86 ± 16*	476 ± 97	
Egg galleries/m^2	255 ± 31	281 ± 43	
Offspring/egg gallery	0.46 ± 0.11*	1.72 ± 0.28	
Egg gallery length (mm)	47.1 ± 1.6*	52.9 ± 1.7	

[a] Within each row, means that are significantly different from values for uncaged, unbaited bolts are indicated with asterisks, two-tailed paired-sample *t* test (experiments 1 and 3), and two-way analysis of variance followed by Tukey test (experiment 2), $P < 0.05$. All values log $(x + 1)$-transformed.

Thanasimus larvae and 94% of the *Rhizophagus* larvae had emerged from the baited bolts, whereas corresponding values for unbaited bolts were 30% and 86%. There was no significant difference in the numbers of emerging larvae for any of the three species between baited and unbaited bolts in experiment 3 (Figure 3). Of the *Thanasimus* larvae that emerged from the unbaited bolts, 82% were more than 10 mm long, whereas the corresponding value for baited bolts was 34%.

In experiment 2, numbers of larvae of *Thanasimus*, *Rhizophagus*, and *Phloeonomus* caught in emergence traps were higher for unbaited than baited bolts, but, as demonstrated in experiment 3, the results for *Thanasimus*, *Rhizophagus*, and *Epuraea* are misleading because many larvae had apparently already left the bolts by the time they were placed in the emergence traps in experiment 2 and the course of larval emergence differed between the baited and unbaited treatments. Adults of *A. aedilis*, *Plegaderus vulneratus* (Panzer), and *Cylister linearis* (Er.) emerged almost exclusively from the baited bolts. Large numbers of *Phloeonomus* and low numbers of other staphylinids were

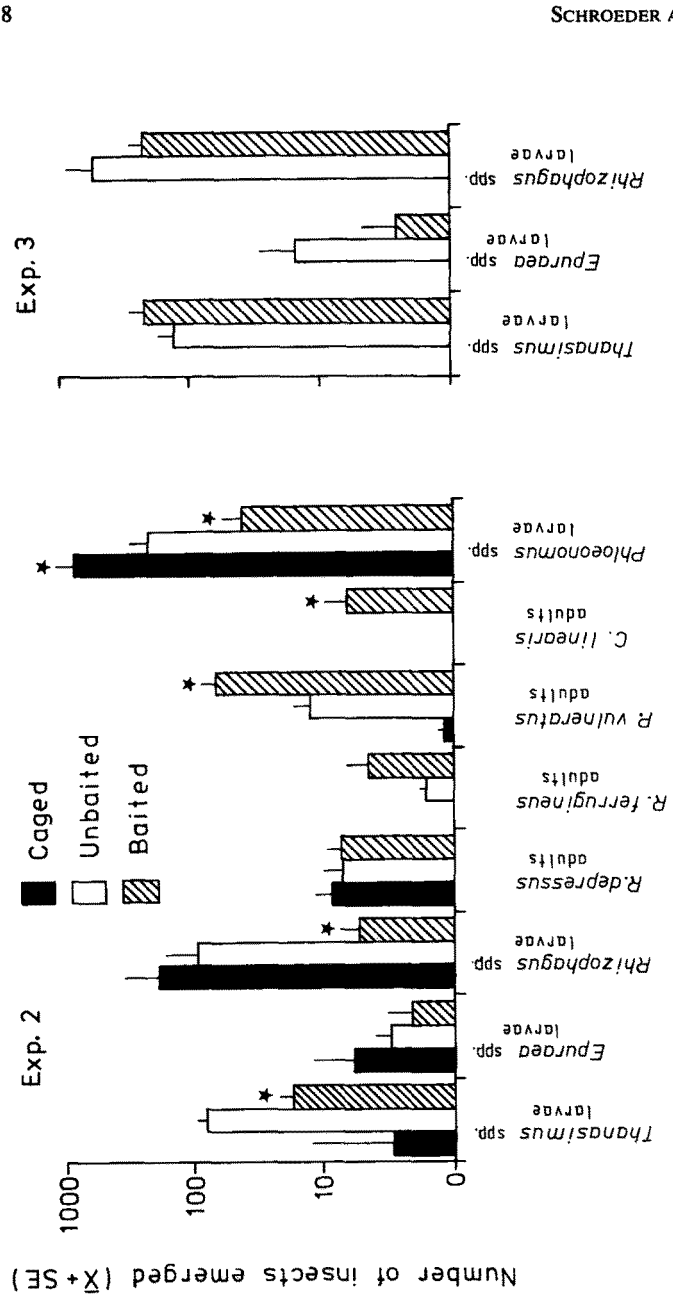

FIG. 3. Insects emerging per square meter from baited, unbaited, or caged Scots pine bolts attacked by *T. piniperda*. Within species, means significantly different from that of unbaited bolts are signified with asterisk, two-tailed paired-sample *t* test (experiment 3) and two-way analysis of variance followed by Tukey test (experiment 2), $P < 0.05$. All values log $(x + 1)$-transformed.

caught in the emergence traps. Five parasitic wasps emerged from the baited bolts, whereas 45 emerged from the unbaited ones. Larvae of *Rhizophagus* and *Phloeonomus* emerged from most of the caged bolts, and larvae of *Thanasimus* and *Epuraea* emerged from about half of the caged bolts.

Autumn inspection of the debarked bolts in all three experiments revealed egg galleries of *H. palliatus* and *Trypodendron lineatum* (Oliv.), in addition to those of *T. piniperda* (Figure 4). Of the 25 baited and 25 unbaited bolts, respectively, 11 and 11 were attacked by *H. palliatus* and five and one by *T. lineatum*. Four of the caged bolts were attacked by *H. palliatus*. Only a few emergence holes of *H. palliatus* were found, indicating that most of the collected adults of this species had colonized the bolts as adults. *R. inquisitor* larvae were present in baited and unbaited bolts in about the same numbers, while larvae of *P. depressus* were present in all 25 baited bolts but in only one of the unbaited bolts. Emergence holes of *A. aedilis* were present almost exclusively on baited bolts. A few larvae of *Plegaderus* were present in the baited bolts. Diptera larvae were found in low numbers. Larvae of *Xylophagus* spp. (Xylophagidae) and Stratiomyidae were found in both baited and unbaited bolts, while larvae of *Medetera* spp. (Dolichpodidae) were found mainly in the baited bolts. No

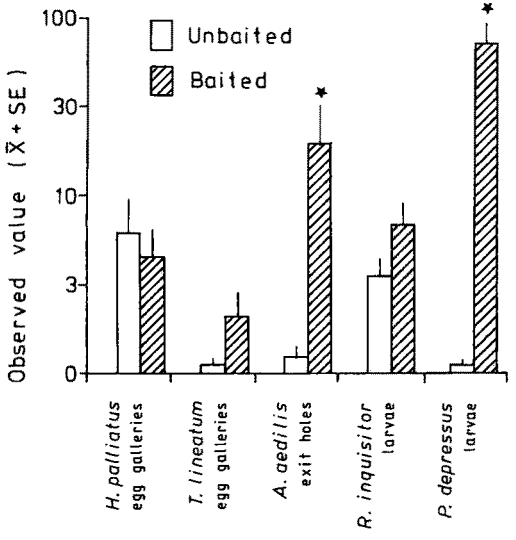

FIG. 4. Insects, egg galleries, and emergence holes found in baited and unbaited bolts in the autumn. For paired bars, values that are significantly different between baited and unbaited bolts are signified with an asterisk, two-tailed paired-sample t test of log $(x + 1)$-transformed values, $P < 0.05$.

exact counts of these genera were made. A few larvae of *Rhaphidia* (Rhaphidioptera) were found in the baited and unbaited bolts.

The subcortical temperature sums were slightly lower in the caged than in the uncaged bolt. The number of degree days above the 5°C threshold for *T. piniperda* development (Salonen, 1973; Saarenmaa, 1985) was 619 (upper side) and 554 (underside) in the uncaged bolt and 602 and 591 (upper side) and 540 (underside) in the caged bolt.

DISCUSSION

Productivity of *T. piniperda* was reduced by 89% in the exposed, unbaited bolts and by 97% in the exposed, baited bolts (Table 3). In exclusion studies of *Ips* and *Dendroctonus* spp., the difference between exclusion and exposure treatments ranged from 80 to 89% (Linit and Stephen, 1983; Miller, 1986; Weslien, 1992; Weslien and Regnander, 1992). The difference in our study might have been even greater had all insect species besides *T. piniperda* been excluded from the caged bolts (Figure 3).

The enhanced attraction of several beetle species to the baited bolts as demonstrated by trap catches and the visual inspection was evidently a result of the high release rates of α-pinene and ethanol from the baited bolts. All species caught in significantly higher numbers at the baited bolts, except *R. inquisitor*, *P. ferrugineus*, and *P. depressus*, have earlier been demonstrated to be attracted by α-pinene and ethanol (Tilles et al., 1986; Schroeder and Lindelöw, 1989; Lindelöw et al., 1993).

T. piniperda productivity was decreased four- to sevenfold in baited bolts, apparently due to the detrimental effect of other insect species. Previous studies indicate that the larva of *T. formicarius* is one of the most important predators on broods of *T. piniperda* in northern Europe (Hanson, 1937; Nuorteva, 1964; Gidaszewski, 1974). In Norway spruce, *T. formicarius* can reduce the progeny production of *I. typographus* by 80% (Weslien, 1994). Adults and larvae of *R. depressus* and *R. ferrugineus* and adults of *Epuraea* spp. have also been reported to prey on eggs and young larvae of *T. piniperda* (Hanson, 1937). Adults of *E. marseuli*, the dominant species of *Epuraea* caught in the present study, fed on bark beetle eggs in laboratory experiments (Nuorteva, 1956). We found adults of these predators to be 6- to 22-fold more abundant on baited than on unbaited bolts, suggesting a corresponding difference in the numbers of eggs laid. The high numbers of adult *Rhizophagus* and *Epuraea* present on the baited bolts may also have caused considerable mortality in eggs and young larvae of *T. piniperda*.

Thanasimus and *Rhizophagus* larvae were involved in the reduction of *T. piniperda* progeny production in both the baited and the unbaited bolts, as

demonstrated by the large numbers of emerged larvae of these species (Figure 3). In experiment 3, covering the whole period of larval emergence, there was no significant difference in the numbers of emerging larvae of these species between baited and unbaited bolts (Figure 3). However, the number of emerged larvae is not necessarily a good measure of the number that have fed under the bark. The larval densities found in experiment 3 are equal to the highest reported larval densities of *Thanasimus* (Thalenhorst, 1958; Weslien and Regnander, 1992; Weslien, 1994). Thus many larvae may have succumbed because of intraspecific competition, which can cause high mortality at high larval densities of *T. formicarius* (Weslien, 1994). The fact that larvae were smaller in the baited than in the unbaited bolts indicates that intraspecific competition actually was more severe in the baited treatment and supports the hypothesis that there were more *Thanasimus* larvae feeding under the bark of baited than unbaited bolts.

Only a few of the potential competitor species caught in traps attacked the bolts. Egg galleries of *H. palliatus* and larvae of *R. inquisitor* were present in both baited and unbaited bolts, while *A. aedilis* reproduced almost exclusively in the baited bolts. The impact of *H. palliatus* was probably minimal as the number of egg galleries was low, and most attacks were unsuccessful. *R. inquisitor* has a two-year life cycle, and its larvae are still small and probably also of low impact by the time the new generation of *T. piniperda* is ready to emerge. In contrast, the new generation of *A. aedilis* may emerge as early as the autumn of the first year. Thus, large larvae of this species will be present with the *T. piniperda* brood. In an earlier cage experiment, *A. aedilis* reduced the productivity of *T. piniperda* by 84% (Nuorteva, 1962). The density of *A. aedilis* larvae was comparable to that in our experiments.

Adults of *P. vulneratus* and *C. linearis* as well as larvae of *Plegaderus* spp. and *P. depressus* were also almost exclusively confined to the baited bolts. The larvae of *P. depressus* feed on the phloem that remains after most other phloem-feeding insects have left (Andersen and Nilssen, 1978). Adults of *P. vulneratus* fed on bark beetle eggs in laboratory experiments (Nuorteva, 1956). In our study adults were caught at the baited bolts about one month after *T. piniperda* had attacked the bolts, i.e., after most of the *T. piniperda* eggs had hatched. The *Plegaderus* larvae were found at the autumn inspection of the bolts, more than two months after the emergence of the new generation of *T. piniperda*. Not much is known about the feeding habits of *C. linearis* adults, but since members of this species were low, it could not have markedly influenced *T. piniperda* reproduction.

The fact that the productivity of *T. piniperda* in the unbaited bolts was somewhat lower in experiment 2 than in experiments 1 and 3 could have been caused by the high breeding density in experiment 2 (Table 3). In *T. piniperda*, breeding density is inversely related to both egg gallery length and productivity

(Eidmann and Nuorteva, 1968; Salonen, 1973; Saarenmaa, 1983; Sauvard, 1989). The breeding densities of *T. piniperda* in the present study (Table 3) were high compared with levels generally observed in the field, where densities rarely exceed 300 galleries/m^2 (Nuorteva, 1964; Saarenmaa, 1983; Långström, 1984).

Mean egg gallery length was highest in the caged, unbaited bolts and lowest in the uncaged, baited bolts, despite the fact that breeding densities were higher in the caged and uncaged, unbaited bolts than in the uncaged, baited bolts. The shorter galleries in baited, uncaged bolts may have been a result of other insects disturbing the parent bark beetles.

Our study demonstrates that bark beetle progeny production can be strongly reduced by increasing the activity of natural enemies and competitors by means of attractants. Adults of *Rhizophagus* and *Epuraea*, together with larvae of *Thanasimus* and *A. aedilis*, appear to be the prime candidates responsible for the low progeny production of *T. piniperda* in the baited bolts. In the future, the proposed strategy of reducing bark beetle progeny production could be an important complement to already existing measures of reducing bark beetle-caused damage.

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ECDYSTEROIDS FROM *Pycnogonum litorale* (ARTHROPODA, PANTOPODA) ACT AS CHEMICAL DEFENSE AGAINST *Carcinus maenas* (CRUSTACEA, DECAPODA)

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Abstract—*Pycnogonum litorale* (Ström) is unpalatable to the common shore crab *Carcinus maenas*, a generalist predator in the pycnogonid's habitat. A feeding bioassay reveals that the crabs are deterred by ecdysteroids that occur in high levels in all developmental stages of *P. litorale*. The total ecdysteroids in the pycnogonids reach 5.9×10^{-4} M. The 20-hydroxyecdysone 22-acetate (20E22A), which is the predominant ecdysteroid in the pycnogonids, and 20-hydroxyecdysone (20E), the arthropod molting hormone, were tested for their antifeedant effect on *C. maenas*. When contained in food pellets in homogeneous concentrations, 20E and 20E22A significantly reduced food consumption at 1.25×10^{-4} and 5.0×10^{-4} molar levels, respectively. The present results demonstrate for the first time chemical defense in arthropods in a marine predator–prey relationship. Furthermore, they provide evidence that ES contained in one animal can act as feeding deterrents on another animal.

Key Words—Pycnogonids, *Pycnogonum litorale*, Pantopoda, Crustacea, *Carcinus maenas*, ecdysteroids, 20-hydroxyecdysone, 20-hydroxyecdysone 22-acetate, chemical defense, predator–prey interactions.

INTRODUCTION

Chemical feeding deterrents have been isolated from a variety of terrestrial and freshwater arthropods (see reviews in Blum, 1981; Pasteels and Grégoire, 1983; Dettner, 1987), whereas chemical defense in marine arthropods is largely uninvestigated. The present paper reports on the discovery of chemical defense in an archaic marine arthropod, *Pycnogonum litorale*.

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The pycnogonids (pantopods) are, apart from the xiphosurans, the only extant primitive marine chelicerates. Their fossil history can be traced back to the Devonian (Bergström et al., 1980). *P. litorale* is unique in that it contains the highest ecdysteroid concentrations ever found in animals. In juvenile males the total ecdysteroid content is up to 0.1% of their body dry weight (Tomaschko and Bückmann, 1993), corresponding to a 5.9×10^{-4} molar concentration in living animals. Not only are the high levels of the ecdysteroids (ES) unusual, but are their chemical structures. Apart from 20-hydroxyecdysone (20E), the following seven ES have been found in all developmental stages of *P. litorale* (for chemical characterization see Bückmann et al., 1986): 20-hydroxyecdysone 22-glycolate, the 25 *R* and 25 *S* isomers of 20,26-dihydroxyecdysone 22-acetate, 22-deoxy-20,26-dihydroxyecdysone, 20-hydroxyecdysone 22-acetate (20E22A), 22-deoxy-20-hydroxyecdysone, and ecdysone 22-glycolate. The predominant compound is 20E22A. It has been found only during in vitro incubations in *Drosophila melanogaster* (Maróy et al., 1988) and in a snail (Garcia et al., 1986). The other six ES are unique in the animal kingdom.

One function of the ES in *P. litorale*, which is common to all arthropods, is the control of molting (Bückmann and Tomaschko, 1992). This, however, does not explain the presence of ES in nonmolting stages, i.e., in embryos and adults. Regardless of sex, ES concentrations are so high in all developmental stages that additional functions have been suspected (Tomaschko and Bückmann, 1993).

An important hint at a possible allelochemical function of ES is contained in the results of Takahashi and Kittredge (1973). For seawater containing 20E in 10^{-6}–10^{-9} molar concentrations, the normal feeding response in males of the lined shore crab, *Pachygrapsus crassipes*, is suppressed. This led to the idea that pycnogonid ES may serve as protection against predation by crustaceans. The assumption that *P. litorale* possesses some kind of chemical defense seemed reasonable, as all developmental stages, except the planktic first larvae, have very limited locomotor ability. The animals are found on wave-washed shores and live ectoparasitically in exposed positions on coelenterates or attached to hard substrates (Arnaud and Bamber, 1987). Having no physical defensive weapons, they would be easy prey for potential predators.

In order to study the defensive properties of the pycnogonid ES, a feeding bioassay with the common shore crab *Carcinus maenas* has been developed. *C. maenas* is a widespread generalist predator in the northeast Atlantic littoral (Crothers, 1968), easily capable of coping with prey of the size of *P. litorale*. It reaches high abundance in the interstices of rocky shores and jetties, where it is often closely associated with *P. litorale* (K.-H. Tomaschko, personal observation).

In a first experiment, living pycnogonids were offered to *C. maenas* in order to find out whether they are eaten or rejected by the crabs. Next, the

inhibition of crab feeding by lyophilized and powderized pycnogonids was tested. Finally, the feeding deterrent properties of 20E and 20E22A were investigated by adding the ES in different amounts to standardized food pellets. 20E22A is the most abundant ES in *P. litorale*, representing between 66.2% and 85.5% of the total ES in all developmental stages (Tomaschko and Bückmann, 1993). 20E makes up only a few percent of the pycnogonid ES, yet it is of interest because it represents the active form of the molting hormone in both *C. maenas* and *P. litorale*.

METHODS AND MATERIALS

Preparation of Experimental Diet. The semiartificial basic diet for *C. maenas* consisted of water, gelatin, and, as a flavoring agent, lyophilized and ground mantle tissue of *Mytilus edulis* (20:2:1; w/w/w). Ingredients were stirred at 90°C for 5 min, in order to dissolve the gelatin and disperse the mussel powder. Pellets were prepared by pipetting 15-μl portions of the mixture into holes (2.5 mm diameter) in a Plexiglas plate (3 mm thick). Pellets congealed within a few minutes and could be easily removed.

Pycnogonid powder was obtained by lyophilizing 30 adult females and thoroughly grinding them in a mortar. The powder was mixed with the basic diet at 38°C for 5 min at concentrations of 40%, 20%, 10%, and 5% (w/w), respectively. ES-treated pellets were prepared by mixing the basic diet with 20E (Sigma) and 20E22A (Sigma), respectively. From 2×10^{-3} M stock solutions, lower concentrations were obtained by adding adequate amounts of the basic diet and mixing it for 5 min at 38°C.

Carcinus *Feeding Bioassay.* Following 24 hr of starvation, adult male and female crabs were placed individually in glass aquaria ($25 \times 15 \times 10$ cm) containing 500 ml artificial seawater, where they were randomly assigned to treatment. Control pellets (basic diet) and treated pellets were presented singly and alternately to the individual crabs. Initial responses of the crabs after pellet presentation were scored as either 100% acceptance (pellet swallowed completely), partial acceptance (part of the pellet swallowed, estimated in percent), or rejection (pellet first mouthed and then completely rejected, 0% acceptance). In the case of partial or complete rejection, the pellet was immediately removed from the aquarium in order to avoid repeated mouthing, which otherwise occurred at a rate of approximately 20%. Water was renewed after each offering of treated pellets. The number of pellets for each crab was restricted to 20 per day. Crabs ignoring a control pellet were regarded as saturated and not taken into account for that day. Significances were calculated with the Wilcoxon, Mann, and Whitney test.

Biological Material. P. litorale was reared as described previously (Bück-

mann and Tomaschko, 1992). Adult males and females of *C. maenas* had a carapace width of 30–40 mm and were collected near Wilhelmshaven, North Sea, Germany, at low tide. They were reared in a 400 liter tank, each animal isolated in a cubic container (10 × 10 × 10 cm) in artificial seawater (Tropic Marin) at a salinity of 2.7% and a constant temperature of 15°C in a 12L:12D photoperiod. Every other day the crabs were fed to saturation with mantle tissue of *Mytilus edulis*.

RESULTS

Feeding Experiments with Intact Pycnogonids. Intact, living adult pycnogonids (25 males and 25 females) were each offered to an individually caged *C. maenas*, 16 pycnogonids (nine males and seven females) were not attacked (Figure 1). In response to the presentation of the pycnogonids, the crabs intensified ventilation movements of their mouth parts for 10–60 sec. However, they did not touch the pycnogonids and eventually moved away. Twenty-nine pycnogonids (14 males and 15 females) were released unharmed after having been briefly seized with the chelipeds and palpated with the mouth parts for about 5–30 sec. Five pycnogonids (two males and three females) were released after suffering injuries from intensive mouthing for 5–30 sec by the crabs' mandibles. The cuticle of a walking leg was punctured and body fluid leaked out. However, in no case were discernible parts of the pycnogonids ingested. All pycnogonids were still alive 30 days after the experiment. It is obvious that *P. litorale* is not consumed by *C. maenas*.

Predator Deterrence of Pycnogonid Powder. This experiment was performed in order to exclude the possibility that the nonacceptance by *C. maenas*

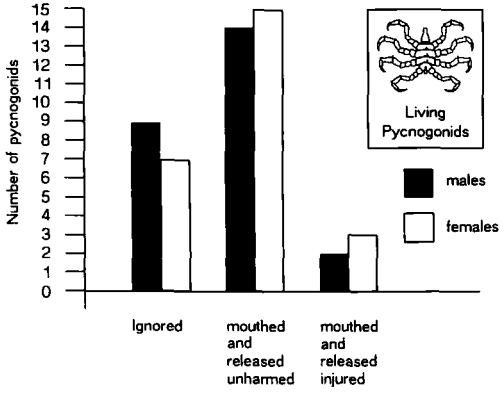

FIG. 1. Fate of 50 adult pycnogonids offered to *C. maenas*.

could be due to the behavior of living pycnogonids and/or to the physical protection by their thick cuticles. Lyophilization of 30 adult female pycnogonids reduced their weight (3.1 g) to 29% (0.9 g). Food pellets containing 40% (w/w) pycnogonid powder were completely rejected by all crabs. At the 30%, 20%, 10%, and 5% levels, the food consumption was inhibited significantly by 92.3%, 87.2%, 59.1%, and 19.3%, respectively (Figure 2). These results clearly show that the feeding deterrency for the crab is most likely due to chemical constituents in *P. litorale*.

Predator Deterrence of Pycnogonid Ecdysteroids. Both 20E and 20E22A dose-dependently reduced artificial food acceptance by *C. maenas*. There was no significant difference in food acceptance between male and female crabs. Control pellets as well as all ES-treated pellets were seized by the crabs with their chelipeds and carried to the mouth. All control pellets were ingested within 5–10 sec. ES-treated pellets were, in case of rejection, either released intact after a few seconds or lacerated and cast out by ventilatory movements of the exopodites within 10–30 sec. Even in cases of acceptance, the ecdysteroid-treated pellets were often mouthed for 30–60 sec before being partially or completely ingested.

At a 20.0 $\times$ 10^{-4} molar level, 20E completely inhibited artificial food consumption (Figure 3, for natural concentrations see Discussion). All pellets

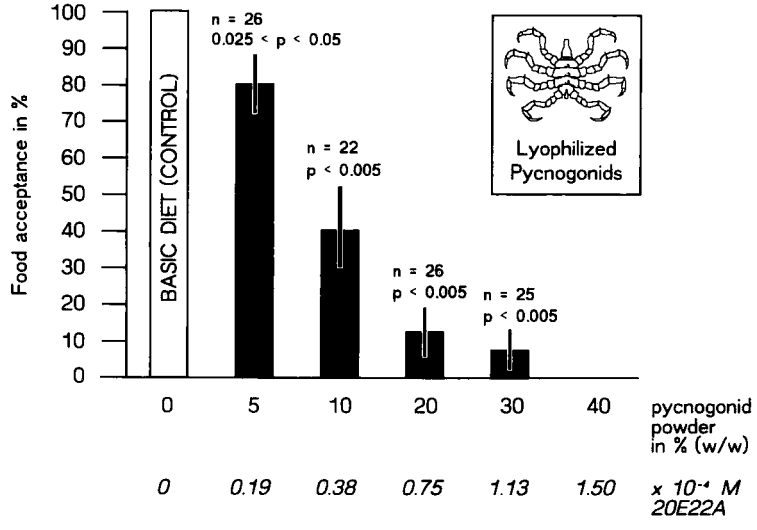

FIG. 2. The effect of lyophilized pycnogonid powder on food acceptance by *C. maenas* (mean values $\pm$ SEM) and the levels of 20E22A (calculated according to Bückmann et al., 1986) within the pellets.

were rejected after being palped with the mouth parts. 20E22A caused 95.0% inhibition at the same molar concentration as compared to the controls (Figure 4). At concentrations of 10.0×10^{-4} M and 5.0×10^{-4} M, both 20E and 20E22A reduced food acceptance significantly. However, 20E was approximately twice as effective as 20E22A (Figures 3 and 4). At molar concentrations of 2.5×10^{-4} and 1.25×10^{-4}, only 20E significantly inhibited artificial food consumption by 61.1% and 22.9%, respectively (Figure 3).

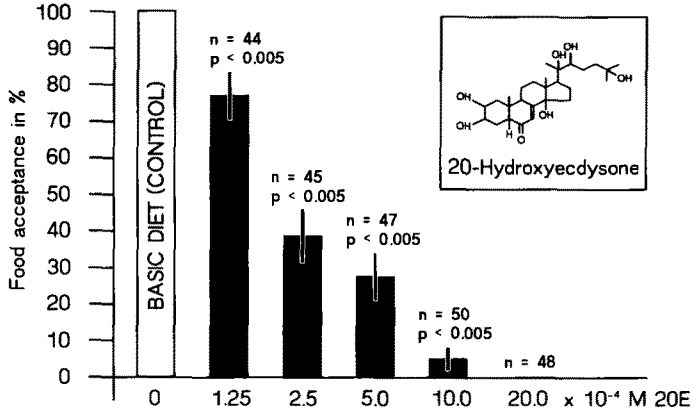

FIG. 3. The effect of homogeneous concentrations of 20-hydroxyecdysone (20E) on food acceptance by *C. maenas*. Mean values ± SEM.

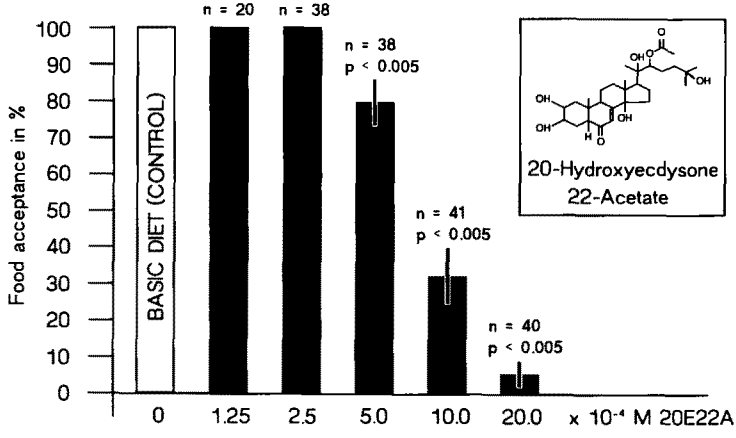

FIG. 4. The effect of homogeneous concentrations of 20-hydroxyecdysone 22-acetate (20E22A) on food acceptance by *C. maenas*. Mean values ± SEM.

DISCUSSION

Significance of Ecdysteroidal Defense in P. litorale. It is obvious that *P. litorale* is not consumed by *C. maenas* and that its unpalatability is due to chemical constituents. Furthermore, the assay with artificial food pellets demonstrates the feeding deterrent effect of 20E and 20E22A on *C. maenas*. This dose-dependent effect is significant when a critical concentration is exceeded. In food pellets with homogeneous ES concentrations, the critical levels for 20E and 20E22A are at about 1.25×10^{-4} M and 5.0×10^{-4} M, respectively (see Figures 3 and 4). Comparing the ES content with the degree of unpalatability of pycnogonid powder (Figure 2), it seems likely that, in addition to 20E and 20E22A, other compounds contribute to the pycnogonid's unpalatability. In this respect, the contribution of the other six ES that have been identified from *P. litorale* (Bückmann et al., 1986) remains to be investigated. However, these ES are present in all developmental stages in low levels only (Tomaschko and Bückmann, 1993) and thus should play only a minor role in chemical defense, unless they are much more potent feeding deterrents that 20E22A. On the other hand, a possible synergistic effect of the ES among one another or with other chemicals must be taken into account, as has been described for other defensive compounds in staphylinid beetles (Dettner, 1993). Further feeding assays, using and combining different fractions of pycnogonid powder, should show whether other factors than ES (or other ES) are involved in pycnogonid chemical defense.

The present experiments were restricted to 20E and 20E22A, due to the fact that both compounds can be purchased in large amounts. In *P. litorale*, the highest ES-levels have been found in juvenile males (Tomaschko and Bückmann, 1993), where the 20E22A reaches concentrations of 1600 nmol/g dry weight (total ES: 1965 nmol/g dry weight) corresponding to a 4.8×10^{-4} M (total ES: 5.9×10^{-4} M) concentration in living animals. When assuming homogeneous concentrations in living pycnogonids, the 20E22A level in juvenile males would suffice to significantly reduce acceptance by *C. maenas* (see 20E22A levels of food pellets in Figure 4), whereas in adult females homogeneously distributed 20E and 20E22A could not fully account for their unpalatability. However, in living pycnogonids, the ES are not distributed homogeneously, but are concentrated in the integument and can be secreted through cuticular pores (Tomaschko and Bückmann, 1990). These pores occur in high numbers (about 10,000–20,000 per adult animal) and are evenly distributed over the body surface.

Recent experiments have shown that *P. litorale* secretes high amounts of ES in response to disturbance (K.-H. Tomaschko, unpublished data). It seems likely that when a pycnogonid is mouthed, it will release locally very high ES levels in solution, which may well be above the efficient concentration for food pellets. Secreted defensive chemicals can be perceived by a predator before it

seriously injures its prey. Thus, ES secretion in response to disturbance could explain why in the present experiments most pycnogonids were released unharmed by the crabs.

Unusual ES as Feeding Deterrents. The origin of the ES in *P. litorale* is still unclear. An endogenous source seems more likely than an exogenous one (see discussion in Bückmann et al., 1986). The formation of unusual ES could be an archaic feature of primitive arthropods that has been retained by the pycnogonids. On the other hand, it could also be a special evolutionary adaptation to the function of ES as feeding deterrents. The formation of a spectrum of different unusual ES may represent a way to avoid counteradaptations by predators. Pasteels (1993) suggests that it is probably more difficult for predators to overcome a mixture of toxins with various physicochemical properties, and hence biological activities, than a single compound.

Semiochemical Functions of Arthropod Ecdysteroids. In arthropods, three major processes are under the control of ES: molting, growth, and gametogenesis (Koolman, 1990). Apart from these hormonal functions, semiochemical properties of ES have been suggested by several authors. Kittredge and Takahashi (1972) postulated a sex pheromone function of 20E in crustaceans. This suggestion, however, is not consistent with findings by Atema and Gagosian (1973), Adelung et al. (1980), and Seifert (1982). During electrophysiological recordings from chemoreceptors of spiny lobsters, Spencer and Case (1984) found that 20E elicits sensory responses at levels of 10^{-13} M with a latency of less than 1 sec. However, as this is true for adult females and males as well as for juveniles, it can not corroborate the pheromone hypothesis. Thus, the evidence for a pheromonal function of ES in crustaceans is still inconclusive.

A defensive function of steroids has been demonstrated for pregnene and pregnadiene derivatives that occur in numerous arthropods (Blum, 1981). Many of these compounds are identical to well-known vertebrate hormones and appear to function primarily as defensive agents against vertebrate predators (Gerhart, 1991). Defense by steroids of the "true" ecdysteroid type, as defined by Lafont and Horn (1989), has been suggested in a zoanthid (Sturaro et al., 1982, Guerriero and Pietra, 1985) and in tick eggs (Connat et al., 1987), but has never been substantiated. Real evidence for a defensive ecdysteroid function has been restricted to plants. Many plants produce high amounts of phytoecdysteroids (up to 3.2% of dry weight) (Bandara et al., 1989) that can act as a chemical defense against "nonadapted" phytophagous insects by disturbing the insect's larval development when ingested (Lafont et al., 1991).

The apparently rare occurrence of ecdysteroidal defense in arthropods may be due to the fact that the high ES concentrations required are potentially dangerous to the arthropod producer itself. Thereby, the question arises how *P. litorale* manages this problem, especially in developmental stages where exogenous ES can seriously disturb the normal molting cycle and lead to death

(Bückmann and Tomaschko, 1992). The hormonal activity of 20E22A in *P. litorale* is still unknown. However, its molt-inducing potential has been demonstrated in a tick species, where it induced supermolting at a 500-ppm level (Savolainen et al., 1991). *P. litorale* must have found a way to separate the ES against a concentration gradient in compartments in the integument (see above) where they cannot interfere with its basic metabolism.

Several marine arthropods contain venoms that are poisonous to humans and other terrestrial vertebrates and in vitro show toxic effects on frog and crayfish nerves (Hashimoto and Konosu, 1978). The present paper provides the first evidence for chemical defense in marine arthropods against predators in their natural environment. Chemical defense is presumably of vital significance for *P. litorale*, considering its lack of physical defensive weapons and its immutability. The use of ES as feeding deterrents is also a novel feature for arthropods. Apart from their hormonal function as molting hormones, the pycnogonid ES act exogenously as allelochemicals. They produce a nongenomic effect in both sexes of *C. maenas*, leading within seconds to a behavioral change by inhibiting food consumption. This may help the crabs to avoid ingestion of "overcritical" doses of ES that might disturb their physiological processes. The short latency of this response to ES strongly implicates interaction with a membrane-associated component. With regard to the classical scheme of steroid hormone action on the gene expression (Ashburner, 1980), it seems of interest to localize the site of exogenous ES reception in *C. maenas* and to investigate the specificity and the chemical structure of this ES receptor.

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VOLATILE SHELL-INVESTIGATION CUES OF LAND HERMIT CRABS: Effect of Shell Fit, Detection of Cues from Other Hermit Crab Species, and Cue Isolation

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Abstract—Land hermit crab responses to volatile shell-investigation cues from land hermit crabs and from marine hermit crabs are analogous to the responses of marine hermit crabs to shell-investigation cues from marine crabs and from snails. Land hermit crabs attracted to shell cues are in worse-fitting shells and are more likely to investigate conspecifics' shells than are crabs attracted to feeding cues. Moving land hermit crabs from worse shells to better shells decreases the number of crabs investigating shells, while moving crabs from better shells to worse shells increases the number of crabs investigating shells. Gravid females have better-fitting shells than nongravid females or males. Crabs from two different populations in Panama have different shell fits and show different levels of responses to shell-investigation cues. Land hermit crabs respond to volatile shell-investigation cues from both land and marine hermit crabs, but marine hermit crabs do not respond to cues from land hermit crabs. A cue detection system for volatile cues most likely evolved in land hermit crabs during their transition from a marine to a terrestrial existence. Thus, the cues found in land hermit crabs and marine hermit crabs may be chemically similar. Volatile compounds collected from hermit crabs onto Tenax columns can be eluted with ethanol and act as shell-investigation cues in field assays.

Key Words—Land hermit crabs, *Coenobita*, *Calcinus*, *Clibanarius*, Crustacea, chemical cues, shell fit, shell acquisition, behavior.

INTRODUCTION

A critical activity for hermit crabs is locating gastropod shells. Hermit crabs use shells to obtain protection from predators and desiccation (Hazlett, 1981). Shell

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type and quality can influence a hermit crab's reproductive success (Bertness, 1981; Hazlett, 1989; Hazlett and Baron, 1989). The supply of shells can limit the size of hermit crab populations (Provenzano, 1960; Childress, 1972; Vance, 1972; Bach et al., 1976; Kellogg, 1976; Spight, 1977).

Marine hermit crabs can locate shells by detecting two types of chemical cues. Gastropod predation sites provide an intact gastropod shell whose occupant has been digested by a predatory gastropod (McLean, 1974; Rittschof, 1980a). Marine hermit crabs are attracted to these sites by peptides released during the digestion process (Rittschof, 1980b; Rittschof et al., 1990; Kratt and Rittschof, 1991). Marine hermit crabs are also attracted to cues released from the hemolymph of damaged, dead, or dying conspecifics (Rittschof et al., 1992). Marine hermit crabs attracted to either gastropod cues or marine hermit crab cues actively search for shells at cue sources and vigorously investigate the shells of other hermit crabs that are attracted to cue sources (McLean, 1974; Rittschof, 1980a; Rittschof et al., 1992).

Land hermit crabs, *Coenobita* spp., are attracted to volatile chemical cues released from dead conspecifics and actively investigate shells at cue sources by touching the shells with their chelipeds and walking legs (Small and Thacker, 1994). Unlike marine hermit crabs, land hermit crabs do not respond to dead gastropod odors with shell-investigation behaviors, but rather with feeding behaviors (Rittschof and Sutherland, 1986).

In this paper, I describe experiments that characterize the nature of land hermit crab shell-investigation behavior and the cues that stimulate this behavior. I differentiate between the effects of visual and olfactory stimuli and suggest that some land hermit crabs are more attracted to olfactory feeding cues, while others are more attracted to olfactory shell cues. A physical factor that could influence shell-investigation behavior in land hermit crabs is shell fit. I report that land hermit crab attraction to shell cues and execution of shell-investigation behaviors are correlated with poor shell fits. I show that shell fit varies between sexes and that differences between populations in shell fit are correlated with differences between populations in shell-investigation behavior.

As land hermit crabs have colonized the terrestrial environment, they have retained the ability to locate shells by detecting cues from dead conspecifics. The transition from a marine existence to a terrestrial existence has required a change in this signaling system from a nonvolatile peptide cue to a volatile cue. The pattern of this change was examined by determining if two species of land hermit crabs respond to shell cues from each other or from marine hermit crabs. I also determine if marine hermit crabs respond to shell cues released from land hermit crabs. A procedure was devised for isolating land hermit crab shell-investigation cues to chemically compare them to marine hermit crab cues.

METHODS AND MATERIALS

Study Sites and Organisms

Coenobita compressus H. Milne Edwards, a land hermit crab found on the Pacific coast of the Americas, was studied at two locations. The work was conducted at the Achotines Laboratory of the Inter-American Tropical Tuna Commission (Los Santos Province, Republic of Panama) between January and April, the dry season, of 1992 and 1993. Achotines Bay is bordered by a sandy and rocky beach that is shaded by a tropical dry forest until midday. *C. compressus* are found on the beach and in the forest and are extremely abundant. These crabs are active in the morning until the beach is no longer shaded and at night, with maximum activity during falling tides at disk and dawn. The experiments were performed between 0600 and 1030 hr and between 1730 and 1930 hr.

C. compressus was also studied at the Naos Laboratory of the Smithsonian Tropical Research Institute (STRI), near Panama City, Republic of Panama, from March to May 1993. The site was Culebra Beach, a stretch of sandy beach between two rocky outcrops. *C. compressus* were less abundant at this location than at Achotines and were most active at night, as there was no daytime shading of the beach. Maximum activity occurred on falling tides near dusk. Experiments were conducted between 1800 and 2100 hr. *Calcinus obscurus* (Stimpson), a Pacific marine hermit crab that lives in rocky tidepools, is found at both Achotines and Naos and was studied at each location between 2 hr before and 2 hr after daytime low tides.

Bombacopsis sessilis (Bentham, spiny cedar) flowers are a common food item for *C. compressus* at Achotines. These flowers fall onto the beach in the mornings during the dry season and attract large numbers of feeding crabs. A brachyuran ghost crab, *Ocypode gaudichaudii* Milne Edwards and Lucas, is also found at Achotines and Naos and was used as an odor source at each location. The shells of *Nerita scabricosta* Lamarck, an abundant gastropod on rocky substrates at each site, were the most common shells used by *C. compressus* and were also used by *C. obscurus*. *N. scabricosta* was used as an odor source at each location.

In April 1993, I studied *Coenobita clypeatus* (Herbst), a Caribbean land hermit crab, and *Clibanarius antillensis* Stimpson, a Caribbean marine hermit crab, at STRI's Galeta Marine Laboratory, near Colon, Republic of Panama. *C. clypeatus* was active during the day and night at low abundance on a sand and coral rubble beach. *C. antillensis* occurred in large numbers on a reef flat, intermixed with a large snail population. Both hermit crabs inhabited the shells of *Nerita fulgurans* Gmelin, a common gastropod in this area, as well as several

other species of snails. Experiments on both species were conducted between 0700 and 1700 hr.

Hemolymph from *O. gaudichaudii*, *C. clypeatus*, and *C. compressus* was obtained by inserting a 22-gauge needle on a 1-ml tuberculin syringe into the sinus located on the major chelae of the crab. Over 1 ml of hemolymph could be drawn from large individuals. Tissue extracts were made from *O. gaudichaudii*, *C. obscurus*, *C. antillensis*, *C. compressus*, and *C. clypeatus* with a ratio of 1 g tissue to 3 ml seawater, and from *N. scabricosta* and *N. fulgurans* with a ratio of 1 g tissue to 1 ml seawater. After weighing tissue into a 30-ml vial, an appropriate amount of seawater was added and the tissue was homogenized. The mixture remained at ambient temperature for 1 hr before storing it in a refrigerator at 10°C.

Attraction to Feeding Cues and Shell Cues

Land hermit crabs could be stimulated to investigate shells by visual cues after arriving at an odor source and finding an empty shell or conspecifics, or they could be stimulated to investigate shells by olfactory cues before arriving at an odor source. To differentiate between visual and olfactory shell cues at odor sources, odor sources were placed in shallow pits to prevent visual cues from affecting crabs as they approached an odor source and to prevent social facilitation by visual cues (Kurta, 1982). The presence or absence of an empty shell at the odor sources controlled for the visual stimulation of an empty shell.

At Achotines, a 30-cm-diameter, 5-cm-deep pit was made in the sand on the beach, and all land hermit crabs within 1 m were removed. An odor stimulus was then placed in the center of the pit, with or without a clean, empty *N. scabricosta* shell. The test stimuli were a blank control, *B. sessilis* flower, *O. gaudichaudii*, *N. scabricosta*, *C. obscurus*, and *C. compressus*, which were crushed with a rock to facilitate the release of odors. For 4 min after the first crab arrived in a pit, the number of crabs arriving, the number of feeding, and the number investigating the shells of conspecifics that had come to the pit were recorded. An observation period of 4 min was used because, after than time, such a large number of crabs was present at the pit that individual investigation events could not be recorded accurately. If an empty shell was present, the number of crabs investigating the empty shell and the number of shell exchanges were also recorded. No shell exchanges occurred without an empty shell present. Eight trials with each stimulus were performed in a random order and consecutive trials were conducted in pits at least 10 m apart. The percent of crabs feeding, percent investigating conspecifics, percent investigating the empty shell, and percent exchanging shells were calculated and analyzed by analysis of variance (ANOVA), transforming percent investigating conspecifics, percent investigating the empty shell, and percent exchanging shells with an arcsine

transformation to meet the assumptions of normality and homogeneity of variances (Neter et al., 1990). Pairwise comparisons were made of significant effects using the Scheffé method of multiple comparisons (Neter et al., 1990).

Behavior of Crabs Attracted to Different Stimuli

The previous experiment suggested that *C. compressus* attracted to feeding cues behave differently than crabs attracted to shell-investigation cues. To test this suggestion and to determine if shell fit was correlated with shell-investigation behavior, the behavior of crabs attracted to different odor sources, using a method similar to that of Small and Thacker (1994) was assayed. At Achotines, land hermit crabs in *N. scabricosta* shells that were attracted to a crushed conspecific (*C. compressus*) or a *B. sessilis* flower were collected. Crabs were also collected haphazardly on the beach and under driftwood. For each of these three groups, 10 crabs were individually marked on their shells with paper correction fluid and placed in a 30-cm-diameter plastic bucket. After 3 min, a crushed *C. compressus* was added, and 3 min later an empty *N. scabricosta* shell large enough for the largest crab in the bucket to use. The crabs were then observed for an additional 10 min (Small and Thacker, 1994). During each time period, the identity of crabs that investigated conspecifics or exchanged shells were recorded. Before starting and after completing this experiment, the shell fit was measured of each crab using Abrams' (1978) extension–withdrawal categories. This method ranks shell fit into 12 categories, one being a crab most withdrawn into its shell and 12 being a crab most extended from its shell; thus, a larger number indicates a worse shell fit. This experiment was repeated five times for each of the three groups of crabs. The number of crabs investigating conspecifics and exchanging shells in each group was compared using G tests. I compared the shell fits of different crab groups, of investigators and noninvestigators, and of exchangers and nonexchangers using Kilmogorov–Smirnov (KS) test (Conover, 1980). Also compared were the proportion of crabs investigating conspecifics in each shell fit category using a G test, pooling every two categories to obtain larger cell values (Sokal and Rohlf, 1981).

Effect of Shell Fit on Shell-Investigation Behavior

After finding a correlation between shell fit and attraction to feeding cues or shell cues, the probability of an individual's shell fit influencing the probability of that individual engaging in shell-investigation behaviors was determined. At Achotines, crabs in *N. scabricosta* shells were collected that had good (extension–withdrawal category <6) or bad (extension–withdrawal category >7) shell fits and, to control for possible effects of manipulation, each crab was removed from its shell and then allowed to return to its shell. The previously described procedure (see above) was then performed with 10 crabs

with good shell fits or 10 crabs with bad shell fits. Upon completion of the procedure, each crab was removed from its shell and given access to a shell that worsened (good to bad) or improved (bad to good) its shell fit. The new shell fit was measured and the procedure repeated. Knowing the identity of each individual in the two trials, the number of crabs investigating conspecifics and exchanging shells were analyzed using the McNemar test for significance of changes (Sokal and Rohlf, 1981).

Comparison of Shell Fits between Sites and Sexes

To test Abrams' (1978) report of shell fit varying between land hermit crab populations and to determine if shell fit varies between sexes, land hermit crabs in *N. scabricosta* shells were collected at Achotines and at Naos, the carapace length and shell length of each crab were measured and its sex determined. For each crab, a shell fit ratio was calculated, defined as carapace length divided by shell length (Rittschof, 1980a). Analysis of covariance (ANCOVA) (Neter et al., 1990) was performed on the shell fit ratios, using carapace length as a covariate and sex and site as grouping variables. Juveniles were analyzed separately from adults, using the smallest-sized gravid female as a size cutoff for the adult category. Juvenile sex categories were male and female, while adult sex categories were male, female, and gravid female. Pairwise comparisons were made of significant effects using the Scheffé method of multiple comparisons (Neter et al., 1990).

Response Time to Shell-Investigation Cues at Different Sites

Populations in better-fitting shells might be expected to respond less to shell-investigation cues than populations in worse-fitting shells. To test this hypothesis, I determined if differences in shell fit between populations at different sites were correlated with differences in response times to shell-investigation cues. The number of crabs attracted per minute to a 1.85-ml vial containing 250 μl of *C. compressus* hemolymph placed in the center of a 30-cm-diameter shallow pit at Achotines and Naos was calculated and data were compared using a two-tailed t test (Sokal and Rohlf, 1981).

Distribution of Shell-Investigation Cues

Shell-investigation cues released from marine hermit crabs are present in their hemolymph (Rittschof et al., 1992). The following bioassay was performed to determine if land hermit crab shell-investigation cues are present in *C. compressus* hemolymph and to determine if shell cues could be extracted from *C. obscurus*. At Achotines, 250 μl of stimulus fluid was placed in a 1.85-ml vial in the center of a 30-cm-diameter shallow pit with the lip of the vial 0.5 cm

above the sand, and placed an empty *N. scabricosta* shell 2 cm away from the vial. The stimuli tested were: seawater, a null activity control; *O. gaudichaudii* hemolymph; *N. scabricosta* extract; *C. obscurus* extract; *C. compressus* hemolymph; and *C. compressus* crushed inside a vial, a positive activity control (Table 1). The number of crabs arriving in the first 4 min after the first arrival and the number of crabs investigating conspecifics' shells were recorded. Five replicates of this procedure were performed and the data pooled before analyzing the proportion of crabs investigating conspecifics' shells using a *G* test for independence (Sokal and Rohlf, 1981). The stimuli were then separated into one group homogenous with the null activity control and one group homogeneous with the positive activity control, using a simultaneous test procedure for unplanned comparisons (Sokal and Rohlf, 1981).

Detection of Shell-Investigation Cues of and by Other Species

The shell-investigation stimuli of different species were compared by determining if cues from land and marine hermit crabs from both the Pacific and Caribbean coasts of Panama stimulated shell-investigation behaviors in land and marine hermit crabs. Additional bioassays were performed with *C. compressus* to determine if this species responds to shell-investigation cues from *N. fulgurans*, *C. antillensis*, and *C. clypeatus* (Table 1). Bioassays on *C. obscurus*, *C. antillensis*, and *C. clypeatus* were also performed to determine if they respond

TABLE 1. STIMULI TESTED IN SHELL-INVESTIGATION ASSAYS WITH FOUR SPECIES OF HERMIT CRABS[a]

Stimulus	Assay[b]					
	COM-A	COM-N	OBS-A	OBS-N	CLY	ANT
Seawater	+	+	+	+	+	+
O. gaudichaudii hemolymph	+		+			
N. scabricosta extract	+		+		+	+
N. fulgurans extract		+		+	+	+
C. obscurus extract	+		+	+	+	+
C. antillensis extract		+		+	+	+
C. compressus hemolymph	+	+	+		+	+
C. compressus crushed	+					
C. clypeatus hemolymph		+		+	+	+

[a] A + indicates that a given stimulus was used in assays with the respective hermit crab species.
[b] COM-A = *C. compressus* cue distribution assay, Achotines; COM-N = *C. compressus* filled-shell assay, Naos; OBS-A = *C. obscurus* assay, Achotines; OBS-N = *C. obscurus* assay, Naos; CLY = *C. clypeatus* assay; ANT = *C. antillensis* assay.

to shell-investigation cues from each other, *N. scabricosta*, *N. fulgurans*, and *C. compressus* (Table 1).

C. compressus *Filled-Shell Bioassay.* At Naos, land hermit crab densities were considerably lower than at Achotines, and crabs were also less active during daylight hours. Crabs responding to a stimulus often did so in the absence of any other crabs, making the observation of investigating conspecifics less frequent. To compensate for these conditions, a different bioassay was used for *C. compressus* at Naos to test responses to seawater, *N. fulgurans* extract, *C. antillensis* extract, *C. compressus* hemolymph, and *C. clypeatus* hemolymph (Table 1). Three 30-cm-diameter shallow pits were made 1 m apart on Culebra Beach and a 1.85-ml vial containing 250 μl of stimulus was placed in the center of each pit; 2 cm away from the vial was placed a *N. scabricosta* shell filled with concrete to mimic a land hermit crab withdrawn into its shell. For a 20-min interval, the number of crabs arriving and the number investigating the filled shell at each vial were counted. This longer time interval was used to obtain a greater number of observations of individual crabs. The stimuli were then changed and observations repeated for 20 min, using seawater as a control in each observation period. This procedure was performed on three different nights and the data pooled before analysis using *G* tests (Sokal and Rohlf, 1981).

C. obscurus *and* C. antillensis *Bioassay.* To examine the shell-investigation behavior of marine hermit crabs, the bioassay described by Rittschof et al. (1992) was used: 10 crabs were placed in 3 liters of seawater in a 30-cm-diameter bucket. The crabs were separated manually and mixed with seawater; after 15 sec the crabs were observed for 1 min, and the number of hermit crabs that investigated another hermit crab's shell was counted. I repeated this procedure, adding 100 μl of seawater before mixing the seawater, and then performed a third trial, adding 100 μl of stimulus before mixing the seawater. At Achotines, test stimuli for *C. obscurus* were *O. gaudichaudii* hemolymph, *N. scabricosta* extract, *C. obscurus* extract, and *C. compressus* hemolymph (Table 1). At Naos, test stimuli for *C. obscurus* were *N. fulgurans* extract, *C. obscurus* extract, *C. antillensis* extract, and *C. clypeatus* hemolymph (Table 1). At Galeta, test stimuli for *C. antillensis* were *N. scabricosta* extract, *N. fulgurans* extract, *C. obscurus* extract, *C. antillensis* extract, *C. compressus* hemolymph, and *C. clypeatus* hemolymph (Table 1). Five replicates of each stimulus were performed at Achotines and four replicates of each stimulus at Naos and at Galeta, using fresh crabs for each replicate at all locations. The replicates were pooled and the data analyzed by comparing the proportion of crabs responding before addition to the proportion of crabs responding after addition of seawater, and the proportion of crabs responding after addition of seawater to the proportion of crabs responding after addition of test stimulus using *G* tests, with the proportion responding before addition as an expected value for the proportion responding after addition (Sokal and Rohlf, 1981).

C. clypeatus *Bioassay.* Since land hermits at Galeta (*C. clypeatus*) occurred at a lower density than *C. compressus* at Achotines and Naos, I used a bioassay based on the previously described bucket assay to examine shell-investigation behavior. Ten individuals were placed in a 30-cm-diameter bucket and allowed 10 min to acclimate to the bucket. Next, a plastic bottle cap (15 mm diameter) was placed in the center of the bucket. After 1 min, the crabs were observed for 1 min and the number of individuals that investigated another crab's shell recorded, scoring each individual only once. I then placed 250 μl of extract (*N. scabricosta*, *N. fulgurans*, *C. obscurus*, *C. antillensis*) or hemolymph (*C. compressus*, *C. clypeatus*) in the bottle cap and again observed investigation behavior for 1 min (Table 1). Each stimulus was tested with each group of 10 crabs, removing the crabs, and rinsing the buckets three times between trials. Four replicates of this experiment were performed, the stimuli presented in a different order in each replicate, and the replicates pooled for analysis by *G* test, with the control population fondling used as an expected value for the experimental proportion fondling for each stimulus (Sokal and Rohlf, 1981).

Volatile Cue Isolation and Bioassay

Volatile shell-investigation cues were isolated by collecting them on Tenax-TA (20/35 mesh size), using a method similar to that of Ciccioli et al. (1976) and Patt et al. (1988). Tenax-TA is a porous polyester based on 2,6-diphenyl-*p*-phenylene oxide and can be used as an adsorbent to collect organic volatile compounds or as a gas chromatography stationary phase to separate these compounds. A battery-powered pump pushed air through a sealed cup containing an odor source, then through a sampling column, which consisted of a glass pipet tube filled with 300 mg of Tenax, held in place with plugs of steel wool at either end of the tube. Before use, the columns were conditioned by rinsing them with 10 ml (approximately 10 column volumes) of 95% ethanol and heating them to 200°C overnight.

Volatile compounds were collected at 0°C by suspending the column in an ice bath. To collect volatiles at 40°C and higher, the column was suspended by copper tubing in a drying oven that could be heated to temperatures ranging from ambient to over 200°C. For any given temperature reported, the oven temperature was within 5°C of that temperature. To sample volatile compounds, I first heated or cooled the column to a target temperature and then started airflow through the sealed cup and the column at 20 ml/min for 15 min. The odor source was then added to the cup and airflow resumed for 30 min. After 30 min, the oven was turned off, the column allowed to cool to ambient temperature and then eluted with 10 ml of 95% ethanol.

Eluents of *C. compressus* odor made at different temperatures were compared to *C. compressus* hemolymph (positive control) and ethanol (negative

control) to determine if they contained molecules that stimulated shell-investigation behavior of *C. compressus* at Naos. Three 30-cm-diameter shallow pits were made 1 m apart, a 1.85-ml vial containing 250 μl of stimulus placed in the center of each pit, and a filled shell placed 2 cm from the vial. For a 20-min interval, the number of crabs arriving and the number investigating the filled shell were counted. The positions of the vials were then changed, observations repeated for 20 min, the vials' positions changed a third time, and observations repeated for an additional 20 min. Data were pooled from the three 20-min observation periods and the proportion of crabs investigating the filled shell at the test eluent compared to the proportion at *C. compressus* hemolymph and to the proportion at ethanol using *G* tests (Sokal and Rohlf, 1981).

To determine if eluents made from the collection of odors from an empty cup, *O. gaudichaudii*, *C. compressus*, *C. clypeatus*, *C. obscurus*, and *C. antillensis* at 130°C and 150°C stimulated shell investigation, the previous assay was modified. Four 30-cm-diameter shallow pits and three 20-min observation periods were used to test 12 different fractions each night, repeating the procedure on six different nights, changing the positions and observation periods of the 12 stimuli each night. The data from each night were pooled and the proportion of crabs investigating filled shells compared using a *G* test (Sokal and Rohlf, 1981).

The previously described *C. obscurus* assay was repeated using the 130°C *C. obscurus* eluent as a stimulus and seawater and ethanol as controls to determine if the molecules that were shell-investigation cues for *C. compressus* were also cues for *C. obscurus*.

RESULTS

Attraction to Feeding Cues and Shell Cues

Since no feeding was possible at empty pits, the control treatment was not included in the analysis of the percent of crabs feeding. Percent feeding varied significantly between different stimulus odors ($F_{4,69} = 11.00$, $P < 0.001$, Figure 1), while the presence of an empty shell had no effect on feeding ($F_{1,69} = 0.25$, $P = 0.621$, Figure 1). Pairwise comparisons of stimulus effects showed that significantly less feeding occurred at *C. compressus* odors (all other odor stimuli different from *C. compressus*, $P < 0.02$; not different from each other, $P > 0.33$). The percent of crabs investigating conspecifics varied significantly between different stimuli ($F_{5,81} = 19.33$, $P < 0.001$, Figure 2) and significantly more investigation of conspecifics occurred in the presence of an empty shell ($F_{1,81} = 13.88$, $P < 0.001$, Figure 2). Pairwise comparisons of stimulus effects showed that *C. compressus* odors stimulated significantly more investigation activity than the other odors and that *C. obscurus* odors also stimulated a high

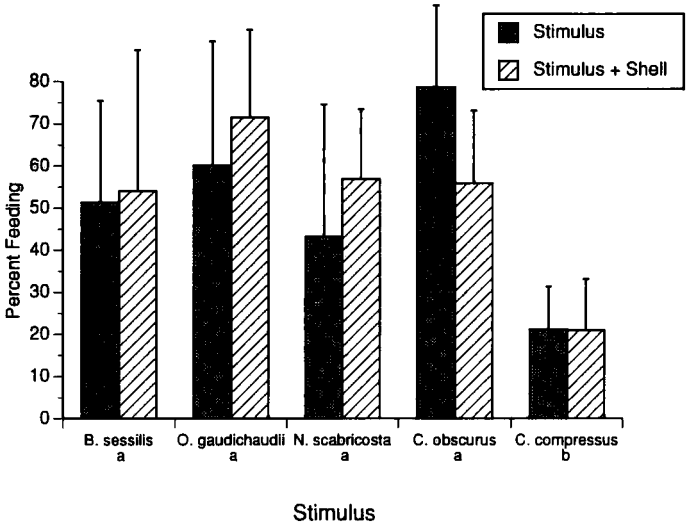

FIG. 1. The percent of *C. compressus* arriving at pits that fed on different odor stimuli. Shaded bars indicate odor stimuli alone, striped bars indicate odor stimuli combined with an empty shell. Identical letters under stimulus names indicate pairs of stimuli that are not significantly different ($P > 0.05$) by Scheffé's multiple comparison test of the effect of stimuli.

amount of investigation activity in the presence of an empty shell, while little investigation occurred at *B. sessilis* sites (Figure 2). The percent of crabs investigating an empty shell did not differ significantly between stimuli ($F_{5,41} = 1.89$, $P = 0.117$, Figure 3). The percent of crabs exchanging shells also did not significantly vary between stimuli ($F_{5,41} = 1.98$, $P = 0.102$, Figure 4).

Behavior of Crabs Attracted to Different Stimuli

When the shell-investigation behavior of different groups of crabs attracted to different odors was examined, the number of crabs investigating conspecifics' shells varied significantly between crab groups ($G = 10.74 > 9.21$, $2df$, $P < 0.01$, Figure 5), with more investigation shown by crabs that had been attracted to a dead conspecific than to haphazardly collected crabs or crabs that had been attracted to a flower. The number of crabs exchanging shells also differed significantly ($G = 6.23 > 5.99$, $2df$, $P < 0.05$, Figure 5) with the same pattern between groups. The distribution of crabs in shell-fit categories varied significantly between crab groups, with the group attracted to dead conspecifics having significantly worse shell fits than the group attracted to flowers (conspecific-flower: maximum difference = 0.380, $P = 0.001$; flower-random and conspe-

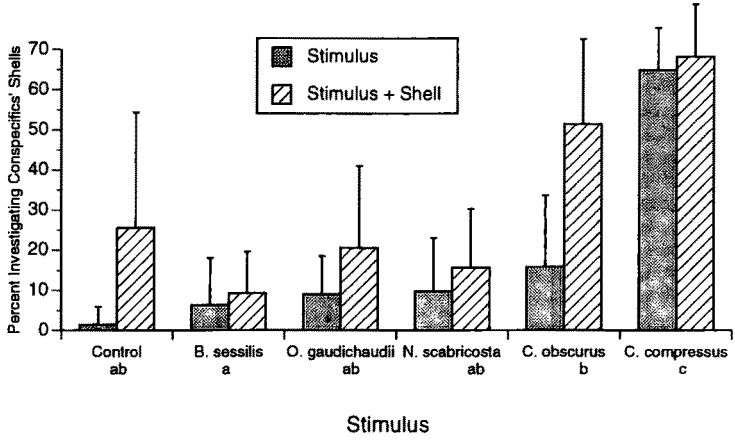

FIG. 2. The percent of *C. compressus* arriving at pits that investigated conspecifics' shells at different odor stimuli. Shaded bars indicate odor stimuli alone, striped bars indicate odor stimuli combined with an empty shell. Identical letters under stimulus names indicate pairs of stimuli that are not significantly different ($P > 0.05$) by Scheffé's multiple comparison test of the effect of stimuli.

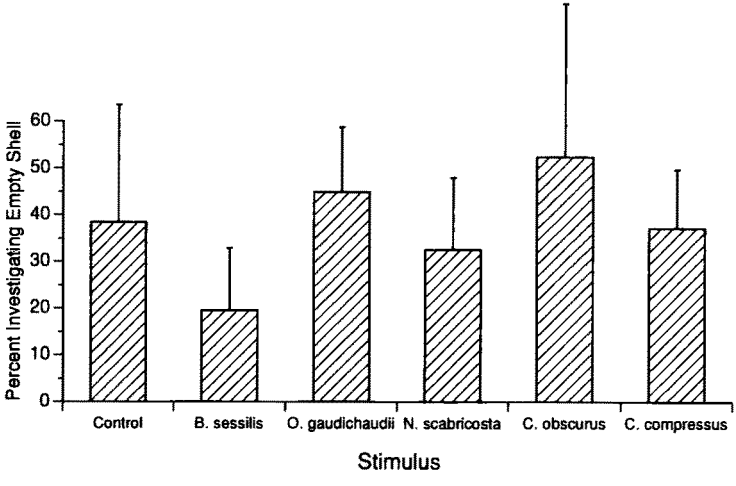

FIG. 3. The percent of *C. compressus* arriving at pits that investigated the empty shell placed at each odor stimulus.

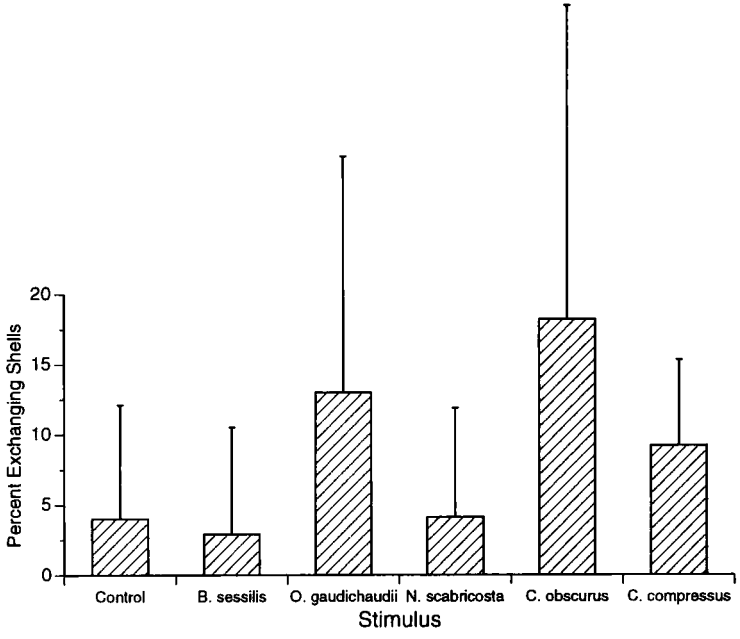

FIG. 4. The percent of *C. compressus* arriving at pits that exchanged shells at each odor stimulus provided with an empty shell.

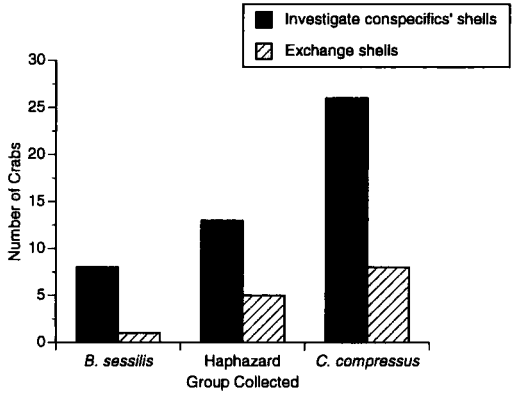

FIG. 5. The number of *C. compressus* investigating conspecifics' shells (shaded bars) and the number exchanging shells (striped bars) in groups of crabs attracted to a dead conspecific (*C. compressus*), a *B. sessilis* flower, or collected haphazardly.

cific-random: maximum difference = 0.140, $P = 0.660$, Figure 6). The distribution of shell fits also differed significantly between crabs that investigated conspecifics and crabs that did not, with the investigating crabs having significantly worse shell fits (maximum difference = 0.281, $P = 0.013$, Figure 7). The same pattern was found in crabs that exchanged shells and crabs that did not, with exchanging crabs having significantly worse shell fits (maximum difference = 0.509, $P = 0.002$, Figure 8). The proportion of crabs investigating conspecifics' shells increased as the shell-fit category increased ($G = 18.01 > 13.28$, $4df$, $P < 0.01$, Figure 9); thus, crabs in poorly fitting shells were more likely to investigate conspecifics' shells than were crabs in better-fitting shells.

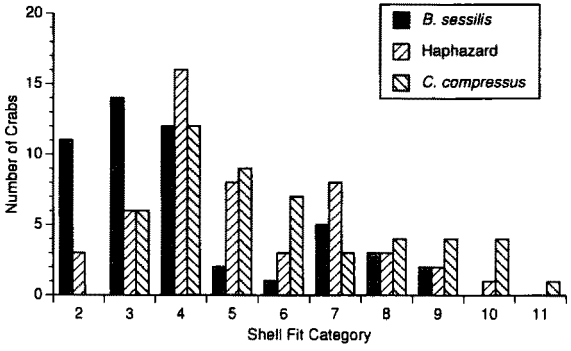

FIG. 6. The number of *C. compressus* in each shell-fit category for groups of crabs attracted to a dead conspecific (*C. compressus*), a *B. sessilis* flower, or collected haphazardly.

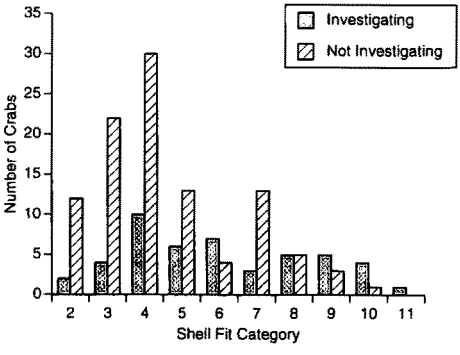

FIG. 7. The number of *C. compressus* in each shell-fit category investigating conspecifics' shells or not.

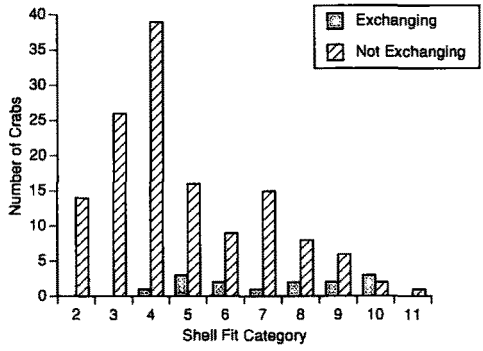

FIG. 8. The number of *C. compressus* in each shell-fit category exchanging shells or not.

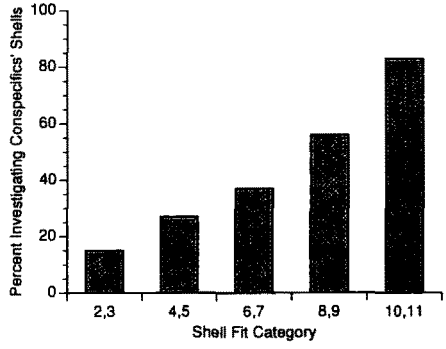

FIG. 9. The percent of *C. compressus* in paired shell fit categories that investigated conspecifics' shells.

Effect of Shell Fit on Shell-Investigation Behavior

More crabs investigated conspecifics' shells when they were in a worse-fitting shell than when they were in a better-fitting shell (Table 2). Shell fit significantly influenced shell investigation in crabs that started with a good shell fit (binomial test of 18:4, $P = 0.0022$) and in crabs that started with a bad shell fit (16:2, $P = 0.0007$). Since the starting shell fit did not affect this pattern of behavior, a pooled test of the results is possible and confirms that shell investigation was significantly influenced by shell fit (34:6, $P < 0.0005$). Similarly, more crabs exchanged shells when they were in a worse-fitting shell than when they were in a better-fitting shell (Table 3). This effect is not significant when crabs that started with a good shell fit (binomial test of 8:2, $P = 0.0547$)

TABLE 2. NUMBER OF CRABS INVESTIGATING CONSPECIFICS' SHELLS WHEN PLACED IN GOOD-FITTING OR BAD-FITTING SHELLS[a]

		Bad shell				Bad shell	
A		INV	NOT	B		INV	NOT
Good shell	INV	5	4	Good shell	INV	3	2
	NOT	18	23		NOT	16	29

[a] A: crabs originally in good-fitting shells, later placed in bad-fitting shells; B: crabs originally in bad-fitting shells, later placed in good-fitting shells. INV = crabs that investigated conspecifics' shells; NOT = crabs that did not investigate.

TABLE 3. NUMBER OF CRABS EXCHANGING SHELLS WHEN PLACED IN GOOD-FITTING OR BAD-FITTING SHELLS[a]

		Bad shell				Bad shell	
A		EXCH	NOT	B		EXCH	NOT
Good shell	EXCH	2	2	Good shell	EXCH	0	1
	NOT	8	38		NOT	6	43

[a] A: crabs originally in good-fitting shells, later placed in bad-fitting shells; B: crabs originally in bad-fitting shells, later placed in good-fitting shells. EXCH = crabs that exchanged shells; NOT = crabs that did not exchange shells.

are examined separately from crabs that started with a bad shell fit (6:1, $P = 0.0625$). However, the starting shell fit did not affect the pattern of this behavior, and a pooled test of the results does reveal a significant effect of shell fit (14:3, $P = 0.0064$).

Comparison of Shell Fits between Sites and Sexes

The results of the ANCOVAs of shell-fit ratios for juveniles and adults are displayed as adjusted least-squares means (Table 4). For juveniles, no significant effects on shell-fit ratios were found due to sex ($F_{1,89} = 0.87$, $P = 0.354$, Table 4) or site ($F_{1,89} = 0.53$, $P = 0.468$, Table 4). Thus, the shell fits of juveniles at Achotines and Naos, males and females, were not significantly different. For adults, significant effects were found due to sex ($F_{2,454} = 18.42$, $P < 0.001$, Table 4), site ($F_{1,454} = 89.91$, $P < 0.001$, Table 4), and an interaction between site and sex ($F_{2,454} = 3.19$, $P = 0.042$, Table 4). At Achotines, the shell-fit ratios of males and females were not significantly different, but both males and

TABLE 4. ADJUSTED LEAST-SQUARES MEANS OF SHELL FIT RATIOS FOR JUVENILE MALES AND FEMALES AND ADULT MALES, FEMALES, AND GRAVID FEMALES AT ACHOTINES AND NAOS[a]

	Achotines	Naos
Juveniles	0.508	0.519
Males		
Females	0.522	0.484
Adults		
Males	0.645 a	0.599 b
Females	0.637 a	0.571 c
Gravid females	0.603 b	0.569 bc

[a] Identical letters after adult means indicate pairs that are not significantly different ($P > 0.05$).

females had significantly higher shell-fit ratios than gravid females. Thus, gravid females were in better-fitting shells than males or females. At Naos, the shell-fit ratios of males and females were not significantly different and the shell-fit ratios of gravid females were not significantly different and the shell-fit ratios of gravid females were not significantly different than those of females. However, gravid females did have significantly lower shell-fit ratios than males. The shell-fit ratios of males and females at Achotines were significantly higher than those of males and females at Naos, but the shell-fit ratios of gravid females at Achotines were not significantly different than those of gravid females at Naos.

Response Time to Shell-Investigation Cues at Different Sites

At Achotines, land hermit crabs were attracted to conspecific hemolymph at a rate of 1.98 crabs/min (SD 1.11), while at Naos, crabs were attracted at a rate of 0.29 crabs/min (SD 0.15), a significantly lower rate of attraction ($t = 5.230$, $22df$, $P < 0.001$).

Distribution of Shell-Investigation Cues

In test of hemolymph and extracts from stimulus species, rather than whole crushed stimuli, the proportion of crabs investigating conspecifics' shells varied significantly between different stimuli ($G = 57.68 > 15.09$, $5df$, $P < 0.01$). Seawater, *N. scabricosta*, and *O. gaudichaudii* formed a homogeneous group that did not stimulate shell investigation ($G_H = 2.878 < 11.07$, $5df$, $P > 0.05$), while *C. obscurus*, *C. compressus* hemolymph, and *C. compressus* crushed in a vial formed a homogeneous group that did stimulate shell investigation ($G_H = 7.758 < 11.07$, $5df$, $P > 0.05$). Shell-investigation cues are therefore present in *C. obscurus* extract, *C. compressus* hemolymph, and crushed *C. compressus*.

Detection of Shell-Investigation Cues of Other Species

C. compressus *Filled-Shell Bioassay.* The proportion of crabs investigating the filled shell varied significantly between odor stimuli ($G = 29.44 > 13.28$, $4df$, $P < 0.01$). Seawater and *N. fulgurans* formed a homogeneous group that did not stimulate shell investigation ($G_H = 0.008 < 9.488$, $4df$, $P > 0.05$), while *C. clypeatus*, *C. antillensis*, and *C. compressus* formed a homogeneous group that did stimulate shell investigation ($G_H = 4.57 < 9.488$, $4df$, $P > 0.05$).

C. obscurus *Bioassays.* For all stimuli at Achotines and Naos, the number of crabs responding after the addition of seawater did not change significantly from the number of crabs responding before the addition of seawater (Table 5). The number of crabs responding after the addition of stimuli did not change significantly for *O. gaudichaudii*, *C. compressus*, or *C. clypeatus* (Table 5). *C. obscurus* extract significantly increased the number of crabs investigating conspecifics' shells at Achotines and at Naos (Table 5). Shell investigation was also significantly stimulated by *N. scabricosta*, *N. fulgurans*, and *C. antillensis* (Table 5).

C. antillensis *Bioassays.* For all stimuli, the number of crabs responding after the addition of seawater did not change significantly from the number of crabs responding before the addition of seawater (Table 6). The number of crabs responding after the addition of stimuli did not change significantly for *C. compressus* or *C. clypeatus* (Table 6). *C. antillensis* extract significantly increased the number of crabs investigating conspecifics' shells, as did extracts of *N. fulgurans*, *N. scabricosta*, and *C. obscurus* (Table 6).

C. clypeatus *Bioassays. C. clypeatus* investigated conspecifics' shells significantly more often in response to cues from conspecific hemolymph, *C. obscurus* extract, *C. antillensis* extract, and *C. compressus* hemolymph (Table 7). The number of crabs investigating did not significantly change after the addition of *N. fulgurans* extract or *N. scabricosta* extract (Table 7).

Volatile Cue Isolation

Eluents of *C. compressus* odors collected at 0°C, 100°C, and 130°C stimulated investigation of filled shells, while eluents from collection at 150°C and 200°C did not (Table 8). Tests of eluents from several species of crabs showed that collections at 130°C from *C. compressus*, *C. clypeatus*, *C. obscurus*, and *C. antillensis* were all effective in generating investigation of filled shells, while 150°C collections from these species and both collections from the control and *O. gaudichaudii* were not stimulating (overall $G = 70.71 > 33.14$, $11df$, $P < 0.005$; multiple comparison $G = 65.17 > 33.14$, $11df$, $P < 0.005$). Tests of 130°C *C. obscurus* eluent, which stimulated shell investigation in *C. compressus*, showed no stimulation of shell investigation in *C. obscurus* (seawater addi-

TABLE 5. PROPORTION OF *C. obscurus* THAT INVESTIGATED CONSPECIFICS' SHELLS IN RESPONSE TO VARIOUS STIMULI[a]

Stimulus	Percent investigating	*G*	*P*
Control	36		
Seawater	30	0.80	>0.05
O. gaudichaudii	36	0.83	>0.05
Control	34		
Seawater	36	0.09	>0.05
N. scabricosta	62	13.89	<0.001
Control	40		
Seawater	40	0.00	>0.05
C. obscurus	62	9.82	<0.005
Control	34		
Seawater	36	0.09	>0.05
C. compressus	40	0.34	>0.05
Control	30		
Seawater	28	0.12	>0.05
N. fulgurans	60	18.42	<0.001
Control	30		
Seawater	33	0.12	>0.05
C. obscurus	70	23.50	<0.001
Control	43		
Seawater	30	2.66	>0.05
C. antillensis	73	30.62	<0.001
Control	34		
Seawater	32	0.09	>0.05
C. clypeatus	34	0.09	>0.05

[a]The first four assays shown were conducted at Achotines, the remaining at Naos.

tion: $G = 0.00 > 3.841$, $1df$, $P > 0.05$; ethanol addition: $G = 0.056 < 3.841$, $1df$, $P > 0.05$; eluent addition: $G = 0.056 < 3.841$, $1df$, $P > 0.05$).

DISCUSSION

Land hermit crabs locate dead conspecifics by detecting volatile chemical cues (Small and Thacker, 1994). Land hermit crabs attracted to a shell source by odors may be attracted by feeding cues rather than by shell cues. The visual stimulus of a shell at an odor source might then stimulate shell-investigation

TABLE 6. PROPORTION OF *C. antillensis* THAT INVESTIGATED CONSPECIFICS' SHELLS IN RESPONSE TO VARIOUS STIMULI

Stimulus	Percent investigating	*G*	*P*
Control	40		
Seawater	43	0.10	>0.05
N. scabricosta	60	4.94	<0.05
Control	35		
Seawater	40	0.43	>0.05
N. fulgurans	58	4.97	<0.05
Control	40		
Seawater	40	0.00	>0.05
C. obscurus	58	4.97	<0.05
Control	43		
Seawater	33	1.68	>0.05
C. antillensis	60	12.69	<0.001
Control	45		
Seawater	38	0.92	>0.05
C. compressus	40	0.11	>0.05
Control	40		
Seawater	38	0.10	>0.05
C. clypeatus	38	0.00	>0.05

behavior (Hazlett, 1982; Orihuela et al., 1992), with the visual cues acting separately from the olfactory cues that initially attracted a crab to the area. Crabs attracted to dead conspecifics show less feeding and more investigation of conspecifics' shells than crabs attracted to other odor sources. The presence of an empty shell at an odor source visually stimulates shell-investigation behaviors, generating the same levels of investigation of empty shells and shell exchange at all odor sources. Since intact empty shells are rarely encountered on beaches (personal observation; Ball, 1972), it is not surprising that they would generate a high amount of shell-investigation activity when encountered. In the absence of shells, odors from conspecifics and a marine hermit crab stimulated shell investigation in *C. compressus*, while the odor of *B. sessilis* flowers and other foods did not.

Odors that attracted crabs to food sources or shell sources separated responding crabs into two response categories prior to visual stimulation by an empty shell or conspecifics' shells. Crabs attracted to food odors are in better-fitting shells and show less shell-investigation behavior than crabs attracted to

TABLE 7. PROPORTION OF *C. clypeatus* THAT INVESTIGATED CONSPECIFICS' SHELLS IN RESPONSE TO VARIOUS STIMULI

Stimulus	Percent investigating	*G*	*P*
Control	28		
N. scabricosta	40	2.91	>0.05
Control	30		
N. fulgurans	33	0.33	>0.05
Control	28		
C. obscurus	55	13.33	<0.001
Control	35		
C. antillensis	60	10.34	<0.005
Control	38		
C. compressus	58	6.55	<0.025
Control	20		
C. clypeatus	53	20.72	<0.001

TABLE 8. PERCENT OF *C. compressus* INVESTIGATING FILLED SHELL IN RESPONSE TO ELUENTS OF *C. compressus* VOLATILES COLLECTED AT DIFFERENT TEMPERATURES, *C. compressus* HEMOLYMPH CONTROL, AND ETHANOL CONTROL

Temperature (°C)	Eluent (%)	Hemolymph (%)	*G*	*P*	Ethanol (%)	*G*	*P*
0	87	70	1.41	>0.20	39	10.26	<0.005
100	64	79	0.68	>0.40	40	3.12	<0.10
130	76	67	0.55	>0.40	37	8.81	<0.005
150	5	39	7.12	<0.01	20	1.92	>0.10
200	43	78	5.94	<0.05	48	0.10	>0.70

dead conspecifics. Crabs collected haphazardly display an intermediate level of shell investigation and are in intermediate shell fits. This correlation of shell fit and likelihood of displaying shell-investigation behaviors is causative. Moving crabs from worse-fitting shells to better-fitting shells reduces the amount of shell investigation they display, while moving crabs from better-fitting shells to worse-fitting shells increases the amount of shell investigation they display. A similar pattern is found in the marine hermit crab *Clibanarius vittatus*, for which small changes in shell fit result in large changes in behavior (Rittschof, 1980a; Ritt-

schof et al., 1992; Katz and Rittschof, 1993). Moving crabs into smaller shells increases the amount of shell investigation observed in *C. vittatus*, while moving crabs into larger shells decreases the amount of shell investigation observed (Katz and Rittschof, 1993). Shell fit, therefore, not only influences the probability of shell-investigation behavior being displayed at a given odor source, but whether or not an individual is attracted to feeding cues or shell cues. Attraction to feeding cues may also be related to the limitations that shell fit places on growth rate. Hermit crabs in poorly fitting shells grow more slowly than crabs in better-fitting shells (Markham, 1968; Fotheringham, 1976). Crabs in poorly fitting shells may therefore eat less and be less attracted to food odors, while crabs in better-fitting shells may seek food more frequently to attain a higher growth rate.

C. compressus gravid females are in better-fitting shells than males and nongravid females at Achotines. The same pattern is found at Naos, but the crabs at Naos are in better-fitting shells overall than the crabs at Achotines, demonstrating that shell fit does vary between land hermit crab populations (Abrams, 1978). The difference in shell fit between gravid and nongravid females suggests that only females in better-fitting shells invest in reproduction. The eggs of land hermit crabs are protected from desiccation by a properly fitting shell (De Wilde, 1973). Poorly fitting shells may limit clutch size (Wilber, 1989) in addition to increasing the risk of desiccation. Bertness (1981) has reported that some marine hermit crabs (*Clibanarius albidigitus*, *C. obscurus*, and *Pagurus* sp.) in relatively smaller shells reproduce more frequently and have larger clutch sizes than those in relatively larger shells. In contrast, Bach et al. (1976) reported that *Clibanarius tricolor* females in better-fitting shells have a larger clutch size than females in worse-fitting shells. Shell fit may therefore exert different influences on reproduction in different groups of hermit crabs. Since female land hermit crabs in better-fitting shells are more likely to reproduce successfully and male land hermit crabs manipulate a female' shell during courtship (Page and Willason, 1982; Dunham and Gilchrist, 1988), I hypothesize that male land hermit crabs may prefer to mate with females in better-fitting shells. Alternatively, females may not mate unless they are in better-fitting shells.

Differences in shell fit between populations of land hermit crabs at Achotines and Naos are evident in differences in the responses of these populations to shell-investigation cues. The lower levels of shell-investigation activity and better shell fits observed at Naos could be related to a larger supply of large *N. scabricosta* shells, higher predation rates on crabs in poorly fitting shells, or lower juvenile recruitment. These factors would decrease the number of crabs in poorly fitting shells, which would in turn decrease the number of crabs actively searching for shells. Crabs at Naos are less active in general than crabs at Achotines, coming out onto the beach only at dusk and returning to shelter well

before dawn. In contrast, crabs at Achotines are active on the beach from one to two hours before dusk, all through the night, returning to shelter by 1100 hr, at which time the beach is no longer shaded and begins to heat up rapidly. Peak activity at both sites occurs on falling tides near dawn and disk, after new and full moons.

C. compressus responds to shell-investigation cues in the hemolymph of conspecifics and in extracts of a local marine hermit crab, *C. obscurus*, but not from a snail whose shell these two hermit crabs use, *N. scabricosta*, nor from a brachyuran crab found on the same beach, *O. gaudichaudii*. When tested with similar species from the Caribbean coast of Panama, *C. compressus* responds to shell-investigation cues from a congener, *C. clypeatus*; a marine hermit crab, *C. antillensis*; but not from a snail whose shell is inhabited by these two species, *N. fulgurans*. Likewise, *C. clypeatus* responds to shell-investigation cues from both species of land hermit crabs and marine hermit crabs, but not from either of the snail species. The marine hermit crabs from the Pacific and Caribbean coasts of Panama, both diogenids, respond to shell-investigation cues from both marine hermit crab species and both snail species, but not from the land hermit crabs or the brachyuran crab.

Since land hermit crabs can detect volatile shell-investigation cues from marine hermit crabs, these volatile compounds were most likely present in the ancestral hermit crabs that first moved onto land. A detection system for these cues probably evolved during the transition to a terrestrial environment. The nonvolatile peptide cues from gastropod tissue used to locate shell sources from a distance in water (Kratt and Rittschof, 1991) can not be used in this manner on land. However, land hermit crabs have retained a detection system for peptide cues from gastropod tissue, since newly metamorphosed juveniles can find their first shell underwater by detecting these cues (Gilchrist, 1991). The shell-investigation cues released from the hemolymph of marine hermit crabs are small (<500 Da), water-soluble, and could be volatile (Rittschof, personal communication; Rittschof et al., 1992).

Land hermit crabs do not respond to shell-investigation cues from snails, possibly because there are no volatiles found uniquely in snails and not in other carrion sources, preventing the differentiation of carrion likely to have shells and carrion not likely to have shells. Predators of snails on land (e.g., birds, *Gecarcinus*) are also less likely to leave an intact shell than are predatory marine gastropods (McLean, 1974). The cost of a detection system may not be balanced by the frequency of encountering an intact shell at the source of the cue. Dead or dying marine hermit crabs could frequently be left in the intertidal zone with their shells as the tide recedes, making it profitable for land hermit crabs to be able to detect them.

Although land hermit crabs are derived from marine hermit crabs (McLaughlin, 1983), diogenid marine hermit crabs do not respond to shell-

investigation cues from land hermit crabs, suggesting that the particular cues found in the hemolymph of marine hermit crabs are not present in the hemolymph of land hermit crabs. Land hermit crabs may have lost these cues, indicating that marine hermit crabs may maintain shell-investigation cues in their hemolymph at some cost. This idea of a cost associated with maintaining cues in hemolymph would not be supported if these cues serve another, primary function in the crab.

In addition, two separate cue detection systems may exist. First, marine hermit crabs may detect a nonvolatile peptide-based cue released from gastropod tissues that land adult hermit crabs can not detect because it can not be transmitted in air. Second, marine and land hermit crabs may detect another, unidentified type of cue from hermit crab hemolymph that is sufficiently water soluble and volatile to be transmitted in both water and air.

The shell-investigation cues of land hermit crabs may have diverged from those of marine hermit crabs such that the diogenid marine hermit crabs tested in these experiments can not detect land hermit crab cues. Rittschof et al. (1992) have shown that *C. vittatus*, a diogenid marine hermit crab, does not respond to shell-investigation cues from *Pagurus longicarpus* and *Pagurus pollicaris*, two pagurid marine hermit crabs, while *P. longicarpus* and *P. pollicaris* do respond to cues from *C. vittatus*. This evidence suggests that the evolution of hermit crab shell-investigation cues can be studied by determining if cue detection abilities are correlated with hermit crab phylogeny. Identification of the cues of several species of hermit crabs could reveal how shell-investigation cues have changed and which groups of hermit crabs share similar cues.

Isolation of volatile shell-investigation cues is possible by collecting volatile compounds onto Tenax at different temperatures. Biologically active compounds are present in 130°C collections from land hermit crabs and marine hermit crabs, but not in 150°C collections, suggesting that the cues from the four species that stimulated *C. compressus* shell-investigation behavior are similar. The 130°C collection of *C. obscurus* volatiles do not stimulate shell investigation in *C. obscurus*, indicating that the stimulant molecules of land hermit crabs are different from those of marine hermit crabs. No volatiles collected from controls or *O. gaudichaudii* stimulate shell investigation. The next step in this procedure will be to analyze each collection of volatiles using gas chromatography. Compounds present in the control, *O. gaudichaudii*, or 150°C collections can be ruled out as potential shell cues, leaving compounds present in 130°C collections from marine hermit crabs and land hermit crabs as candidate cues. These candidate cues can then be identified by mass spectroscopy.

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MALE-PRODUCED AGGREGATION PHEROMONE OF *Carpophilus obsoletus* (COLEOPTERA: NITIDULIDAE)[1]

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Abstract—Males of *Carpophilus obsoletus* Erichson produce an aggregation pheromone to which both sexes respond. The pheromone was identified by GC-MS as (2*E*,4*E*,6*E*,8*E*)-3,5,7-trimethyl-2,4,6,8-undecatetraene (**1**), which is also a minor constituent of the pheromone blends of *C. hemipterus* (L.), *C. freemani* Dobson, and *C. lugubris* Murray. The pheromone was synergized in wind-tunnel bioassays by propyl acetate, a "host-type" coattractant. In a dose–response study, 50 pg of **1**, plus propyl acetate, was significantly more attractive than just propyl acetate. Pheromone emission from groups of 65 males, feeding on artificial diet, averaged 2.2 ng/male/day. Emissions from individual males were larger, averaging 72 ng/day and ranging as high as 388 ng/day. Synthetic **1** was tested in a date garden in southern California (500 μg/rubber septum), using fermenting whole-wheat bread dough as the coattractant. The pheromone plus dough attracted significantly more beetles than dough alone (means were 4.2 and 0.0 beetles per week per trap). Captured beetles were 54% females. Field trap catches were highest during the months of July and August.

Key Words—*Carpophilus obsoletus*, sap beetle, Coleoptera, Nitidulidae, aggregation pheromone, hydrocarbon, tetraene, date.

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INTRODUCTION

Carpophilus obsoletus Erichson (Coleoptera: Nitidulidae) is a small (ca. 4 mm) dark brown sap beetle that occurs throughout the tropical, subtropical, and milder temperate regions of the world (Dobson, 1954). This insect can be a serious pest of commodities such as dried fruit (Dobson, 1954). *C. obsoletus* also attacks maize (Rathore and Senger, 1969). *C. obsoletus* is a minor species in the nitidulid complex attacking dates in southern California (R.S.V., personal observation; Bartelt et al., 1992b).

Male-produced aggregation pheromones have been identified in other *Carpophilus* species: *C. antiquus* Melsheimer (Bartelt et al., 1993b), *C. freemani* Dobson (Bartelt et al., 1990a), *C. hemipterus* (L.) (Bartelt et al., 1990b, 1992a), *C. lugubris* Murray (Bartelt et al., 1991), and *C. mutilatus* Erichson (Bartelt et al., 1993a). All pheromones isolated to date have been triene or tetraene hydrocarbons, usually in blends. The fact that *C. obsoletus* responded significantly to the pheromone blend of *C. hemipterus* in field tests (Bartelt et al., 1992b) prompted us to characterize its pheromone. Pheromone isolation was guided by wind-tunnel bioassays. Activity of the pheromone was verified under field conditions.

METHODS AND MATERIALS

Beetles. The *C. obsoletus* culture was started from insects captured in a date garden near Oasis, California. The beetles were reared on the diet reported by Dowd (1987), except that additional brewer's yeast replaced the pinto beans. Insects from the culture were used both for pheromone production and for wind-tunnel bioassays.

Pheromone Collections. Beetles were separated by sex and placed with diet medium in volatile-collection flasks as described earlier for *C. hemipterus* (Bartelt et al., 1990b). Initially, each flask contained ca. 65 beetles. Twice as many collectors were set up for males (six flasks for males versus three flasks for females) because all of the previously studied, related species had male-produced pheromones. Volatiles were also collected from three flasks containing just diet, for chromatographic comparisons. Later in the project, volatile collections were also obtained from three flasks containing individual male beetles on diet because pheromone production from individuals has been reported to be higher than that from groups in another species (*C. antiquus*) (Bartelt et al., 1993b). Filters of Super Q porous polymer (Alltech Associates, Deerfield, Illinois) were used to clean the incoming air and to capture the volatiles from the feeding beetles. The temperature during collections was 27°C, the humidity of incoming air was ca. 30%, and the photoperiod was 14L:10D.

Counts were kept so that amounts of volatiles could be expressed in beetle-

days (the average amount of material collected per beetle per day). The pooled collections over a three-week period amounted to ca. 6930 beetle-days from males and 3465 from females. About 10% of this material was set aside as a standard for bioassay tests, and the remainder was used for chromatographic isolation of the pheromone.

Bioassay. Pheromone isolation was guided by wind-tunnel bioassays. The wind-tunnel bioassays were conducted as described by Bartelt et al. (1990b). The wind tunnel contained ca. 500–1000 beetles, and ca. 100 were in flight at any time during bioassays. Two different treatment preparations to be compared were applied to pieces of filter paper, and these were hung side by side in the upwind end of the wind tunnel. When more than two treatments were to be compared, they were tested in pairs with a balanced incomplete block experimental design. Responses by the beetles to an active preparation included an upwind, casting flight followed by alighting on the filter paper. Each test lasted 3 min, and the numbers of landings were recorded. Propyl acetate (20 μl, 1% solution in mineral oil) was added as a coattractant in all bioassay treatments except where otherwise indicated; previous studies have shown that bioassay count is enhanced by the presence of a "host-type" volatile coattractant (Bartelt et al., 1990a,b). The control consisted of filter paper containing only propyl acetate.

Existence of Male-Specific Pheromone. Seven one-week volatile collections from groups of male beetles were compared with the corresponding volatile collections from female beetles in the wind-tunnel bioassay in order to establish the existence of a male-specific pheromone in *C. obsoletus* (7 beetle-days per test; total of 14 tests).

Chromatography and Pheromone Isolation. High-performance liquid chromatography (HPLC) was initially conducted on pooled male-derived and pooled female-derived collections using a Spectra Physics model SP8700 solvent delivery system equipped with a silica column (Microsorb Si 80-125-C5, Rainin Instruments, Woburn, Massachusetts). Hexane was run for 5 min, at a flow rate of 1 ml/min, followed by a solvent gradient programmed from 100% hexane to 100% diethyl ether in 15 min. Effluent was collected as 1-ml fractions. Each male-derived chromatographic fraction, plus coattractant, was bioassayed four times against a control consisting of only the coattractant. Active male-derived fractions were then compared with the corresponding female-derived fractions and control in the wind-tunnel bioassay (eight replications per treatment). All collected fractions were later analyzed by GC.

The silica fraction containing maximal male-specific activity (3–4 ml after injection) was rechromatographed using a 25-cm $\times$ 0.46-cm (ID) Lichrosorb Si60 column (5-μm particle size) (Alltech), coated with $AgNO_3$ as described by Heath and Sonnet (1980). The column was eluted isocratically with a solution of 10% toluene in hexane at a flow rate of 1 ml/min. A Waters Associates model

6000 pump and R401 refractometer detector were used for this analysis. The beetle-derived samples were not concentrated enough to be detected by the refractometer, so effluent was collected as 1-ml fractions, which were also bioassayed (four times per fraction) and later analyzed by GC. The corresponding female-derived chromatographic fraction was processed similarly, then active male-derived fractions were again compared with the corresponding female-derived fractions and control in the wind tunnel bioassay as described above.

Analysis. Volatile collections and chromatographic fractions were analyzed by gas chromatography (GC). GC was performed with a Hewlett-Packard (HP) 5890 Series II instrument equipped with flame ionization detector, splitless injector, a HP 7673 autosampler, and interfaced to a HP 3396A integrator. The oven temperature was programmed from 50°C to 270°C at 10°C/min; the injector temperature was 220°C, and the detector temperature was 270°C. A 15-m × 0.25-mm capillary column (DB-1, with 1.0-μm film thickness, J&W Scientific, Folsom, California) was used with 2-μl sample injections. An internal standard (*n*-nonadecane) was used for pheromone quantitation.

For confirmation of peak identities, positive ion, electron impact mass spectra (70 eV) were obtained with a HP 5970 MSD instrument, with sample introduction through a DB-1 capillary GC column. An oven temperature program similar to that described above was used.

Chemicals. Synthetic (2*E*,4*E*,6*E*,8*E*)-3,5,7-trimethyl-2,4,6,8-undecatetraene (**1**) was available from earlier research (Bartelt et al., 1990a). It was purified to >95% purity by $AgNO_3$-coated silica HPLC using 10% toluene in hexane as the isocratic elution solvent, for use in wind-tunnel bioassay tests. For field bioassay studies, technical grade **1** was used without HPLC purification; this was 72% all-*E* **1**, 19% *Z* isomers, and 9% various uncharacterized minor products (each <1% of total mixture).

Additional Wind-Tunnel Experiments. The importance of the coattractant was verified after the identification of the *C. obsoletus* pheromone (**1**). In this experiment, treatments were: synthetic pheromone at 0.5 ng alone, synthetic pheromone at 0.5 ng with propyl acetate, propyl acetate alone (control), and a blank consisting of only filter paper. All comparisons were done, two at a time, in a balanced incomplete block experiment (six replications per treatment).

Synthetic **1** was compared with beetle-derived preparations of **1** at various stages of purification so that the existence of other attractant compounds might be revealed. These stages included: crude pheromone extracts (before any chromatography), pheromone extracts after only hexane–ether gradient silica HPLC, and pheromone extracts after hexane–ether silica gradient HPLC and HPLC using the silver nitrate-coated column. Each beetle-derived sample (which contained **1**) was tested against an equal amount of synthetic **1** and a control consisting of filter paper plus coattractant. In all cases, the amount of beetle-derived or synthetic pheromone was 0.5 ng/test and the coattractant was propyl acetate

(pairwise tests, balanced incomplete block design, eight replications per treatment). In additional experiments, the silica–HPLC-purified pheromone from beetles was compared to synthetic pheromone at doses of 0.05 ng and 5.0 ng.

Pheromone Production: Group vs. Individual Beetles. Pheromone production by a group of 65 male beetles was compared with pheromone production by individual male beetles, on the basis of the amount of pheromone emitted (nanograms) per beetle per day. Mature beetles (2-week-old adults) were used in this study so that consistent pheromone production could be expected. Pheromone was collected twice a week for a period of two months. The collections from individual beetles were then continued as long as the beetles remained alive.

Field Bioassay. Field tests were conducted in a date garden near Oasis, California. The location and methodology were as described previously for an experiment with *C. hemipterus* (Bartelt et al., 1992b). The wind-directed pipe traps (Dowd et al., 1992) were hung 1 m above the ground, and trap spacing was ca. 20 m. The traps were baited either with fermenting whole-wheat dough only, or a combination of pheromone plus dough. Whole-wheat bread dough is a commonly used nitidulid attractant, and its major volatile emissions have been characterized (Lin and Phelan, 1991). There were two replications of each treatment in a randomized block design. Beetles were collected from the traps weekly from April 21 to September 29, 1992. The pheromone septa (containing 500 μg **1**) were replaced every two weeks, and the dough baits were replaced weekly. Field bioassay results presented in this paper are part of a larger study to be published at a later date.

Statistical Analysis. Wind-tunnel and field data were transformed to the log $(X + 1)$ scale before analysis to stabilize variance. Balanced incomplete block experiments involving comparisons among three or more treatments were analyzed by the method of Yates (1940). Analysis of variance (ANOVA) was used to analyze field trap catch data. ANOVA was also used to compare pheromone production rates from individuals and groups of beetles.

RESULTS AND DISCUSSION

Evidence for Male-Specific Pheromone. Volatile collections from male beetles were significantly more attractive in the wind-tunnel bioassay than those from female beetles, establishing the existence of a male-specific pheromone. Mean bioassay counts (7 beetle-days per test, $N = 14$) for male-derived volatiles and female-derived volatiles were 37.6 and 2.1, respectively ($F = 106$, 1 and 13 *df*, $P < 0.001$). Only male-produced aggregation pheromones have been discovered so far in *Carpophilus* species.

Isolation of Hydrocarbon Pheromone. After HPLC on the silica column,

the most active male-derived fractions (3–5 ml after injection, Table 1) had very low polarity. Although several fractions with higher polarity also had significant activity, attempts to isolate the active compounds were unsuccessful because of low and inconsistent bioassays. However, these polar, male-derived fractions (18–21 ml) were similar in activity to the corresponding female-derived fractions (data not shown); thus, these materials appear not to be male-specific attractants and may emanate from the diet.

Rechromatography of the most active hydrocarbon fraction (3–4 ml after injection) on the $AgNO_3$-coated silica column resulted in a group of active fractions 7–10 ml after injection (lower section of Table 1).

GC analysis of the male-derived and female-derived fractions 7–10 ml after injection revealed the presence of a single peak at a retention time of 13.82 min in the male-derived fractions. Amounts of the peak in each of the active fractions were: 7–8 ml after injection (trace), 8–9 ml after injection (0.12 ng/beetle-day, 11%), and 9–10 ml after injection (0.95 ng/beetle-day, 89%). The 13.82-min peak was not observed in the corresponding female-derived $AgNO_3$-coated silica column fractions.

The activity of the 4 to 5-ml silica column fraction (Table 1) was probably due to the same compound. By GC, the 13.82-min peak was present in the fraction in the amount of 0.03 ng/beetle-day.

Identification of Hydrocarbon Pheromone. The GC retention time and mass spectral fragmentation pattern of the male-specific compound matched that of (2*E*,4*E*,6*E*,8*E*)-3,5,7-trimethyl-2,4,6,8-undecatetraene **1**, shown in Figure 1 (Bartelt et al., 1990b).

This compound was previously reported as a component of the *C. hemipterus* pheromone blend (Bartelt et al., 1990b, 1992a) and the *C. freemani* pheromone blend (Bartelt et al., 1990a). It has recently been identified as a component of the *C. lugubris* pheromone blend (R.J.B., unpublished results). The fact that *C. obsoletus* was caught in field traps, baited with only the *C. hemipterus* pheromone blend, also supports our identification (Bartelt et al., 1992b).

Minor amounts (<5% as abundant as **1**) of other male-specific compounds were also detected when the silver-coated silica chromatographic fractions were carefully compared by GC. By GC retention (elution before **1**), mass spectrometry (spectra similar to **1**), and experience with other tetraenes (Bartelt et al., 1992a), these other compounds were believed to be *Z* isomers of **1**. Some isomerization/degradation has invariably occurred with all of the tetraenes (Bartelt et al., 1992a), and it is unknown whether the minor isomers are actually in emissions from *C. obsoletus*. These were not analyzed further in this study.

Effect of Coattractant. The synthetic pheromone was active in wind-tunnel tests, and it was synergized by the host-type attractant, propyl acetate. Mean bioassay counts ($N = 8$) for blank filter paper, control (propyl acetate, 20 μl, 1% in mineral oil), pheromone alone (0.5 ng), and pheromone (0.5 ng) with

TABLE 1. MEAN BIOASSAY COUNTS ($N = 4$) FOR CHROMATOGRAPHIC FRACTIONS DERIVED FROM MALE *C. obsoletus*[a]

Volume after injection (ml)	Beetle-derived	Control
A. Initial separation by polarity (hexane–ether gradient on silica column)		
2–3	3.1	5.1
3–4	104.4***[b]	1.7
4–5	50.2***	3.1
5–6	10.7	5.1
6–7	9.2*	4.1
7–8	7.0**	1.5
8–9	10.0***	1.7
9–10	3.8	3.6
10–11	3.0	3.0
11–12	5.2	4.2
12–13	6.3	5.0
13–14	4.8	3.5
14–15	7.8	4.0
15–16	6.5*	2.3
16–17	10.1*	3.9
17–18	3.7	3.2
18–19	30.8***	2.0
19–20	11.2***	2.3
20–21	15.3***	1.4
21–22	7.0*	0.9
22–23	3.7	1.5
23–24	1.6	1.4
24–25	4.8	2.3
25–26	3.4	1.4
B. Rechromatography of the active, 3 to 4-ml fraction from A (above) on silver-nitrate coated silica.		
2–3	1.4	0.7
3–4	2.1	2.0
4–5	1.0	1.4
5–6	1.9	0.5
6–7	1.5	2.2
7–8	10.8***	0.9
8–9	42.3***	0.9
9–10	50.0***	0.0
10–11	8.0*	2.7
11–12	6.0	2.3
12–13	4.4	3.1
13–14	4.6	1.6

[a] Initial chromatographic fractions were used at 30 beetle-days per test and subsequent chromatographic fractions were used at 27 beetle-days per test. Bioassay counts are the numbers of beetles flying upwind to the filter paper baits and alighting during the 3-min tests. Coattractant was propyl acetate (1% in mineral oil, 20 μl/test).

[b] Differences from the control at the 0.05, 0.01, and 0.001 levels denoted by *, **, and ***, respectively [t tests in $\log(X + 1)$ scale, using pooled error].

propyl acetate (1% in mineral oil, 20 μl) were 0.1, 5.1, 5.7, and 26.1, respectively. The combination of pheromone and propyl acetate was significantly more attractive than either alone. Both single attractants were significantly more attractive than the control but did not differ from each other (LSD, 0.05 level). The idea of synergism is supported by the fact that the total activity of the combination of pheromone and coattractant is greater than the sum of the activities of pheromone alone plus coattractant alone.

Beetle-Derived vs. Synthetic Pheromone. The presence of **1** accounted for all of the observed activity of beetle-derived materials at three stages of purification (Table 2). Thus **1** was the dominant attractant in the volatile collections, and there was no evidence for additional important sex-specific attractants having been overlooked. We found no significant differences in the activity of the beetle-derived pheromone vs. the synthetic pheromone. The beetles were very sensitive to **1** in the wind tunnel, responding significantly to a 50-pg dose.

Pheromone Production: Group vs. Individual Beetles. Pheromone production was significantly lower for the group of males than for individual males.

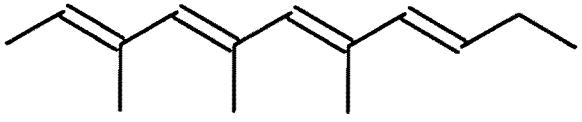

FIG. 1. Male-produced aggregation pheromone of *Carpophilus obsoletus* Erichson: (2*E*,4*E*,6*E*,8*E*)-3,5,7-trimethyl-2,4,6,8-undecatetraene **1**.

TABLE 2. COMPARATIVE ACTIVITY OF SYNTHETIC **1** AND BEETLE-DERIVED **1** AT VARIOUS STAGES OF PURIFICATION

Step of purification[a]	Amount of **1** per test (ng)	Mean bioassay count (N = 8)[b]		
		Beetle-derived **1**	Synthetic **1**	Control
Whole collection	0.5	14.5a	14.2a	0.1b
After hexane–ether gradient	0.05	5.3a	4.3a	0.2b
	0.5	8.3a	8.3a	0.0b
	5.0	15.6a	14.6a	0.2b
After silver-coated silica HPLC	0.5	9.4a	9.9a	0.0b

[a] Coattractant was propyl acetate (1% in mineral oil, 20 μl/test).

[b] Bioassay counts are the numbers of beetles flying upwind to the filter paper baits and alighting during the 3-min tests In each row, means followed by the same letter are not significantly different [LSD, 0.05 level, balanced incomplete block analysis, log(X + 1) scale].

Overall means were 2.2 ng/male/day for the group ($N = 15$ volatile collections) and 72 ng/day for individuals ($N = 45$; 15 volatile collections from each of three beetles); the t statistic for the difference was 6.92 ($P < 0.0001$). However, there was also significant variability among the three individuals tested: Their means (standard deviation) were 28.4 (33.5), 45.3 (27.4), and 141 (90.2) ng/day. For six of the 45 collections, no pheromone was detected, but daily production was as high as 388 ng/day. Except for brief lapses, pheromone production occurred throughout the entire two-month study. The reason for occasional nonproduction of pheromone by mature individual beetles is unknown but may involve environmental conditions such as condition of diet. Group pheromone production never stopped entirely, possibly because beetles vary somewhat in their perception of an unfavorable environment.

Monitoring of pheromone production by the individual males was continued until beetle death; one individual survived 9.5 months and produced pheromone during all but the last month. Over this period, pheromone production decreased gradually, in approximately linear fashion until ceasing at 8.5 months [regression model: daily pheromone production (ng) = 152 − 0.6 × age (days), $R^2 = 0.3586$, $F_{1,50} = 27.96$, $P < 0.001$]. Considerable day-to-day variation in pheromone production was observed; the standard deviation about the regression line was 55 ng/day.

Given a maximum pheromone-production level of 388 ng/beetle-day and the observation that beetles will respond in the wind-tunnel bioassay to a level of only 50 pg of the pheromone (with propyl acetate as a host-type synergist), only one hundredth of one percent of the total maximum daily pheromone emission is required for the biological response of aggregation.

Field Trap Data. Synthetic **1** was active in the test at the date garden. The pheromone, in combination with fermenting whole-wheat bread dough, attracted a total of 200 *C. obsoletus* (54% female); the dough by itself attracted no *C. obsoletus*. Mean trap catches ($N = 48$; two traps, 24 weeks) for pheromone plus fermenting bread dough and dough only were 4.2 and 0, respectively ($F = 316$, $P < 0.001$). Weekly trap catches varied (Figure 2). Seasonal activity peaked during the months of July and August, but substantial catches were also recorded in late April. A similar pattern of trap catch over time was observed for *C. obsoletus* previously (Bartelt et al., 1992b).

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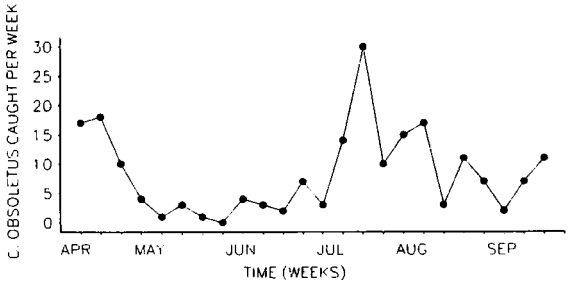

FIG. 2. Trap catch pattern over time for *C. obsoletus* in a date garden near Oasis, California, April 21 to September 29, 1992. Each point is the weekly catch, totaled over two traps baited with a combination of pheromone (500 μg **1**) plus fermenting bread dough. (No *C. obsoletus* were caught in the two control traps baited with dough only.)

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DERMAL GLAND SECRETIONS OF TROPICAL BONT TICK, *Amblyomma variegatum* (ACARINA: IXODIDAE): BIOLOGICAL ACTIVITY ON PREDATORS AND PATHOGENS

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Abstract—When they are mechanically disturbed, all instars of the tropical bont tick *Amblyomma variegatum* exude droplets of a liquid on the dorsal, lateral, and ventral cuticle. These spread out and quickly evaporate. In this study, the possible role of these secretions was investigated in relation to predators and pathogens. In laboratory bioassays, it was demonstrated that the secretions from engorged larvae, nymphs, and females have an antibiotic activity against the bacteria species *Bacillus thuringiensis* and *Serratia marcescens*, combined with a repellent effect on a potential predator, the fire-ant *Solenopsis geminata*.

Key Words—*Amblyomma variegatum*, Acarina, Ixodidae, dermal glands, *Solenopsis geminata*, Hymenoptera, Formicidae, defensive secretion, entomopathogenic bacteria, antibiotic.

INTRODUCTION

The tropical bont tick *Amblyomma variegatum* has been introduced from Africa during the last centuries to islands of the Indian Ocean and also to numerous islands of the Lesser Antilles and to Puerto Rico (Uilenberg et al., 1984). It is responsible for important economic losses in goats and cattle, is associated with

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the bacterial skin infection dermatophilosis, and it is a vector of a rickettsiosis of ruminants i.e., heartwater disease.

Preliminary studies on potential biological control agents in Guadeloupe (Barré et al., 1991) have led to the conclusion that this species has very few natural enemies: the main predators are birds (the cattle egret, *Bubulcus ibis*, preying mainly on the host) and the fire-ant *Solenopsis geminata* (Barré et al., 1991). In Guadeloupe, release of different tick instars into various pasture systems failed to indicate the existence of organisms pathogenic to ticks (Barré, unpublished results). Moreover, laboratory bioassays with numerous strains of entomopathogenic nematodes (Mauléon et al., 1993), fungi and bacteria (Mauléon, unpublished results) were always negative. In the literature, the only reports of an effective biological control agent on a tick species are the studies of Samish and Glazer (1991, 1992): *Boophilus annulatus* can be infected in the laboratory by nematode strains.

Concurrent with these observations, we noticed that when *A. variegatum* are roughly handled, or when their legs are pinched with forceps, larvae, nymphs, and adults immediately produce droplets of a transparent liquid on the dorsal, lateral, and ventral cuticle. These droplets spread out and dry after a few minutes. Yoder et al. (1993a,b) described this phenomenon in *Dermacentor andersoni*, *D. variabilis*, *Amblyomma americanum*, and *A. maculatum* and showed that the secretions have a repellent activity against predatory ants.

One hypothesis for the resistance of ticks to natural enemies could be the existence of defensive secretions. The aim of this study was to investigate the possible role of these secretions, particularly towards predators and pathogens. Thus, repellency, toxicity and antibiotic effects were evaluated on different species.

METHODS AND MATERIALS

Tick Rearing. The ticks used in this study were the descendants of engorged females collected on cattle in Gardel (Guadeloupe, Grande-Terre), in December 1982. Wild ticks, coming from different areas of Guadeloupe, were regularly added to the strain to avoid inbreeding. Larvae were fed in cotton bags placed on the ears of goats. Nymphs and adults were applied in cotton jersey bags to the flanks of the goats. These bags were glued to the animal's skin after it had been shaved and washed with acetone, and the free end of the bag was closed with a rubber band. After the blood meal, ticks were placed in vials, which were kept in a Plexiglas box with a double floor. The lower part of the box was filled with a saturated Na_2CO_3 solution to produce a constant relative humidity of 90–95%. The ticks were submitted to the natural photoperiod (photophase ranging from 12 to 14 hr) and the temperature varied from 22°C (at night) to 26°C.

Collection of Secretions. The different instars, engorged or not, were manipulated under a binocular microscope until they discharged their gland contents. The secretions were collected by touching the droplets with a piece of filter paper, which was then immersed in a glass vial containing a solvent. After 10 min, the filter papers were removed from the solvent and the solutions stored at −18°C until use. When pure secretions were tested, the droplets were allowed to touch the body of the potential prey used in the tests (see below).

Test of Repellent Activity. We studied the predatory behavior of a common ant of Guadeloupe, namely the fire ant *Solenopsis geminata*, which has been shown to be a predator of *A. variegatum*. Fragments of colonies were collected in the field by digging up a nest and placing soil containing large numbers of ants in boxes. The sides of the boxes were lined with sticky paper to prevent ants from escaping. They were fed with *Galleria mellonella* (Lepidoptera) larvae. One preliminary series of experiments on predation was carried out by testing the activity of fourth-instar *G. mellonella* larvae, moistened under the microscope with pure secretions of unfed male ticks. Bristol paper with 10 treated and 10 untreated *G. mellonella* larvae were presented to the ants, and the number of predated larvae was recorded after 1, 3, 5, 7, 9, and 15 min. Ten replicates were made and results analyzed using the Wilcoxon nonparametric test.

A second series of tests on predation was made using extracts of the secretions from engorged larvae, nymphs, and females, detached from their host for one or six days. Secretions were extracted with hexane, methanol, and water. Engorged larvae were also extracted by washing for 2 hr (160 larvae in 1 ml of solvent); the solution was then evaporated under argon to reach a concentration of one engorged larva equivalent per microliter (1 ELE/μl). Engorged nymphs were stressed with forceps under the dissecting microscope, and the secretions absorbed with pieces of filter paper, previously washed with *n*-hexane. After 2 hr in the solvent, the papers were removed and the solution evaporated to a volume of 100 μl to give a concentration of 0.5 engorged nymph equivalent (ENE)/μl. The engorged females were extracted following the same method, but only three females were used for the same volume of solvent [concentration of 0.03 engorged females equivalent (EFE)/μl]. For each test, four fourth-instar *G. mellonella* larvae were placed on Bristol paper (3 × 3 cm, 1.5 cm between two larvae), one treated with 10 μl of extract and three controls treated with 10 μl of solvent alone. The Bristol paper was placed on the ant nest and, after 3 min, the number of predated larvae noted. Ten replicates were carried out for each extract. For statistical analyses, the chi-square test was used.

Toxicity Tests. Pure secretions or aqueous extracts were tested on last-instar larvae of *G. mellonella* by contact and through injection. The larvae were maintained in continuous culture in plastic boxes (26 × 13.5 × 7.5 cm). Egg masses were placed between beeswax sheets, and all the instars were fed with pollen.

The boxes were kept at 30 ± 2°C in a room with a constant dim light and relative humidity of 90%. For contact toxicity, larvae were directly moistened with the secretion of engorged female ticks, detached either for one day or for six days [0.5 engorged female secretion equivalent per larva (0.5 EFSE/L)]. The control consisted of larvae rubbed with damp filter paper. For each treatment (secretion or control), six replicates were made with five last-instar *G. mellonella* larvae, placed individually in holes of a microtitration box, closed with a plastic wire-net lid. For statistical analysis, the nonparametric Mann & Whitney test was used. For injection tests on toxicity, the engorged female secretion was extracted in water and sterilized with a filter (Millipore, 0.45 μm) and concentrated to give an extract of 0.5 EFSE/15 μl. For each replicate, 10 larvae were injected with 15 μl of the extract under the cuticle and 10 larvae with 15 μl sterile water; each batch of 10 larvae was placed in a 9-cm-diameter Petri dish, the floor of which was covered with filter paper. Six to 10 replicates were carried out. For both contact and injection tests, the mortality was recorded after 24, 48, and 72 hr.

Test of Antibiotic Activity. We used the method of gelose diffusion antibiograms, utilizing filter-paper disks moistened with the secretions of different instars of *A. variegatum*. The antibiotic activity towards entomopathogenic bacteria (*Bacillus thuringiensis* and *Serratia marcescens*), fungi (*Beauveria bassiana*, *Metarhizium anisopliae*, and *Paecilomyces fumosoroseus*), and entomoparasitic nematodes (*Heterorhabditis* sp.) was investigated. Mueller-Hinton gelose was placed in 9-cm-diameter Petri dishes, dried for 1 hr at 25°C under a laminar flow, and then was kept at 4°C. Pure cultures of *B. thuringiensis* (LM2174 strain, from the Institut Pasteur, Paris), *S. marcescens* (colored biotype), *B. bassiana* (strain 252, Abbott), *M. anisopliae* (strain 131, INRA la Minière), *P. fumosoroseus* (strain 56, INRA la Minière), and *Heterorhabditis* sp. (strain Saint-François, INRA Guadeloupe, concentration 10,000 nematodes/ml) were inoculated on the gelose (1 ml/Petri dish). The dishes were dried for 15 min at 28°C. Seven filter-paper disks, each 6 mm in diameter, were then placed in each Petri dish. A reference disk was placed at the center with three control and three experimental disks arranged around it. Reference disks for the bacteria were impregnated with 30 μg kanamycin standard; those for fungi were impregnated with an antifungal agent, Actidione (14 mg/ml); for the nematodes, the reference disk was untreated. The control disks were untreated. The experimental disks had been moistened with the secretions of engorged females and nymphs, which had been detached for 0, 6, 12, 18, 24, 30, 36, 42, or 48 hr, or for 4, 6, 8, or 10 days. The secretions of one female or 10 nymphs were used to impregnate one disk, and the quantity of secretion on a disk was measured by weighing the paper before and after application of the secretion. The Petri dishes were incubated for 24 hr at 28°C, with the lid up. After incubation, the effect of the secretions on bacterial or fungal growth was determined by

measuring the diameter of the area around each treated disk, which was free of either bacteria or fungi. Results were assessed by expressing this diameter (D_s) as a proportion of the diameter of the area around the reference disk (D_r) in which bacterial or fungal growth had been inhibited. In the case of *Heterorhabditis* sp., the dishes were observed under the binocular microscope to detect dead nematodes.

RESULTS

Test of Repellent Activity. The predatory behavior of the ants, in response to the pure male secretions, is represented in Figure 1. Throughout the observation period, the *G. mellonella* larvae that were treated with the male secretions suffered significantly less predation than did controls. However, the predation pattern depends on the solvents used to collect the extracts and the tick instar. In the case of engorged females, the repellent activity is quite clear: extracts obtained with hexane, methanol, or water are very active for females detached from the host for either one or six days (Figure 2c). As shown in Figure 2a and 2b, nymph and larval extracts have a significant level (5%) of repellency only with ticks detached for six days; when they are detached for less than one day, the hexanic and aqueous extracts are not active, particularly in the case of larval extracts. The low level of repellent activity in larvae and nymphs detached for less than one day could be explained by qualitative (variation of the secretion contents after the detachment) or quantitative aspects (low production after early detachment). However, the results indicate that for larvae and nymphs detached for less than one day the polar compounds extracted by methanol but not by hexane have a repellent effect, even at low doses. All other hexane extracts are active, probably due to the higher amounts of products extracted.

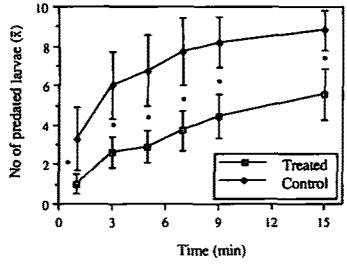

FIG. 1. Predatory behavior of the fire ant *Solenopsis geminata* towards *Galleria mellonella* fourth-instar larvae, moistened with pure cuticular secretions of unfed male *A. variegatum*. For each replicate ($N = 10$) 10 larvae were presented to the ant nest; values with an asterisk are significantly different from the control (Wilcoxon test, $\alpha = 5\%$).

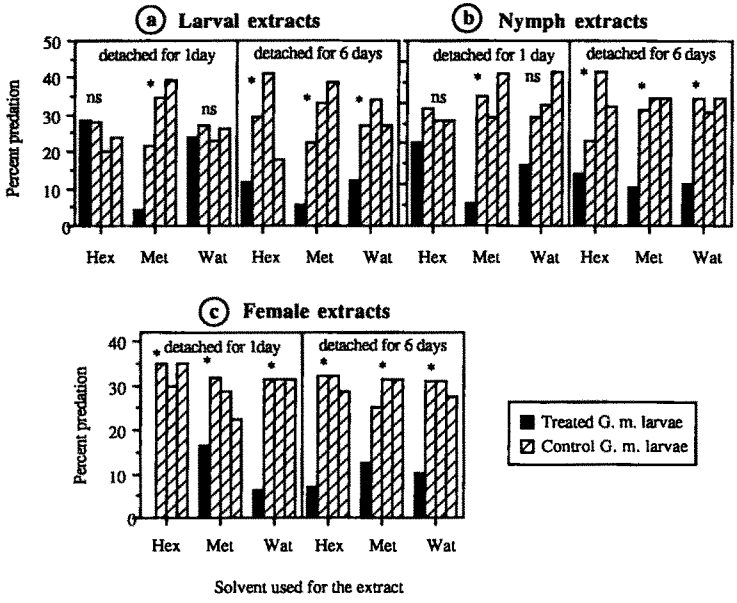

FIG. 2. Predatory behavior of the fire ant *Solenopsis geminata* towards *Galleria mellonella* fourth-instar larvae treated with extracts of cuticular exocrine gland secretions of *A. variegatum*. Hex: hexanic extract, Met: methanolic extract, Wat: aqueous extract, G. m.: *Galleria mellonella*. For each replicate ($N = 10$) one treated and three control larvae were exposed to the ants. Values with an asterisk are significantly different from the control at the 5% level (chi-square test).

Toxicity Tests. At any observation time, the mortality was very low for both the control and the treated larvae in contact toxicity tests (Figure 3). The mortality was null for all the replicates carried out in injection tests with the extracts. We can conclude that these secretions are not toxic for the tested insects.

Test of Antibiotic Activity. The results are summarized in Figure 4. For all situations, the negative control (no secretion on the filter paper) did not inhibit bacterial or fungal growth. The tests carried out with fungi and nematodes were all negative. The secretions of engorged females had an antibiotic activity on both *B. thuringiensis* and *S. marcescens*, with D_s/D_r ranging from 0.25 to 0.6. The quantity of secretion present on the filter paper (0.3–3.6 mg) did not seem to influence the extent of the effect. This antibiotic activity began just after the detachment of the females from the host and remained relatively constant. The antibiotic activity of nymphal secretions is less obvious: for some periods of detachment (0–6 and 6–12 hr in the case of *B. thuringiensis*), the antibiosis

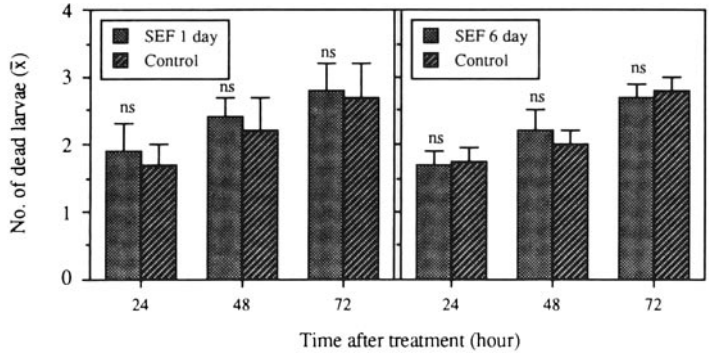

FIG. 3. Contact toxicity of the secretions of engorged female *A. variegatum* (SEF), detached for one or six days, towards *Galleria mellonella* last-instar larvae. For each treatment (secretions and control), six batches of five larvae were used. Values with an asterisk are significantly different from the control at the 5% level (Mann–Whitney test).

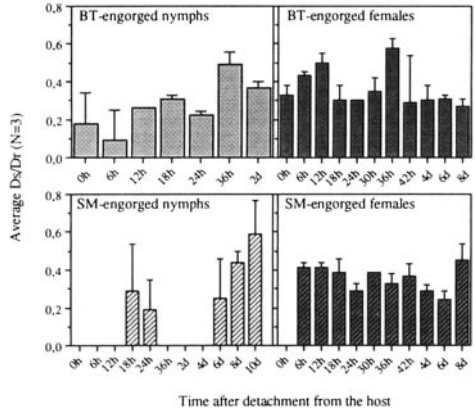

FIG. 4. Antibiotic activity of *A. variegatum* cuticular exocrine gland secretions extracted from engorged nymphs and females and tested on *Bacillus thuringiensis* (BT) and *Serratia marcescens* (SM). D_s/D_r: diameter of the bacteria growth inhibition disk obtained with the secretion/diameter of the bacteria growth inhibition disk obtained with the reference (kanamycin).

pattern is not clear; the area sometimes looks like a halo. In the case of *S. marcescens*, only three detachment periods (18–24, 24–36 hours, 10 days) show a clear antibiosis pattern, with D_s/D_r values ranging from 0.19 to 0.59.

Thus, we can conclude that the secretions have an antibiotic effect on the two tested bacteria. *Bacillus thuringiensis* seems more sensitive than *Serratia*

marcescens, particularly in the case of engorged nymphs, and ticks detached for short periods.

DISCUSSION

We report here the production by larvae, nymphs, and adults of a tick species of an allomonal secretion, which may act as a defensive secretion against different natural enemies. The secretion seems to be produced by dermal glands. In Ixodidae, three types of dermal glands are described (Lees, 1947), differing in size, which increase in volume during the blood meal and which excrete their contents after detachment from the host, before the molt. The function of dermal glands is uncertain and has been reviewed by Hackmann (1982). Some hypotheses have been proposed for different tick species and instars: sensory functions, production of waterproof lipids, activity in relation to secretion of the molting fluid, and deposition of a cement layer. In *Ixodes holocyclus*, Kock [1967, in Hackmann (1982)] has described a pink fluid secreted from dermal glands of all engorged instars. In female *Boophilus microplus*, Binnington [personal communication, in Hackman (1982)] reported the secretion of a clear fluid when they were handled. The composition and function of these secretions were not investigated. In *Dermacentor* species, Yoder et al. (1993a,b) report the presence of a pressure-stimulated secretion, deriving from the large pores occurring in lines on the dorsolateral surface (sagittiform sensilla); chemical analyses demonstrated squalene as a major component; large amounts of synthetic squalene act as a repellent towards the fire ant *Solenopsis invicta*.

In *A. variegatum*, Diehl et al. (1991) described the presence of innervated dermal glands in ventral, lateral, and dorsal position of both sexes. The defensive secretion that we have described should be produced by these glands.

Preliminary analyses with polyacrylamide gel electrophoresis (Diehl et al., in preparation) showed the presence of proteins in the secretions. These analyses were performed with engorged females and nymphs, at different periods after detachment from the host. No qualitative differences were observed between these secretions, but the extraction and preparation of the samples have to be standardized to obtain repeatable results.

In other arthropods, such as insects, defense mechanisms consisting of exocrine secretions in response to aggression or perturbation are known, particularly in the case of Heteroptera. Various organic compounds have been reported to play a role in repellency or toxicity towards predators, but proteins are rarely mentioned.

Soluble proteins are present on the surface of the honey bee cuticle, probably secreted by the tarsal glands (Zupko et al., 1993). The authors suggest that these surface proteins may possess antibacterial activity or may be involved in the pheromone degradation process.

Bacterial infections are known to induce production of a variety of hemolymph proteins (Dunn, 1986), but a recent study seems to indicate that these immune responses can be effective against other organisms such as fungi, trypanosomes, yeasts, and *Plasmodium* sp. (Kimbrell, 1991). Nevertheless, it has not yet been demonstrated that the exocrine secretion of proteins plays a role in defense.

We are now focusing on the identification of these semiochemicals, which appear to be very complex in both their function and chemical composition.

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WHY ARE PREDATOR URINES AVERSIVE TO PREY?

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Abstract—Predator odors often repel prey species. In the present experiments, we investigated whether changes in the diet of a predator, the coyote (*Canis latrans*) would affect the repellency of its urine. Furthermore, because predator odors have a high sulfur content, reflecting large amounts of meat in the diet, we investigated the contribution of sulfurous odors to repellency. Our results were consistent with the hypothesis that diet composition and sulfurous metabolites of meat digestion are important for the repellency of predator odors to potential prey.

Key Words—*Aplodontia rufa*, avoidance, *Canis latrans*, *Cavia porcellus*, coyote, guinea pig, mouse, mountain beaver, *Mus musculus*, predator odors, *Peromyscus maniculatus*, urine.

INTRODUCTION

Predator odors are generally aversive to potential prey species, including *Lepus* and *Cuniculus* (Sullivan et al., 1985a; Sullivan, 1986; Sullivan and Crump, 1984, 1986a; Robinson, 1990), *Aplodontia* (Epple et al., 1993; Nolte et al., 1993), *Microtus* (Dickman and Doncaster, 1984; Gorman, 1984; Stoddart, 1976,

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1980, 1982; Sullivan et al., 1988; Merkins et al., 1991), *Cethrionomys* and *Apodemus* (Robinson, 1990; Stoddart, 1980), *Thomomys* (Sullivan et al., 1990; Sullivan and Crump, 1986b), *Marmota* (Swihart, 1991), *Rattus* (Vernet-Maury, 1980; Vernet-Maury et al., 1984), *Capreolus* and *Cervus* (Abbott et al., 1990; Van Haaften, 1963), and *Odocoileus* (Melchiors and Leslie, 1985; Muller-Schwarze, 1972; Sullivan et al., 1985b; Swihart et al., 1991). Avoidance appears to be mediated, at least in part, by urinary constituents that are not species specific. Such compounds may constitute a generalized meat-eater cue (Epple et al., 1993; Abbott et al., 1990) or a predator "leitmotif" (Stoddart, 1980). Although the chemical nature of this leitmotif remains obscure, one possibility is that it features odoriferous constituents that reflect the diet composition of the predator. Such odors might include sulfur-containing metabolites of protein digestion (Mason et al., 1993). We conducted the present experiments to assess whether diet manipulations would affect the repellency of urine from a predator to several potential prey species and to investigate the contribution of sulfurous compounds to its repellency.

METHODS AND MATERIALS

Experiment 1

This experiment was conducted to determine whether diet manipulations would affect the repellency of a predator urine to several prey species.

Subjects. Four rodent species: mountain beaver (*Aplodontia rufa*, $N = 9$), house mice (*Mus musculus*, $N = 40$), deer mice (*Peromyscus maniculatus*, $N = 20$), and guinea pigs (*Cavia porcellus*, $N = 12$) served as subjects. The two species of mice and the guinea pigs were bred and tested at the Monell Chemical Senses Center. Each animal was individually caged (mice, 27 × 21 × 14 cm; guinea pigs, 50 × 50 × 30 cm) under a 12L:12D cycle (light onset at 0700 hr). The mountain beavers were trapped in the vicinity of Olympia, Washington. These animals were housed and tested in outdoor pens (3 × 3 × 1.5 m) at the Denver Wildlife Research Center (DWRC) Olympia, Washington, research facility.

Stimuli. Four male coyotes (*Canis latrans*) at the DWRC research facility in Millville, Utah, were randomly selected from the captive colony to serve as urine donors. During the first two weeks of July 1992, these animals were maintained exclusively on a diet of cantaloupe. On days 15–20 they were placed in metabolism chambers (120 × 70 × 80 cm) for 18 hr and urine samples were collected (FU). These samples were pooled and frozen at 40°C. On day 21, these four coyotes were returned to their normal diet of minced raw meat for a two-week period. After this two-week period, additional urine samples (MU) were collected on each of five days, as described above. These MU samples

were also pooled and frozen. Subsequently, FU and MU samples were shipped to the Monell Chemical Senses Center and the DWRC Olympia, Washington, facility for chemical and behavioral tests.

Procedures. Similar testing methods were used with all species. Urine samples (1 ml) were pipetted onto pieces of absorbent paper placed inside small (38-mm-diam. × 8-mm) perforated plastic containers. For mice, the containers were placed inside aluminum weigh-boats and the assemblies were secured 10 cm apart with thumbtacks to pieces of wood. Forty sunflower seeds were then weighed and placed in each weigh-boat around the plastic containers. For guinea pigs, the perforated plastic containers were put inside the animals' food cups, and surrounded with 20 g of guinea pig chow. The cups were placed 10 cm apart in the guinea pigs' cages. During mountain beaver tests, the perforated containers were placed inside weigh-boats and surrounded with 10 1-cm^3 apple cubes. The assemblies were then secured 10 cm apart to the ground with metal stakes.

Mice and guinea pigs were adapted to an 18-hr food deprivation schedule. On each of two pretreatment days, these animals were given their respective foods for 2 hr in weigh-boats/food cups containing perforated containers with a piece of absorbent paper treated with 1 ml of tap water. On the two treatment days that followed, the animals were given the same foods; however, this time the absorbent paper was treated with 1 ml of either FU or MU. At the end of each test session, the amount of food remaining in each weigh-boat/food cup was assessed.

Unlike the other three species, mountain beavers were not food deprived because they do not readily adapt to deprivation regimes. On two pretreatment days, all animals were presented with apple cubes placed in the weigh-boats with tap-water-treated absorbent paper in perforated containers. On the two treatment days that followed, the animals were given 24-hr, two-choice tests between apple cubes associated with either FU or MU. The number of cubes remaining after 3, 6, 12, and 24 hr was assessed. Since some animals did not respond until late in the 24-hr period, and because the pattern of results was similar at each measurement interval, only the 24-hr results were presented. Apple weights were not evaluated because moisture loss or gain could not be accurately assessed.

For all individuals of each species, the left–right position of FU urine samples was randomly determined on day 1, and then reversed on day 2.

Analyses. The data for each species was evaluated separately in a two-factor repeated measures analysis of variance (ANOVA). In each case, urine type was the main effect, with the animals nested within urine type and the repeated measure was days. Tukey tests (Winer, 1971, p. 201) were used to isolate significant differences among means subsequent to the omnibus procedure ($P < 0.05$).

Results. All species ingested more (mountain beaver, $P = 0.0064$; house mice, $P = 0.0002$; deer mice $P = 0.0082$; guinea pigs $P = 0.0444$) food from bowls containing FU than they did from bowls scented with MU (Figure 1). There were no day effects ($P > 0.25$) and no interactions between urine types and days ($P > 0.25$).

Experiment 2

Differences in animal response between FU and MU samples in the first experiment may have reflected dilution effects. To control for this possibility, FU and MU samples were lyophilized and then rehydrated to a common concentration. Because all species showed similar responses in experiment 1, deer mice were arbitrarily selected to serve as subjects.

Subjects. Experimentally naive deer mice ($N = 14$) served as subjects.

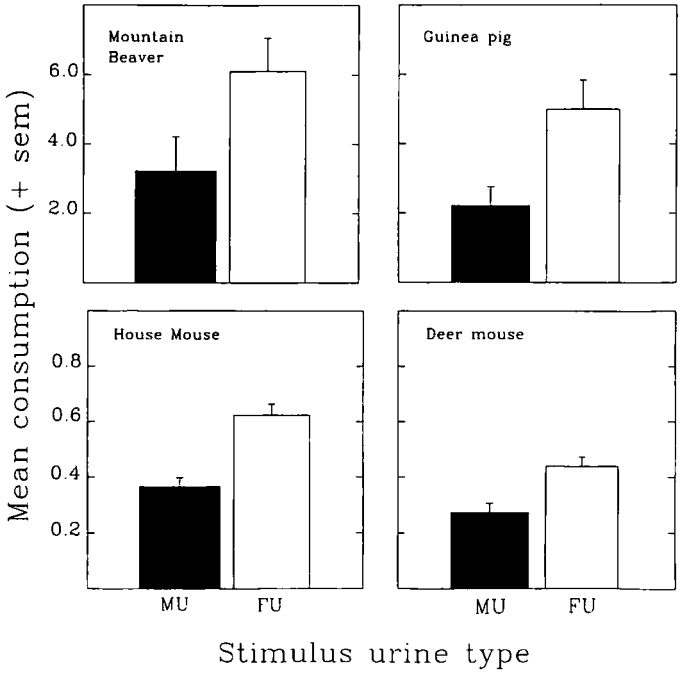

FIG. 1. Intake by mountain beavers, guinea pigs, house mice, and deer mice of food (number of apples cubes, grams of guinea pig chow, grams of sunflower seeds, grams of sunflower seeds; respectively) associated with urine collected either from coyotes on a fruit diet (FU) or from coyotes fed meat (MU).

Animals were individually caged (27 × 21 × 14 cm) under a 12L:12D cycle (light onset at 0700 hr).

Stimuli. FU and MU were the same as used in experiment 1 except, prior to testing, the samples were lyophilized and then rehydrated to a common concentration.

Procedures. Test apparatus was the same as described for experiment 1. On each of four pretreatment days, food-deprived mice (18 hr) were presented with two weigh-boats, each containing 40 sunflower seeds of known weight, and a perforated plastic container with an enclosed absorbent paper treated with 1 ml of tap water. After 2 hr, the weight-boats were removed and the number of seeds remaining were assessed. On the basis of ingestion, animals were then assigned to two counterbalanced groups. Four treatment days immediately followed. One group ($N = 8$) was given 2-hr tests offering choices between sunflower seeds associated with FU or tap water. The other group ($N = 6$) was given 2-hr tests offering choices between sunflower seeds associated with MU or tap water.

Analysis. Difference scores were calculated by subtracting FU or MU ingestion from control (tap-water-scented) ingestion by individual deer mice. Therefore, high positive values indicate avoidance of the urine stimuli relative to the control, while negative values indicate preference for the urine stimuli. These data were evaluated in a two-factor repeated measures ANOVA. Tukey post-hoc tests were used to isolate differences among means ($P < 0.05$).

Results. Mice ingested relatively more ($P = 0.0030$) from FU-scented bowls than from MU-scented bowls (Figure 2). There were no day effects ($P > 0.05$) or interaction between urine types and days ($P > 0.05$).

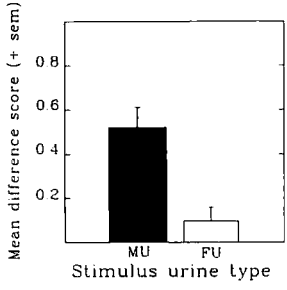

FIG. 2. Difference scores calculated by subtracting ingestion by deer mice of sunflower seeds associated with urine from coyotes on a fruit diet (FU) or urine from coyotes on a meat diet (MU) from ingestion of sunflower seeds associated with a control stimuli (tap water) during two-choice tests.

Experiment 3

Diet manipulation experiments showed that the presence/absence of meat in a donor coyote's diet affected the repellency of the urine. This result led us to hypothesize that by-products of meat digestion in urine, such as sulfur compounds, might contribute to aversiveness. To test this hypothesis, we removed sulfur-containing substances from MU by precipitation with mercuric chloride. The responses of mountain beaver to urine stimuli with and without sulfur compounds were then investigated.

Subjects. Experimentally naive mountain beaver ($N = 10$) were obtained and maintained as described in experiment 1.

Stimuli. MU was the same as described for experiment 1. Sulfur-free urine (SR) was prepared by precipitating MU with mercuric chloride (Golovnya et al., 1972). Briefly, 319 mg of 1 mmol mercuric chloride was dissolved in 2 ml of methanol and added to 25-ml samples of MU. The mixture was agitated for 30 min, stored for 3 hr at room temperature (23°C), and finally, centrifuged. The centrifugate was collected for behavioral testing.

Procedure. Test apparatus and procedures were similar to those described for mountain beaver in experiment 1. Counterbalanced two-choice tests were used to assess ingestion of apple cubes associated with either tap water or MU, and apple cubes associated with either tap water or SR. For each test, apple cubes were available for 12 hr on two consecutive days.

Analysis. Difference scores were calculated by subtracting the number of apple cubes associated with MU or SR ingested by mountain beaver from the number of apple cubes associated with tap water ingested during the same trial. Therefore, high scores indicate a relative avoidance of the urine stimuli, while low scores indicate an indifference. Subsequently, these data were evaluated in a two-factor repeated measures ANOVA. Tukey post-hoc tests were used to isolate differences among means ($P < 0.05$).

Results. Sulfur-free urine samples from meat-fed coyotes were less offensive ($P = 0.0016$) to mountain beavers than whole urine from coyotes fed meat (Figure 3). There were no day effects ($P > 0.05$) or an interaction between urine types and days ($P > 0.05$).

Urine Fractionation and Chromatography

The first two experiments indicated that the donor coyote's diet affected the aversiveness of urine. In the third experiment, urine from meat-fed coyotes precipitated with mercuric chloride was less offensive to mountain beaver than was the whole urine. Chromatograms were subsequently prepared to depict changes in sulfur constituents of these test stimuli.

Stimuli. FU and MU samples were the same as used in experiment 1, and mercury precipitation was as described for experiment 3.

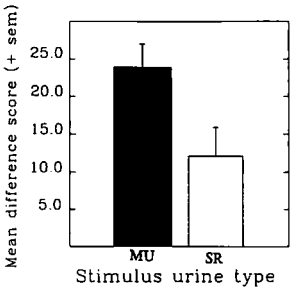

FIG. 3. Difference scores calculated by subtracting ingestion by mountain beavers of apple cubes associated with urine from coyotes on a meat diet (MU) or sulfur-free urine from coyotes on a meat diet (SR) from ingestion of apple cubes associated with a control stimuli (tap water) during two-choice tests.

Methanol Fractionation. Coyote urine (12 ml) was applied to a glass chromatographic column (1 cm ID, 40 cm) packed with Kiselgel RP-18 (40–63 μm; Merck) previously washed with 40 ml of methanol and 40 ml of distilled water. Hydrophilic components of the urine were eluted with distilled water until the current eluate showed a neutral pH; the column was then dried by passing compressed nitrogen through it, and hydrophobic compounds were eluted with methanol (20 ml). The resultant methanol fractions were then removed.

Analytical HPLC. Analytical HPLC of all methanol fractions was performed using a Rainin HPXL two-pump solvent delivery system, Zorbax OS (4.6 × 250 mm) column, and Dynamax UV-M detector. The analytical conditions were: mobile phase: 0–5 min isocratic acetonitrile water 5:95, 5–40 min linear gradient to acetonitrile water 4:6, 40–60 min linear gradient to 100% acetonitrile, 60–80 min isocratic 100% acetonitrile; detection at 204 nm; injection volume, 100 μl.

Results. Among the four major constituents on chromatograms of MU and FU, there were only three peaks common to both urine types (Figure 4). Two of these peaks disappeared from MU after treatment with mercuric chloride (Figure 5).

DISCUSSION

The aversiveness of coyote urine to herbivorous rodents fluctuated with a change in the predator's diet. All species tested ingested more food from bowls scented with urine collected for coyotes fed cantaloupe than they did from bowls scented with urine from coyotes on a meat ration. Deer mice also showed similar tendencies in the second experiment, an indication that reduced repellency reflected a urine solute constituent change rather than merely a dilution effect.

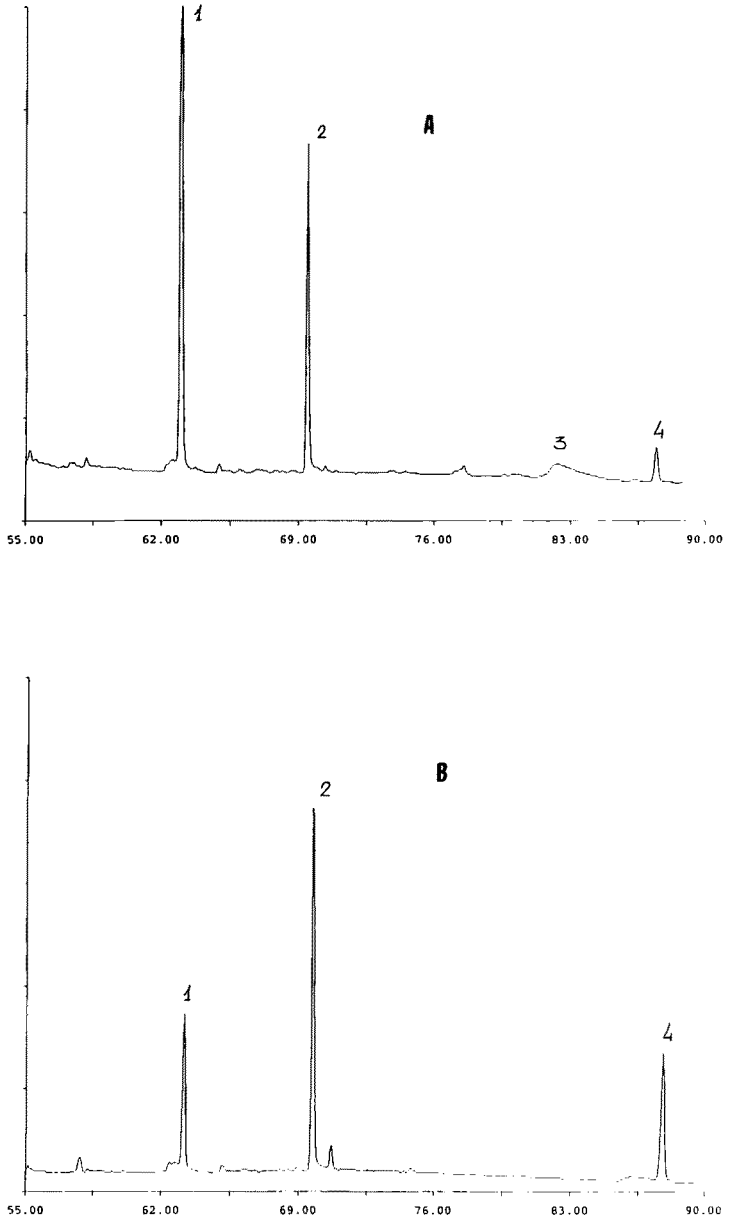

FIG. 4. Chromatograms of urine (methanol fractions) collected from coyotes (A) on a fruit diet (FU) or (B) on a meat diet (MU).

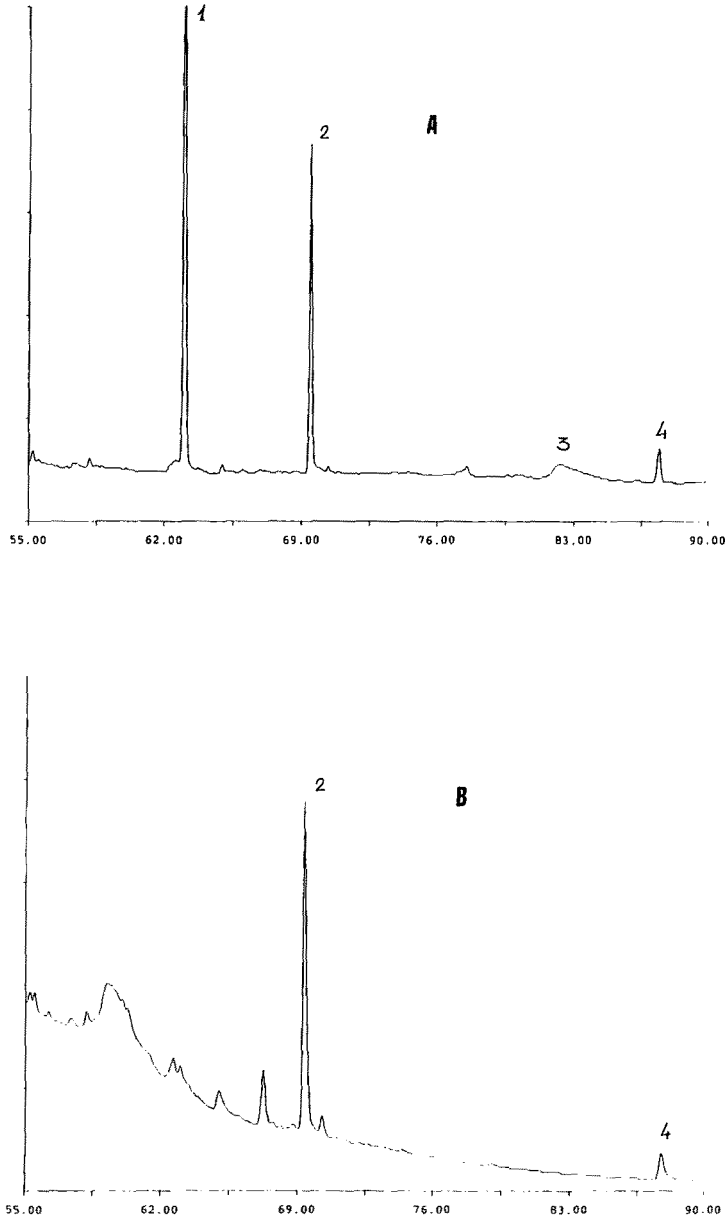

FIG. 5. Chromatograms of urine (methanol fractions) from meat-fed coyotes before (A) and after (B) treatment with mercuric chloride.

The appearance and/or maintenance of sulfur compounds in coyote urine appears to be correlated with repellency. MU treated with mercuric chloride to remove sulfur compounds was less aversive to mountain beavers than untreated samples. Further, sulfur constituents are a primary difference between MU and FU samples. The major component of MU (peak 1, Figure 4) is a minor component in FU, and at least one other constituent of MU (peak 3) is absent from FU. Both of these peaks disappeared from MU after it was treated with mercuric chloride (Figure 5), indicating that they were sulfur-containing.

Nearly half the volatile material in coyote urine is methyl (2-methyl-2-butenyl) sulfide (MMBS) (Schultz et al., 1988), and this substance is common in other predator urines as well (Raymer et al., 1988; Jorgenson et al., 1978). However, the retention time of synthetic MMBS did not correspond to the retention times of peaks 1 or 3 for MU. Although these peaks are probably not MMBS, they are as yet unknown.

FU and SR were both less aversive than MU; however, food intake was reduced in the presence of FU and in the presence of SR relative to control stimuli. Prior experiments indicate that unfamiliar but behaviorally irrelevant odors (e.g., butyric acid, guinea pig urine) do not inhibit feeding by mountain beaver (Epple et al., 1993). Therefore, this weak avoidance may be attributed to other nonsulfurous compounds. For example, acetophenones occur in mustelid (Sullivan and Crump, 1986b) and wolf (Raymer et al., 1986) scents. ortho Aminoacetophenone (OAP) reduces forage intake by mountain beaver (Nolte et al., 1993). Avoidance of plants treated with OAP, however, is considerably less than mountain beaver avoidance of the same plants treated with whole coyote urine (Nolte et al., 1993).

Overall, the present results are consistent with the hypothesis that (as yet unknown) sulfurous odors associated with meat digestion are important for the repellency of predator urines to potential prey. A plausible speculation is that prey use such odors as cues to the diet composition of donors. It may also be that predators attend to sulfurous urinary odors and interpret them as indicators of diet composition; this provides an explanation of the attractiveness of predator urines to these species (Bullard et al., 1983; Fagre et al., 1982).

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INTRA- AND INTERSPECIFIC AVOIDANCE OF AREAS MARKED WITH SKIN EXTRACT FROM BROOK STICKLEBACKS (*Culaea inconstans*) IN A NATURAL HABITAT

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Abstract—The detection of a chemical alarm pheromone may allow receivers to avoid areas where a predator has captured the prey's conspecifics. We marked minnow traps with either brook stickleback (*Culaea inconstans*) skin extract or a control of distilled water and tested whether sticklebacks avoided the skin extract marked traps in a natural habitat. Significantly more sticklebacks were captured in traps marked with control water, thereby demonstrating avoidance of conspecific skin extract. The stickleback captured in traps marked with conspecific extract were significantly smaller than those captured in traps marked with control water, implicating ontogenetic factors (i.e., experience or physiological development) in the development of the response. We also captured significantly fewer finescale dace (*Chrosomus neogaeus*) and fathead minnows (*Pimephales promelas*) in traps marked with skin extract. These data suggest that dace and minnows may benefit by avoiding areas where predators have recently captured sticklebacks.

Key Words—Alarm signaling, antipredator behavior, Schreckstoff, brook stickleback, *Culaea inconstans*, finescale dace, *Chrosomus neogaeus*, fathead minnow, *Pimephales promelas*.

INTRODUCTION

An effective means for prey to reduce the risk of capture is to avoid areas where predation occurs. For many prey fishes this avoidance may be facilitated by

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some type of alarm pheromone system. In the typical Schreckstoff alarm system of cyprinid fishes, for example, exposure to alarm substance results in area avoidance. Fricke (1987) demonstrated that blind cave fish (*Astyanax fasciatus*) avoid the area of a tank where alarm substance is introduced. In field experiments, Von Frisch (1941), Smith (1976), and Mathis and Smith (1993) have demonstrated avoidance of areas marked with the cyprinid alarm pheromone. European minnows (*Phoxinus phoxinus*) abandon artificial feeding stations (Von Frisch, 1941) and breeding male fathead minnows (*Pimephales promelas*) temporarily abandon breeding territories (Smith, 1976) following exposure to conspecific alarm substance. Fathead minnows also avoid traps marked with conspecific alarm substance (Mathis and Smith, 1993). As the Schreckstoff is released only by mechanical damage to the skin, receivers that avoid an area that contains alarm substance effectively avoid sites where a predator has recently captured or injured a conspecific.

In a laboratory experiment, Mathis and Smith (1994) reported that brook stickleback increase shoaling in response to skin extract from conspecifics, but not to a control of swordtail (*Xiphophorus helleri*) skin extract or distilled water. This was the first report of a chemical alarm signal for fishes in the Order Gasterosteiformes. Chemical alarm signals have been reported from a variety of other non-ostariophysan fishes, including darters (Perciformes), gobies (Perciformes), and sculpins (Scorpaeniformes) (reviewed in Smith, 1992), as well as from a variety of other taxa including gastropods (Stenzler and Atema, 1977; Atema and Stenzler, 1977), echinoderms (Snyder and Snyder, 1970), and amphibians (Hews and Blaustein, 1985; Hews, 1988). To our knowledge, however, there have not been any field studies examining the effectiveness of chemical alarm signals in promoting area avoidance in non-ostariophysan fishes. In this study we examine whether brook stickleback avoid areas in a natural habitat that are marked with stickleback skin extract. Specifically, we test the hypothesis that brook sticklebacks will be captured significantly less often in traps marked with conspecific skin extract than in traps marked with a control substance.

In this study we also examine the influence of ontogenetic factors (experience or physiological development) in affecting the ability of fish to respond to skin extract. Given that small fish are generally less developed and less experienced, we would expect a greater proportion of small fish to be caught in skin extract marked traps than in control traps if experienced or more developed fish are more likely to avoid skin extract.

Finally, we examine the possibility that heterospecific prey fishes [finescale dace (*Chrosomus neogaeus*) and fathead minnows] will avoid areas marked with stickleback skin extract. Since all three of these species are small prey fishes that share common predators, individuals should benefit by detecting alarm signals produced by heterospecifics. Cross-species responses to alarm signals are common for closely related fishes (e.g., Pfeiffer, 1963; Smith, 1982; Smith

et al., 1991); however, only one study has demonstrated cross-species reactions by fishes from distantly related taxa. Mathis and Smith (1994) demonstrated that brook sticklebacks respond with antipredator behavior upon exposure to alarm substance from fathead minnows. In this study we test whether finescale dace and fathead minnows (Superorder Ostariophysi) will avoid skin extract from distantly related brook sticklebacks (Superorder Acanthopterygii).

METHODS AND MATERIALS

Stimulus Preparation. We prepared the experimental stimulus from 13 male and 12 female sticklebacks ($\bar{X} \pm$ SD fork length = 5.71 $\pm$ 0.58 cm). We killed the donors by a blow to the head and removed a skin fillet from both sides of each fish. The total area of skin collected was approximately 90 cm^2. Immediately upon removal, we placed the skin samples together in 100 ml of chilled glass-distilled water. We homogenized the skin samples with a polytron homogenizer and filtered the homogenate through glass wool to remove any solid particles. We diluted the skin extract with an additional 300 ml of glass-distilled water (total volume = 400 ml).

We threaded two rectangular cellulose sponges (2.0 × 2.0 × 5.0 cm) onto each of 13 stainless steel wires and saturated the sponges with the skin extract. Since sticklebacks respond with antipredator behavior to stickleback extract but not to swordtail extract or distilled water (Mathis and Smith, 1994), as a control stimulus in this study we saturated identical wire-threaded sponges with glass-distilled water. All sponges were frozen at approximately −20°C for three days. They were removed from the freezer on the day of the experiment and were kept on ice until the beginning of the experiment.

Experiment Protocol. We conducted the trapping experiment in Marshy Creek in south-central Saskatchewan in September 1993. At this site the creek is 1–2 m deep and several meters wide. Previous censuses have indicated that brook stickleback, finescale dace, and fathead minnows occur in large numbers at this site.

We assigned 13 minnow traps to each of the experimental and control treatments. The traps (Gee's Improved Minnow Traps) consisted of roughly cylindrical wire enclosures (43 cm length × 22 cm diameter) with a funnel located at each end leading into the trap. The funnel entrances were approximately 2.5 cm in diameter. We attached the wires to the inside of each trap with the sponges located approximately 4 cm in front of each trap entrance.

We placed control and experimental traps into the water along the north and south shores of the creek, with approximately 5 m between traps. The order of placement of the experimental and control traps was determined randomly with the proviso that no more than two traps in a row could be of the same

treatment condition. Trap pairs (consisting of one control and one experimental trap) were placed into the water at the same time, with the first pair being set at 1030 hr. It took approximately 1 hr to set all the traps. Each trap remained in the water for 3.5 hr, with each pair of traps being removed simultaneously. All fish from each trap were removed and preserved in 10% formalin. The fish were identified to species and their length was measured: fork length for finescale dace and fathead minnows and total length for brook sticklebacks (stickleback tails are rounded not forked).

RESULTS

A total of 1156 fish were captured in the 26 traps. Of these, 822 (71.1%) were brook sticklebacks, 257 (22.2%) were finescale dace, and 77 (6.7%) were fathead minnows. A Wilcoxon–Mann–Whitney test (Siegel and Castellan, 1988) revealed that for each of the three species, there were significantly more fish caught in control traps than experimental traps (brook sticklebacks, $W_x = 139$, $m = 13$, $N = 13$, $P = 0.032$, one-tailed, Figure 1a; finescale dace, $W_x = 140$, $m = 13$, $N = 13$, $P = 0.037$, one-tailed, Figure 1b; fathead minnows, $W_x = 135.5$, $m = 13$, $N = 13$, $P = 0.021$, one-tailed, Figure 1c).

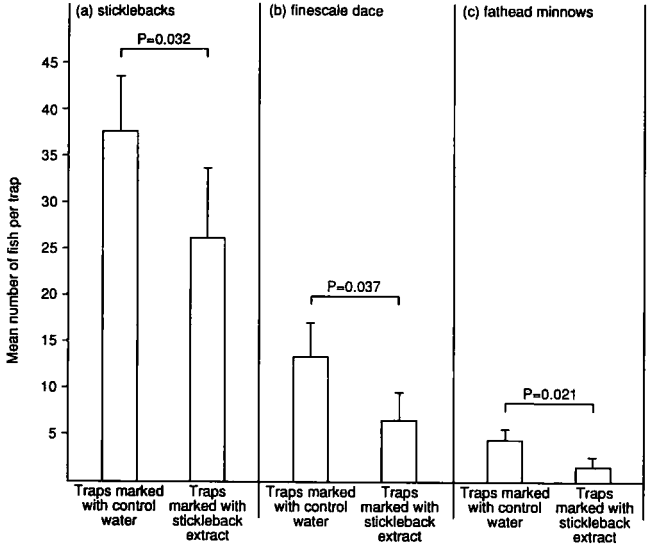

FIG. 1. Mean (+SE) number of: (a) sticklebacks, (b) finescale dace, and (c) fathead minnows captured in traps marked with either brook stickleback skin extract or control water.

One explanation for the difference in the number of finescale dace and fathead minnows caught in control and experimental traps may be interspecific social facilitation. The dace and minnows may be attracted to control traps that contain sticklebacks rather than avoiding the skin extract in experimental traps. To test for social facilitation, we used a Spearman rank-order correlation to test the relationship between the number of sticklebacks and both the number of dace and the number of minnows in the control traps. There was no correlation between the number of sticklebacks and the number of finescale dace in the control traps ($R_s = 0.332$, $N = 13$, $P > 0.10$, one-tailed). However, there was a significant correlation between the number of sticklebacks and the number of fathead minnows in control traps ($R_s = 0.075$, $N = 13$, $P < 0.005$, one-tailed).

A Wilcoxon–Mann–Whitney test revealed that for all three fish species, individuals caught in experimental traps were significantly smaller than individuals in control traps (brook sticklebacks, $W_x = 121$, $m = 12$, $N = 14$, $P = 0.027$, one-tailed, Figure 2a; finescale dace, $W_x = 59$, $m = 8$, $N = 11$, $P = 0.038$, one-tailed, Figure 2b; fathead minnows, $W_x = 13.5$, $m = 4$, $N = 10$, $P = 0.008$, one-tailed, Figure 2c). The observed size differences may be due

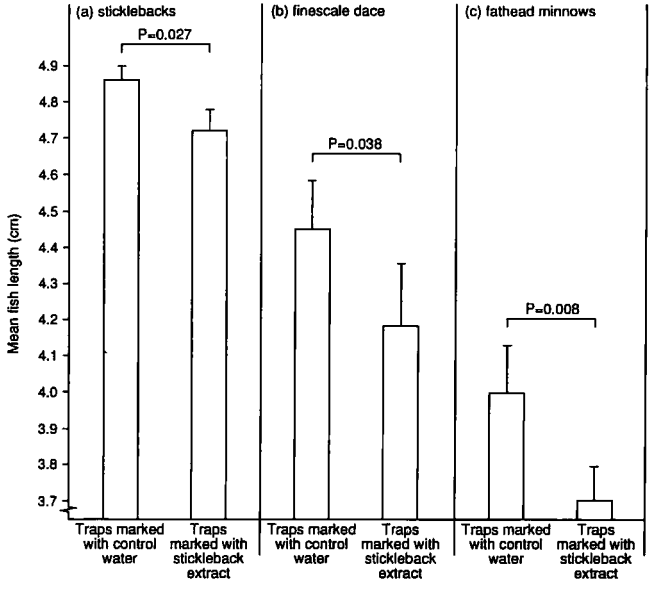

FIG. 2. Mean (+SE) length (cm) of: (a) sticklebacks, (b) finescale dace, and (c) fathead minnows captured in traps marked with either brook stickleback skin extract or control water (length measurements are based on total length for sticklebacks and fork length for finescale dace and fathead minnows).

to either significantly more small sized fishes caught in skin extract marked traps or significantly more large sized fishes caught in control traps or both. For each control and experimental trap, we determined the proportion of sticklebacks belonging to each of three equal size categories (3.2–4.3, 4.4–5.5, and 5.6–6.7 cm total length). A Wilcoxon–Mann–Whitney test shows that there were both significantly more small stickleback caught in experimental traps ($W_x = 139$, $m = 13$, $N = 13$, $P = 0.0495$) and significantly more large stickleback caught in control traps ($W_x = 110$, $m = 13$, $N = 13$, $P < 0.007$). Insufficient numbers of captured fish precluded a similar analysis for finescale dace and fathead minnows.

DISCUSSION

The results of this study demonstrate that brook sticklebacks avoid areas marked with conspecific skin extract in a natural habitat, and thereby provide a field confirmation of chemical alarm signaling by brook sticklebacks. By avoiding conspecific skin extract, sticklebacks likely avoid high risk areas where predators have been successful at capturing conspecifics. In this study 40.75% of the sticklebacks were caught in traps marked with stickleback extract. Despite the fact that the concentration of skin extract, in terms of skin area per trap, in our study was approximately 4.3 times greater than that used in a similar trapping study by Mathis and Smith (1993), they reported that less than 4% of fathead minnows were captured in traps marked with conspecific extract. This apparent difference in the effectiveness of skin extract in promoting area avoidance is consistent with Mathis and Smith's (1994) suggestion that the antipredator behavior associated with skin extract exposure in sticklebacks is weaker than the response of fathead minnows to their conspecific skin extract.

For laboratory-reared cyprinid fishes, responses to alarm substance occur when the fish have no prior experience. Nevertheless, in cyprinids physiological development and experience play a role in determining the strength of the response (Pfeiffer, 1963; Waldman, 1982; Magurran, 1989). In sticklebacks it is unknown if naive fish show antipredator behaviors upon first encountering conspecific extract; however, as we demonstrated that small (and therefore less developed or experienced) fish were more likely to be caught in skin extract-marked traps, experience or physiological development likely plays a role in determining the strength of the response in sticklebacks.

In this study we have demonstrated a cross-superorder response to an alarm signal as finescale dace significantly avoided traps marked with stickleback skin extract. Interpreting the response of fathead minnows to stickleback extract is somewhat more complicated. Minnows were caught significantly less often in traps marked with stickleback skin extract; however, the number of fathead

minnows is positively correlated with the number of sticklebacks in the control traps. Therefore, it is possible that social facilitation is occurring and that the minnows simply are being attracted by the stickleback in the control traps as opposed to avoiding the stickleback extract in the experimental traps. In a laboratory study, Mathis and Smith (1994) failed to detect a fright response by fathead minnows exposed to skin extract from brook stickleback. Nevertheless, for both finescale dace and fathead minnows, it appears that the response to stickleback extract is influenced by experience or development as smaller (younger) fish were more often captured in skin extract-marked traps.

By avoiding areas marked with conspecific skin extract, brook sticklebacks likely are able to lower their risk of capture. By detecting interspecific alarm signals, similar benefits may also extend to finescale dace and fathead minnows that are sympatric with sticklebacks.

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ALARM RESPONSES IN THE CRAYFISH *Orconectes virilis* AND *Orconectes propinquus*

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Abstract—Individuals of two species of crayfish (*Orconectes virilis* and *O. propinquus*) were tested in the laboratory for responses to chemicals released from physically damaged conspecifics. Individuals of *O. propinquus* did not show an alarm response to crushed conspecifics. Individuals of *O. virilis* responded to a water-borne substance released from crushed conspecifics by assuming an intermediate posture and ceasing movement. Similar alarm responses were shown by individuals of *O. virilis* to crushed congeneric individuals (*O. propinquus*), and these responses were not eliminated by either freeze-thawing the crayfish used to prepare the signal or by treating freshly crushed crayfish with the enzyme trypsin. Individuals of *O. virilis* showed strong feeding responses to solutions prepared from frozen fish flesh but showed a mixture of alarm and feeding responses to freshly killed fish. These results indicate that the alarm substance used by *O. virilis* is widespread.

Key Words—Alarm, crayfish, *Orconectes virilis*, *Orconectes propinquus*, chemical signals.

INTRODUCTION

The use of chemical signals by aquatic organisms has been documented in a variety of contexts. Alarm responses shown to substances released from physically damaged conspecific individuals have been reported in a variety of fish (see review by Smith, 1992), amphibians (Pfeiffer, 1963), marine gastropods (Stenzler and Atema, 1976), and echinoderms (Snyder and Snyder, 1970). In addition, responses to substances released from physically undamaged but disturbed conspecific individuals have also been reported for such diverse groups as rats (Valenta and Rigby, 1968), earthworms (Ressler et al., 1968), and crayfish (Hazlett, 1985a,b, 1989). Alarm responses reduce the chances of predation

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(Hews, 1988; Mathis and Smith, 1993), the activity that would most commonly lead to the physical damage releasing the chemical, while disturbance responses increase the chances of a receiver avoiding any of a variety of potentially negative situations (Hazlett, 1985a,b).

Previous work on crayfish (Hazlett, 1985a,b, 1989, 1990a) focused on detection of sex pheromones and on the disturbance response shown by individuals of *Orconectes virilis*. Detection of substances released by disturbed-but-undamaged conspecifics or individuals of other crayfish species was followed by the assumption of a posture that is intermediate between the resting and the highly aroused postures, and by an increase in slow locomotory movements. Similar responses were demonstrated for a species of marine hermit crab (Hazlett, 1990b). However, alarm responses to damaged conspecifics have not been demonstrated for crayfish or indeed for very many crustaceans. Some hermit crabs respond to conspecific hemolymph by an increase in the frequency of gastropod shell manipulation or by fleeing, depending upon shell fit (Rittschof et al., 1992), and the pebble crab *Philyra laevis* is attracted to a food source much less if a crushed conspecific is nearby (McKillup and McKillup, 1992).

The experiments described in this report were designed to determine: (1) if crayfish show an alarm response to damaged conspecifics and, if so, (2) is that response specific to damaged conspecifics? A third set of questions involved initial characterization of the nature of the chemicals involved.

METHODS AND MATERIALS

Observations were done in the laboratory at the University of Michigan Biological Station near Pellston, Michigan, during the summers (June–August) of 1992 and 1993. Pilot studies were done in 1992, while data analyzed in this paper were gathered in 1993. The crayfish studied, adults of *Orconectes virilis* (Hagen 1870) and *Orconectes propinquus* (Girard 1852), were collected from the Maple River (Emmet County, Michigan), and the fish used for preparation of solutions to be tested were collected from Douglas Lake. The basic procedure followed in all tests were similar to that used in the study of other chemical-response systems in crayfish (Hazlett, 1985a,b, 1989, 1990a).

Crayfish were placed in individual 10-gallon aquaria (bottom dimensions 25 × 50 cm) that were visually isolated from one another. The aquaria were continually aerated and contained 12.5 liters of lake water at a depth of 10 cm and a portion of a clay pot as a shelter. After two to three days of acclimation, crayfish were observed individually for 8-min periods during the morning and/or afternoon. The posture of a crayfish (raised, lowered, intermediate) and whether the crayfish was locomoting or not was recorded on a portable computer equipped with an event program. When crayfish are in the lowered posture, the

chephalothorax is in contact with the substrate, the abdomen is curled, and the chelipeds are pulled into the body with the tips of the chelae touching the substrate. The lowered posture is the most common posture of undisturbed crayfish during the day. Locomotion rarely occurs when the crayfish is in the lowered posture. In the intermediate posture, the chephalothorax is elevated slightly above the substrate, the chelipeds are held below the horizontal but not in contact with the substrate, and the abdomen is almost parallel to the substrate but the telson is perpendicular to the substrate. In the raised posture, the chelipeds are held horizontal to the substrate or higher, the chephalothorax is clearly elevated above the substrate by several millimeters, and the telson is horizontal. Crayfish locomote in both the intermediate and raised postures, although rapid locomotion most commonly occurs with the raised posture.

The 8-min observation period was divided into a 2-min early period and a 6-min late period. This division was based on pilot observations that indicated differences in the responses shown under various conditions between these two periods. During the 2-min period, solutions were introduced into the aquarium via a peristaltic pump at the rate of 20 ml/min. The addition of 40 ml into 12,500 ml of lake water represents a dilution of greater than 1:300. The water introduced into the aquaria was either control ("self" water, circulated from the observation aquarium back into the same aquarium) or a test solution. An individual crayfish was tested with a maximum of four different test solutions over a two-day period, and then sex and chephalothorax length were determined.

Individuals of *O. propinquus* were tested with three solutions: the self-water control, a crushed-conspecific solution, and a food solution. For the crushed-conspecific solution, a medium-sized (approx. 3 g wet weight) individual of *O. propinquus* was crushed, placed in 400 ml of distilled water, and the mixture was stirred. All solutions used for both crayfish species were filtered to remove large particles (Fisher P8 Coarse grade filter paper) and introduced to the aquaria of individual crayfish. Introductions were initiated within 10 min after preparation of a solution. Five or fewer individuals were usually tested with one solution type during one observation session; thus, utilization of solutions was completed within an hour after preparation. The food solution was prepared by mixing 4 g of frozen then thawed rock bass flesh (*Amblopolites rupestris*, (Rafinesque 1817)) in 400 ml of distilled water and filtering. Given the results of the tests with these solutions (see Results), no additional solutions were tested with *O. propinquus*.

A total of nine solutions were tested with individuals of *O. virilis*, including the self-water control. The sequence of solutions tested was varied systematically. For tests with *O. virilis*, the crushed *O. propinquus* and food solutions were prepared as described above. The crushed conspecific tests used a 4-g individual of *O. virilis*. In order to initially characterize the nature of the alarm substance, two additional solutions were prepared. The freeze–thawed conspe-

cific solution used a 4-g *O. virilis* that was frozen, thawed shortly before use, and then crushed and mixed in 400 ml of distilled water. The trypsin + conspecific solution was prepared by crushing a live *O. virilis*, mixing it in 400 ml of distilled water, adding 25 mg of trypsin (Nasco), stirring for 5 min, and then filtering the mixture with coarse filter paper.

Observations during pilot studies indicated that individuals of *O. virilis* are much more likely to show a feeding response to freeze–thawed fish flesh than to freshly killed fish. Therefore, the last sets of solutions were prepared with 4 g of muscle tissue from freshly killed fish (rather than frozen), mixed in 400 ml of distilled water, filtered, and tested as soon as possible. Three species of freshly killed fish were tested with individuals of *O. virilis*: rock bass (*A. rupestris*), bluegill sunfish (*Lepomis macrochirus*, Rafinesque 1819) and yellow perch (*Perca flavecens*, (Mitchill 1814)).

The data analyzed were the number of seconds spent in particular postures (raised, intermediate, or lowered) and activities (moving/not moving) during the introduction of the different solutions. The number of transitions from one posture or activity to another (number of acts) was also examined as an overall measure of activity. All analyses were by ANOVA with post-hoc individual comparisons, using the Bonferroni procedure to adjust the critical values for the number of comparisons made (Wilkinson, 1988).

For both *Orconectes virilis* and *O. propinquus*, there were a number of variables that could be compared to examine the responses to the solutions introduced to individuals. In both species, the results of ANOVAs testing for differences among test solutions for particular variables followed the same pattern. There were highly significant differences (P values much less than 0.001) for time spent in different postures and the time spent moving in both the early (first 2 min) and late (last 6 min) portions of the observation period as well as in the sums for the full observation period. However, the statistical differences were almost always much more marked for the late, last-6-min portion. Therefore, all individual comparisons reported below focus on the later period. In addition, since the crayfish had to be in one of the three postures recognized (raised, intermediate, lowered), only two of the three can be statistically analyzed, although descriptions of differences in responses will include all three postures.

RESULTS

In *O. propinquus*, the only significant differences in responses to the three solutions tested were for the raised posture (ANOVA, $F = 4.73$, $P = 0.03$) and time spent moving (ANOVA, $F = 4.77$, $P = 0.012$) during the last 6 min of the observation period. Individual post-hoc comparisons showed that the

differences between responses to crushed conspecific and the food stimulus were significant (raised posture, $F = 9.4$, $P = 0.003$; moving, $F = 9.2$, $P = 0.004$), but there were no significant differences between the crushed conspecific and control solutions (raised $F = 2.4$, $P = 0.121$; move, $F = 1.0$, $P = 0.309$). Thus, although individuals of *O. propinquus* spent less time in the raised position and moved less when a solution of crushed conspecific was introduced compared to a food stimulus (Table 1), their behavior was not different from that shown during a control period of self-water introduction. None of the other variables measured showed a significant overall ANOVA for these crayfish.

In *O. virilis*, there were very significant differences in responses to all nine tests in the time spent in the low posture ($F = 6.1$, $P = 0.0001$), raised posture ($F = 19.4$, $P = 0.0001$), time spent moving ($F = 18.8$, $P = 0.0001$), and in the number of acts executed during the total observation period ($F = 7.0$, $P = 0.0001$). Crayfish spent more time in the intermediate position when a crushed conspecific solution was introduced, more time in the raised position and moving when a frozen fish solution was introduced, and more time in the lowered posture during the control water introduction (Table 2). When crushed conspecific stimulus was introduced, the crayfish spent less time moving than when frozen fish stimuli were presented, but there was no difference in time spent moving between the crushed conspecific stimuli and control (Table 3). Thus, when a signal associated with a physically damaged conspecific was detected, individuals of *O. virilis* assumed an intermediate, "watchful" posture but did not move. While not quantified regularly, it was frequently observed that if a crayfish was outside its clay burrow when the crushed conspecific stimulus was introduced, the crayfish would rapidly back into its burrow and then assume the intermediate posture. In some cases, especially during pilot tests in 1992, individuals that had been moving slowly about the aquarium prior to the introduction of alarm substance seemed to freeze and cease all motion for some minutes.

TABLE 1. TIME SPENT BY INDIVIDUALS OF *Orconectes propinquus* EXPOSED TO VARIOUS SOURCES OF POTENTIAL CHEMICAL SIGNALS[a]

	Seconds, mean (SE)				
	Low posture	Intermediate posture	High posture	Moving	Acts, *N* (SE)
Control	163 (37)	125 (25)	72 (29)	46 (21)	13 (4)
Crushed *O. propinquus*	104 (30)	242 (31)	14 (11)	14 (9)	10 (3)
Freeze-thawed rock bass	72 (26)	163 (37)	125 (30)	107 (28)	15 (3)

[a] The values for postures and time spent moving are for the last 6 min of the observation periods while the number of acts executed is for the full 8-min periods.

TABLE 2. TIME SPENT BY INDIVIDUALS OF *Orconectes virilis* EXPOSED TO VARIOUS SOURCES OF POTENTIAL CHEMICAL SIGNALS.[a]

	Seconds, Mean (SE)				
	Low posture	Intermediate posture	Raised posture	Moving	Acts, *N* (SE)
Control	233 (34)	108 (30)	17 (10)	14 (8)	7 (2)
Crushed *O. virilis*	137 (34)	205 (32)	17 (10)	15 (8)	7 (2)
Freeze–thaw rock bass	36 (18)	111 (23)	211 (29)	192 (27)	21 (3)
Crushed *O. propinquus*	165 (30)	190 (29)	3 (2)	1 (2)	4 (1)
Crushed *O. virilis* + trypsin	211 (32)	142 (31)	5 (5)	5 (5)	4 (1)
Freeze–thaw *O. virilis*	128 (24)	217 (25)	13 (9)	11 (8)	5 (2)
Fresh rock bass	79 (19)	197 (26)	83 (21)	75 (20)	12 (2)
Fresh bluegill	139 (27)	67 (15)	153 (31)	125 (27)	14 (2)
Fresh perch	45 (25)	143 (27)	171 (30)	153 (29)	21 (4)

[a]The values for postures and time spent moving are for the last 6 min of the observation periods while the number of acts executed is for the full 8-min periods.

TABLE 3. INDIVIDUAL COMPARISON VALUES (*F* Value and Associated *P* Values) FROM POST-HOC COMPARISONS OF TIME SPENT BY INDIVIDUALS OF *O. virilis* DURING DIFFERENT STIMULUS PRESENTATIONS

Crushed conspecific vs	Low posture	Raised posture	Moving
Control			
F	7.2	0.001	0.001
P	0.008	0.97	0.99
Frozen rock bass			
F	6.0	62.8	62.6
P	0.015	0.000	0.000
Crushed Congeneric			
F	0.44	0.28	0.35
P	0.51	0.60	0.56
Freeze-Thawed Conspecific			
F	0.42	0.02	0.02
P	0.83	0.88	0.89
Conspecific + trypsin			
F	3.3	0.22	0.20
P	0.07	0.64	0.66

In order to further understand the nature of the alarm signal and test if it was specific to individuals of *O. virilis*, the responses to solutions made from freshly crushed conspecifics were compared to the responses to freshly crushed individuals of congeneric individuals (*O. propinquus*), freshly crushed conspecifics treated with trypsin, and freeze–thawed conspecifics. There were clearly no differences between the responses to solutions made from freshly crushed conspecifics, freshly crushed congenerics, or freeze–thawed conspecifics (Tables 2 and 3). Although there was a slight tendency for the responses to the trypsin-treated conspecific solution to include less time in the intermediate posture and more in the low posture, i.e., closer to the control responses, the differences were not significant. Thus, it appears that the chemical(s) involved in the alarm response shown by individuals of *O. virilis* are (1) present in individuals of a related species and (2) not destroyed by either treatment with the enzyme trypsin or freezing and then thawing.

The use of frozen fish as the basis for a food stimulus was initially motivated by convenience. However, based upon pilot tests, it appeared that frozen fish may be a stronger food stimulus than freshly killed fish. The responses of crayfish to freshly killed rock bass were different from the responses to either frozen rock bass or to crushed conspecifics (Table 4). Because the amount of time spent in raised and intermediate postures and the time spent moving appear to be about halfway between the extremes of alarm response and food stimuli response (Table 2), it appears that a freshly killed rock bass presents both alarm and food stimuli to individuals of *Orconectes virilis*. The responses to solutions

TABLE 4. INDIVIDUAL COMPARISON VALUES (*F* Value and Associated *P*) FROM POST-HOC COMPARISONS OF TIME SPENT BY INDIVIDUALS OF *O. virilis* IN DIFFERENT POSTURES AND ACTIVITIES DURING DIFFERENT STIMILUS PRESENTATIONS

Fresh rock bass vs	Low posture	Raised posture	Moving
Frozen rock bass			
F	1.08	26.7	26.4
P	0.300	0.000	0.000
Crushed conspecific			
F	1.9	7.0	7.2
P	0.17	0.009	0.008
Fresh bluegill			
F	1.3	4.9	2.9
P	0.26	0.027	0.89
Fresh Perch			
F	0.47	8.5	7.7
P	0.49	0.004	0.006

prepared from freshly killed bluegill sunfish were similar to those shown to the freshly killed rock bass. However, the responses shown to fresh perch were different than those shown to fresh rock bass (Table 4) and more closely resembled the feeding responses shown to frozen rock bass.

DISCUSSION

Results from this study demonstrate the presence of an alarm response in the crayfish *Orconectes virilis*. The alarm response (intermediate posture without movement) is similar to the alarm responses shown by some other taxa and presumably functions to reduce the probability of predation. Assumption of the intermediate posture may simply be a correlate of the receiver of the signal no longer being in a relaxed, resting state but rather on alert for potential danger. By not moving, the crayfish reduces the chance of detection by possible predators. This response has been reported for other aquatic animals and demonstrated to reduce the chances of predation (Werner and Anholt, 1993).

The behavior of *O. virilis* is influenced in a wide variety of ways by chemical signals. Pheromones and other chemicals are used to identify sex (Hazlett, 1985a), species (Tierney and Dunham, 1982), maternal condition (Ameyaw-Akumfi, 1976), the presence of food (Tierney and Atema, 1988), the presence of animals that are stressed or disturbed (Hazlett, 1990a,b), and the presence of physically damaged individuals. The detection of the chemical signals associated with each of these situations or conditions contributes to distinct responses by crayfish.

The observation that individuals of the related species *Orconectes propinquus* did not show a statistically significant alteration of behavior when solutions made from crushed conspecifics were introduced, compared to the control solutions, was a bit surprising. However, individuals of this species also did not show a response to disturbance pheromone (Hazlett, 1990a,b) and the responses to sex pheromone appear less well developed in this species (Tierney and Dunham, 1982). The reduced sophistication of chemical communication in *O. propinquus* compared to that of *O. virilis* is puzzling given how close these species are phyletically, although they are in different subgenera (Fitzpatrick, 1987), and how similar they are ecologically. While there are some slight differences in habitat use, individuals can occur almost side by side in many habitats and are faced with a similar array of predators (Hobbs, 1993) and other dangers. Because *O. propinquus* is slightly more active during the day than *O. virilis* (although both are basically nocturnal), *O. propinquus* may rely less upon chemical communication.

The existence of both a disturbance response and a distinct alarm response in the same species leads to a variety of questions. Individuals of *O. virilis*

behave in a low-level alert fashion upon detection of disturbed individuals in the area and move out of their burrow and around their environment as if looking for the source of disturbance. In contrast, the alarm response involves a reduction of locomotion and pulling back into their burrow if the crayfish is out in the open. These responses would appear to be a defense against a particular type of danger, predation. While the exoskeleton of a crayfish could be broken and the alarm substances released by a variety of situations (e.g., a rock falling on the animal, extremes of aggressive interactions, predation), the response shown appears appropriate for this one source of damage.

It is very interesting that for both the disturbance response and the alarm response of *O. virilis*, animals other than conspecifics can be sources of the chemicals involved. In addition to other species and genera of crayfish, other taxa of amenotilic animals can be sources of disturbance chemicals (Hazlett, 1989, 1990a). Similarly, other crayfish as well as fish can be a source of the signal that predatory danger is present. While some predators in aquatic habitats may well specialize on one taxa or another, it is not unusual for predators such as wading birds, snapping turtles, or larger fish to eat a variety of prey that would include both crayfish and small fish. Working with the marine snail *Ilyanassa*, Stenzler and Atema (1976) found some response to damaged congenerics, but this was reduced compared to the response shown to damaged conspecifics—unlike the situation with *O. virilis* where there was no difference in responses to conspecific and congeneric damage.

The alarm responses of *O. virilis* and lack of them in *O. propinquus* may help explain some differences in the feeding behavior of the two species. Individuals of *O. virilis* that are reasonably well fed in the laboratory will not feed upon recently killed conspecifics or upon recently killed fish such as bluegill or rock bass. They will do so if the flesh of these animals has decayed for several days or if the crayfish have not fed recently. In contrast, individuals of *O. propinquus* will readily feed upon conspecifics and freshly killed fish even when well supplied with other sources of food. Traps placed in the field and baited with freshly killed fish often catch more *O. propinquus* initially and catch individuals of *O. virilis* only after a few days of decay (unpublished observations). Differences between the species in the results of field tests of alarm and food signals (Mitchell and Hazlett, in preparation) are consistent with suggestions from the laboratory.

The experiments with crushed congeneric individuals indicate that the chemicals involved are not specific to *O. virilis*. Indeed, the experiments with fresh fish suggest that at least a partial representation of those chemicals that trigger the alarm response may be found in a variety of freshwater animals. The fact that the freeze–thawed preparation of conspecifics resulted in no detectable reduction of alarm substance potency points to the possibility that the chemical(s) involved survived the array of autochthanous enzymes released from the cells

of organisms following this physically damaging technique (Rittschof, 1980). The fact that the enzyme trypsin did not significantly alter the responses shown suggests that if the chemicals involved include proteins or shorter peptides, those molecules are not destroyed (as signals of danger) by cleavage of lysine or arginine peptide bonds. Clearly, much work remains to be done concerning the characterization of the moieties involved in these chemical communication systems and the differences between them, as well as the ways the integration of these multiple inputs affects individuals in nature.

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AGGREGATION PHEROMONE FOR THE PEPPER WEEVIL, *Anthonomus eugenii* CANO (COLEOPTERA: CURCULIONIDAE): IDENTIFICATION AND FIELD ACTIVITY[1]

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Abstract—This study describes the identification of an aggregation pheromone for the pepper weevil, *Anthonomus eugenii* and field trials of a synthetic

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pheromone blend. Volatile collections and gas chromatography revealed the presence of six male-specific compounds. These compounds were identified using chromatographic and spectral techniques as: (*Z*)-2-(3,3-dimethylcyclohexylidene)ethanol, (*E*)-2-(3,3-dimethylcyclohexylidene)ethanol, (*Z*)-(3,3-dimethylcyclohexylidene)acetaldehyde, (*E*)-(3,3-dimethylcyclohexylidene)acetaldehyde, (*E*)-3,7-dimethyl-2,6-octadienoic acid (geranic acid), and (*E*)-3,7-dimethyl-2,6-octadien-1-ol (geraniol). The emission rates of these compounds from feeding males were determined to be about: 7.2, 4.8, 0.45, 0.30, 2.0, and 0.30 μg/male/day, respectively. Sticky traps baited with a synthetic blend of these compounds captured more pepper weevils (both sexes) than did unbaited control traps or pheromone-baited boll weevil traps. Commercial and laboratory formulations of the synthetic pheromone were both attractive. However, the commercial formulation did not release geranic acid properly, and geranic acid is necessary for full activity. The pheromones of the pepper weevil and the boll weevil are compared. Improvements for increasing trap efficiency and possible uses for the pepper weevil pheromone are discussed. A convenient method for purifying geranic acid is also described.

Key Words—Attractant, alcohol, aldehyde, geranic acid, monitoring, aggregation pheromone, *Anthonomus eugenii*, pepper weevil, Coleoptera, Curculionidae.

INTRODUCTION

The pepper weevil, *Anthonomus eugenii* Cano (Coleoptera: Curculionidae), is an important pest of both sweet and hot peppers (*Capsicum* spp.) in the southern United States, Mexico, and Central America (Elmore et al., 1934; Goff and Wilson, 1937). The most important damage is yield reduction resulting from premature abscission of infested fruit. Infested fruit not aborted may contain frass and decaying plant tissue, making them unmarketable. Additionally, the pepper weevil has been implicated in the transmission of internal mold of peppers (Bruton et al., 1989). Because the pepper weevil larvae and pupae are protected within the environment of the pepper pod, insecticide treatments must be directed against the emerged adults. Effective chemical control of adult pepper weevils is hindered by problems associated with detecting adults prior to economic injury (Genung and Ozaki, 1972). Predictive models for pepper weevil adult emergence are unavailable, and decisions regarding adulticide treatments and timing are generally based on classical calendar spraying regimes (Riley, 1990). Although a damage-based threshold has recently been described (Cartwright et al., 1990), visual counts of adults on terminal buds is the most widely accepted sampling method for adult pepper weevils (Andrews et al., 1986; Riley, 1990). Action thresholds for the pepper weevil are low: 5% terminal bud damage (Cartwright et al., 1990) and between 1 adult/400 terminals (Riley et al., 1992) and 1 adult/100 terminals (Andrews et al., 1986). Because both

sampling methods are tedious, time-consuming, and may only detect weevils after they have passed economic levels, a better monitoring system is needed for this pest.

There is previous evidence for a male aggregation pheromone in pepper weevils. Male pepper weevils have been shown to attract females and males in the field (Patrock, 1986; Patrock et al., 1992). In addition, males and dichloromethane extracts of males are reported to attract females and males in a laboratory olfactometer (Coudriet and Kishaba, 1988). A synthetic pepper weevil pheromone, if available, could provide a reliable and economic sampling method for detecting adult pepper weevil presence and determining density for making management decisions.

The objectives of this study were to isolate and identify the male aggregation pheromone and to field test a synthetic pepper weevil pheromone.

METHODS AND MATERIALS

Insects. A laboratory culture of pepper weevils was established from insects collected in Florida and Texas. Pepper weevils were reared according to methods described by Patrock (1986). Fresh jalapeño peppers were purchased locally, grown in a greenhouse, or grown in an outdoor garden. Emerging adult pepper weevils were held individually in 30-ml diet cups and fed sliced fresh jalapeño pepper, black nightshade (*Solanum nigrum*) berries, or a piece of artificial diet (Toba et al., 1969). Adult pepper weevils were sexed using CO_2 anesthetization and characters as described for sexing boll weevils (Agee, 1964). In addition, males can be identified by the presence of a metatibial mucro (Eller, 1994).

Collection of Volatiles. Initially, volatiles were collected using a 50-ml filtering flask and a Tenax porous polymer trap system as described by Bartelt et al. (1990). Later volatile collections were made using a volatile collection system consisting of a 20-cm × 2.2-cm-ID Pyrex glass tube sealed on each end with a No. 11 cork stopper. One cork held a prefilter (7-cm × 4-mm-ID glass tube) with ca. 6 mm of Super Q porous polymer (80/100 mesh; Alltech Associates, Inc., Deerfield, Illinois) held between a stainless steel screen (325 mesh; F.P. Smith Wire Cloth Co., Franklin Park, Illinois) and a glass wool plug. The other cork held a similar filter with ca. 4 mm of Super Q to collect volatiles. Air was drawn through the tube with either the house vacuum system or a vacuum pump at a flow of ca. 130 ml/min.

Volatiles were collected from both male and female pepper weevils to identify male-specific compounds (i.e., the putative aggregation pheromone components). Typically, volatiles were collected from individual pepper weevils on small (i.e., ca. 5-cm or shorter) jalapeño fruit, although occasionally volatiles were collected from groups of weevils, and the plant material was pepper buds

or nightshade berries. Volatiles were also collected from formulations of synthetic pheromones to determine their release rates and component ratios. Collections were made for periods of one to five days, and collected volatiles were extracted using 240-μl hexane for Tenax filters and methylene chloride or hexane for Super Q filters. Ten microliters of a 250 ng/μl solution (i.e., 2500 ng) of α-terpineol was added to each filter extract as an internal standard to quantify collected volatiles and calculate pheromone release rates.

Gas Chromatography. Gas chromatography was performed using a Hewlett-Packard 5890 Series II gas chromatograph (GC) with a Spectra-Physics SP4400 integrator and a Varian model 3700 GC with a Hewlett-Packard 3396A integrator. The columns used were a fused silica Hewlett-Packard HP-5 (0.17-μm film thickness, 25 m $\times$ 0.32 mm ID) (Hewlett Packard Co., Avondale, Pennsylvania) and a fused silica Durabond DB-1 (1.0-μm film thickness, 15 m $\times$ 0.25 mm ID) (J & W Scientific, Folsom, California), respectively. The temperature programs were: 50°C for 3 min then 10°C/min to 220°C and 70°C to 200°C at 10°C/min, respectively. For both gas chromatographs, the injector and detector temperatures were 170°C and 250°C, respectively, and each was equipped with a flame ionization detector with helium as the carrier gas. Injections of 1–2 μl were made in the splitless mode and changed to the split mode after 0.60 min. Retention indices (RI) were calculated relative to *n*-alkene standards according to Poole and Schuette (1984).

GC–Mass Spectrometry (GC-MS). Electron-impact mass spectra (EI-MS) were obtained on a Hewlett-Packard 5970 Mass Selective Detector. An ionizing potential of 70 eV was used for EI spectra. Sample introduction was through a Hewlett-Packard 5890 GC fitted with a DB-1 (0.25-μm film thickness, 15 m $\times$ 0.25 mm ID) capillary column. Chemical-ionization mass spectra (CI-MS) were obtained on a Finnigan 4535 quadrapole mass spectrometer. The reagent gas was isobutane. Sample introduction was through a GC fitted with a DB-1 (0.25-μm film thickness, 15 m $\times$ 0.25 mm ID) capillary column.

Infrared Spectroscopy. Vapor-phase infrared spectra were obtained using a Mattson Instruments Galaxy Series 6020 FT-IR spectrometer with light pipe accessory. Samples were introduced through the Hewlett-Packard GC and column described earlier using the same temperature program. Absorbances are reported in reciprocal centimeters (cm^{-1}).

High-Performance Liquid Chromatography. Prior to proton nuclear magnetic resonance spectroscopy, compounds **1, 2,** and **5** (Figure 1) were purified to ca. 99% pure by high-performance liquid chromatography (HPLC). HPLC separations were performed using a Spectra Physics SP8700 solvent delivery system and Spectra Physics SP8750 pump and Waters R401 refractive index detector. Compounds **1** and **2** were separated using a silica column (5 μm, 4.6 mm diam. $\times$ 250 mm long) and the mobile phase was 25% ether in hexane at a flow rate of 1 ml/min. Their retention volumes were ca. 11.5 and 12.2 ml,

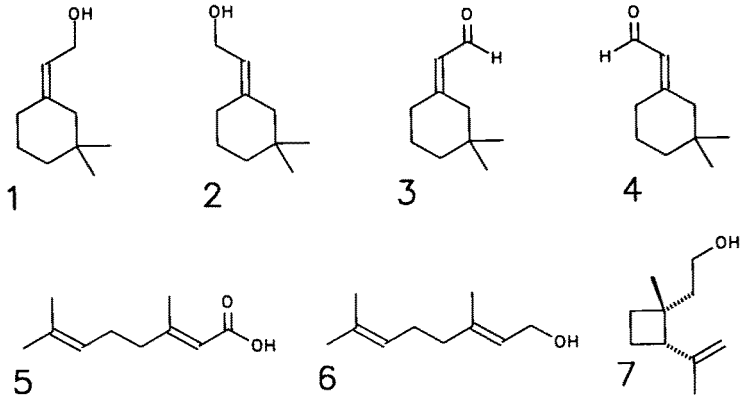


FIG. 1. Structures of compounds discussed in the text and assigned structure numbers.

respectively. Compound **5** was purified using a C-18 reversed-phase column (5 μm, 4.6 mm diam. $\times$ 250 mm long), and the mobile phase was 75:25 methanol–water at a flow rate of 1 ml/min. Compound **5** had an elution volume of ca. 7.0 ml.

Proton Nuclear Magnetic Resonance Spectroscopy (^1H *NMR*). Nuclear magnetic resonance (NMR) proton spectra were obtained on a Bruker 300 MHz instrument using deuterochloroform as the solvent. Shifts are reported in parts per million (δ) relative to tetramethylsilane.

Synthetic Derivatives. The methyl ester derivative of **5** was prepared using diazomethane in ether (Fales et al., 1973).

Synthetic Chemicals. Synthetic **1** was purchased from Frank Enterprises, Inc. (Columbus, Ohio) (95% pure by GC) or Bedoukian Research, Inc. (Danbury, Connecticut) (98% pure by GC). Synthetic **2** (98% pure by GC) and a mixture of **3** and **4** (95% pure by GC) were purchased from Bedoukian Research, Inc. A technical grade of synthetic **5** was purchased from ICN Biomedicals, Inc. (Cleveland, Ohio) (59% pure by GC; major impurity was nerolic acid) and was used without purification in experiments 1 and 2. For experiment 3, synthetic **5** was purified (99% pure by GC) by repeated (ca. 10$\times$) recrystallizations from acetone (70% geranic acid, 30% acetone, by volume) at ca. $-70°C$. Synthetic **6** was purchased from (Aldrich Chemical Co., Milwaukee, Wisconsin) (98% pure by GC).

NCAUR Pheromone Formulation. The synthetic pheromone was formulated using Miraspers (pregelatinized corn starch, pass 100 mesh; A.E. Staley, Decatur, Illinois). The general procedure consisted of adding 0.1% (by weight) 2,6-di-*tert*-butyl-4-methylphenol (BHT) (Aldrich Chemical Co.) as an antioxidant to the pheromone blend (compounds **1, 2, 3, 4,** and **6** in a ratio of

56:38:2:2:2, respectively). This pheromone/BHT blend was subsequently combined with the starch to obtain a mixture containing ca. 8% (by weight) pheromone. Approximately 0.5 g of the pheromone–starch mixture was placed inside a piece of glass tube (ca. 2.5 cm long × 0.5 cm diam.), and the tube was subsequently sealed inside a polypropylene (4 mil) bag (ca. 3.8 cm × 4.4 cm). Compound **5** (geranic acid) was formulated separately and was mixed with an equal amount of mineral oil (Fischer Scientific, Fairlawn, New Jersey) to slow the release of this compound, otherwise the same procedure was used. The pheromone was formulated to release the six components in a ratio of ca. 48:32:2:2:14:2 for compounds **1, 2, 3, 4, 5,** and **6,** respectively, at a total release rate of ca. 13.5 μg/hr.

Field Assays. Field tests of the synthetic pheromone were set up in three separate experiments, reflecting progressive improvements in the pheromone/trap combination. The first experiment was designed to compare commercial boll weevil traps (Great Lakes IPM, Vestaburg, Michigan) with sticky traps (6 in. × 12 in. yellow strips, Olson Products, Medina, Ohio). Previous research has shown that boll weevil traps baited with live male pepper weevils captured pepper weevils; however, pepper weevils were observed to move in and out of the inspection dome (Patrock et al., 1992). In addition, during preliminary tests of synthetic pepper weevil pheromone using boll weevil traps, it was noted on several occasions that, when traps were visually checked without removing captured insects and later rechecked, some pepper weevils had escaped between the two checks. The apparent inefficiency of boll weevil traps at capturing pepper weevils prompted the testing of the sticky traps in experiment 1. The NCAUR pheromone formulation was used (six-component blend with an approximate total release rate of 13.5 μg/hr). Four treatments were compared: unbaited (control) boll weevil traps, boll weevil traps baited with the six-component blend, unbaited (control) sticky traps, and sticky traps baited with the six-component blend. Traps were placed on bamboo stakes just above the tops of the pepper plants and were separated by ca. 10 m. Both baited and unbaited boll weevil traps contained a small piece (ca. 1 cm^3) of Pest Strip (Loveland Industries, Inc., Greely, Colorado) to kill captured insects. Pheromone baits were placed inside the inspection dome of the boll weevil traps and were attached to the sticky traps using a pin and cork. The test was conducted between November and March in Florida and Texas near the cities indicated (Table 2 below). Treatments were set out in random order and the traps were checked at two- to five-day intervals.

A second experiment was set up to compare the attractiveness of the NCAUR formulation to a commercial formulation containing the same components, using yellow sticky traps. The commercial formulation was similar to the Hercon boll weevil lure (Hercon Environmental, Emigsville, Pennsylvania). Ten milligrams per lure of the six-component blend were loaded in ratio of ca.

45:35:3:3:12:2. The NCAUR formulation was the same as described in the first experiment. The test was conducted between March and May in Florida and Texas near the cities indicated (Table 3 below). Treatments were set out in random order and the traps were checked at two- to five-day intervals.

Because the commercial formulation did not release compound **5** as desired, a third experiment was set up to determine whether this compound was a necessary component of the pheromone blend or whether **5** could simply be omitted for practical purposes. A comparison was made of the attractiveness of Hercon lures containing only compounds **1, 2, 3, 4,** and **6,** with and without compound **5** (using the NCAUR formulation). Ten milligrams per lure of components **1–4** and **6** were loaded in ratio of ca. 50:41:3.5:3.5:2. Compound **5** was formulated alone in the NCAUR formulation as described earlier; for this study, highly purified rather than technical grade **5** was used so that any effects of impurities would not confound the experiment. The pheromone treatments were compared using yellow sticky traps as described earlier. The test was conducted during May and June in Florida and Texas and June through October in New Mexico near the cities indicated (Table 4 below). Treatments were set out in random order and the traps were checked at two- to five-day intervals.

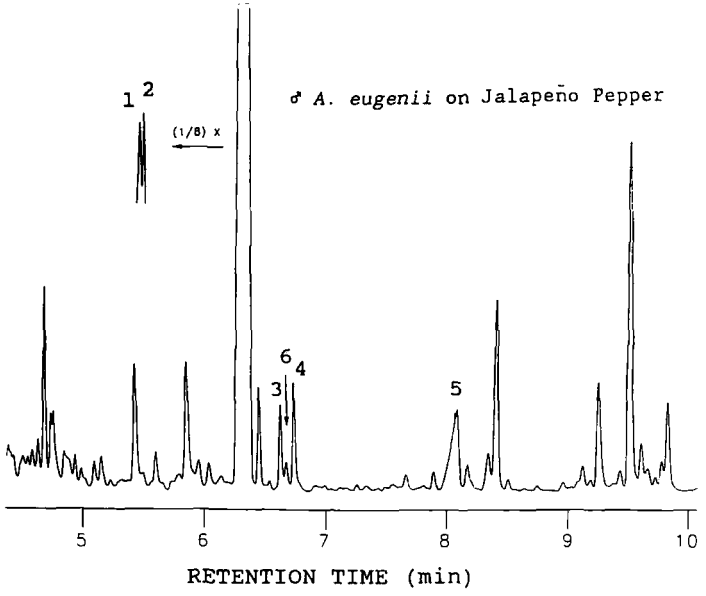
Captured insects were examined using a dissecting microscope to separate pepper weevils from other species of weevils and to determine the sex of the captured pepper weevils.

Statistical Analyses. Trap capture data were analyzed using Statistix 4.0 (Analytical Software, Saint Paul, Minnesota). The analyzed variable was the total trap catch (over test period) after $\log(X + 1)$ transformation for each treatment, location, and replication. Significance levels were 0.05 for all tests.

RESULTS

Male-Specific Compounds. A comparison of the GC profiles of volatile collections of males and females revealed the presence of six male-specific compounds designated 1–6 (Figure 2). Peaks 1–6 were found to correspond to the structures shown in Figure 1 with the same numbers. The GC retention data for these compounds and approximate release rates as determined from Super Q volatile collections are given in Table 1. Analytical results supporting these identifications are summarized in the following paragraphs.

EI-MS spectra for GC peaks 1 and 2 were very similar and had molecular ions at *m/z* 154. A search of the mass spectral library (NBS) gave an essentially perfect match with (*Z*)-2-(3,3-dimethylcyclohexylidene)ethanol **(1),** which is the second most abundant component of the boll weevil pheromone (Tumlinson et al., 1969). At this point, GC peaks 1 and 2 were tentatively identified as (*Z*)-2-(3,3-dimethylcyclohexylidene)ethanol and (*E*)-2-(3,3-dimethylcyclohexyli-



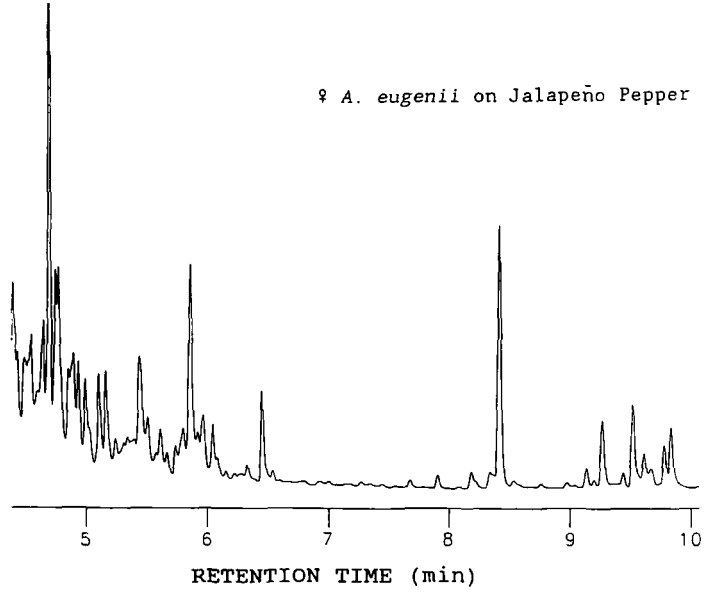


FIG. 2. Gas chromatograms (HP-5 column) of volatile collections of male (top) and female (bottom) pepper weevils feeding on jalapeño peppers.

TABLE 1. RELEASE RATES AND RELATIVE ABUNDANCE OF COMPONENTS OF PEPPER WEEVIL PHEROMONE IN VOLATILE COLLECTIONS, COMPARISON TO BOLL WEEVIL PHEROMONE, AND GAS CHROMATOGRAPHIC RETENTION INDICES (RI) OF THESE COMPOUNDS

	Pepper weevil		Boll weevil	RI	
Compound	Amount emitted (μg/male/day)	Relative abundance	Relative abundance[a]	DB-1	HP-5
1	7.20	48	35	12.14	12.24
2	4.80	32		12.16	12.27
3	0.45	3	14	12.33	12.50
4	0.30	2	15	12.40	12.59
5	1.95	13		13.33	13.62
6	0.30	2		12.35	12.52
7			36	11.90	12.12

[a]Relative abundances as reported by Chang et al. (1989).

dene)ethanol, respectively (compounds **1** and **2,** Figure 2). The GC retention times of the synthetic standards matched those of the pepper weevil-derived compounds on both GC columns. Mass spectral and NMR analyses both gave identical spectra for the weevil-derived and the synthetic compounds **1** and **2,** and the spectra were consistent with those reported earlier (Tumlinson et al., 1971).

GC peaks 3 and 4 both produced mass spectra with molecular ions at *m/z* 152, two units less than alcohols **1** and **2.** By analogy to the boll weevil system, these GC peaks were compared with synthetic standards of the aldehydic boll weevil compounds, (*Z*)-(3,3-dimethylcyclohexylidene)acetaldehyde and (*E*)-(3,3-dimethylcyclohexylidene)acetaldehyde (compounds **3** and **4,** respectively, Figure 2). The GC retention times of the synthetic standards matched those of the pepper weevil-derived compounds on both GC columns. Mass spectral analyses gave identical spectra for the weevil-derived and the synthetic compounds **3** and **4,** and the spectra were consistent with those reported earlier (Tumlinson et al., 1971). The small quantities of natural aldehydes precluded NMR analysis.

EI-MS analysis of GC peak 5 did not reveal an obvious molecular ion; however, CI-MS analysis gave a base peak at *m/z* 169, which was presumably the M+1 ion. FTIR analysis and a search of the EPA vapor-phase library suggested that this compound was an unsaturated carboxylic acid, specifically because of absorptions at 3585, 1752, and 1652 (cm^{-1}). The compound represented by GC peak 5 reacted with diazomethane to give the corresponding methyl ester. The methyl ester gave a much sharper GC peak than did the

original compound, and subsequent EI-MS analysis gave a presumptive molecular ion at *m/z* 182. A search of the mass spectral library (NBS) gave an essentially identical match for this derivative with methyl (*E*)-3,7-dimethyl-2,6-octadienoate (geranic acid methyl ester). Therefore, GC peak 5 was tentatively identified as (*E*)-3,7-dimethyl-2,6-octadienoic acid (geranic acid) **(5).** The GC retention time of the synthetic standard matched that of the pepper weevil-derived compound on both GC columns. Mass spectral and NMR analyses both gave identical spectra for the weevil-derived and synthetic compound **5,** and the mass spectrum was consistent with that reported earlier (Renhold et al., 1974).

EI-MS analysis of GC peak 6 revealed a molecular ion at *m/z* 154, and a subsequent search of the mass spectral library (NBS) gave an essentially perfect match with (*E*)-3,7-dimethyl-2,6-octadien-1-ol (geraniol). The GC retention time of synthetic geraniol matched that of the pepper weevil-derived compound on both GC columns. Mass spectral analyses of pepper weevil-derived compound **6** and synthetic geraniol gave identical spectra. The small quantity of natural material precluded NMR analysis.

Field Assays. The results of experiment 1 and statistical analysis are shown in Table 2. Because the treatment × location interaction was significant, treatments are only compared within a given location. Both the baited and unbaited boll weevil traps captured very few pepper weevils. Overall, pheromone-baited sticky traps caught the most pepper weevils, significantly more than pheromone-baited boll weevil traps at all seven locations, and significantly more than unbaited sticky traps at five of the seven locations. Considering all treatment–location combinations, females accounted for 50–67% of the captured pepper weevils, with an overall average of 60% females.

The results of experiment 2 and statistical analysis are shown in Table 3. Sticky traps baited with either the NCAUR or Hercon formulation captured significantly more pepper weevils than did unbaited sticky traps at five of the six locations. Overall, the two formulations (i.e., NCAUR and Hercon) captured about equal numbers of pepper weevils. The two formulations were statistically equal at three locations, the NCAUR caught significantly more weevils at two locations, and the Hercon caught significantly more weevils at one location. Considering all location–treatment combinations, females accounted for 50–100% of the captured pepper weevils, with an overall average of 84% females.

The results of experiment 3 and statistical analysis are shown in Table 4. The geranic acid (i.e., compound **5**) treatment caught the smallest number of pepper weevils and was statistically equivalent to controls at all seven locations. The Hercon lure alone was statistically equivalent to the controls at five locations but caught significantly more weevils than controls at two locations. The Hercon lure plus geranic acid captured the most pepper weevils and caught significantly more weevils than did the Hercon lure alone or controls at five locations and

TABLE 2. FALL 1992 AND WINTER 1993 PHEROMONE EXPERIMENT COMPARING OLSON STICKY TRAPS AND BOLL WEEVIL TRAPS WITH AND WITHOUT PEPPER WEEVIL PHEROMONE (NCAUR FORMULATION) (EXPERIMENT 1)

Location (test period)	Mean trap catch (range)[a]				Replications (*N*)
	Sticky, pheromone	Sticky, control	Boll weevil, pheromone	Boll weevil, control	
Weslaco, Texas (11/18–12/2)	8.2 a (1–14)	0.4 b (0–1)	0.0 b	0.0 b	5
Loxahatchee, Florida (1/18–2/10)	6.7 a (1–14)	0.0 b	0.0 b	0.0 b	3
Boyton Beach, Florida (1/19–1/29)	5.6 a (1–10)	8.0 a (1–15)	0.0 c	1.6 b (0–3)	5
Immokalee, Florida (1/27–3/4)	2.8 a (0–4)	1.8 b (0–5)	0.4 b (0–2)	0.0 b	5
Bradenton, Florida (1/29–2/19)	4.7 a (1–10)	2.3 a (1–7)	0.3 b (0–1)	0.0 b	3
Delray Beach, Florida (2/9–3/1)	1.2 a (0–3)	0.0 b	0.0 b	0.0 b	5
Immokalee, Florida (2/18–3/29)	17.8 a (2–59)	6.2 b (0–14)	0.4 c (0–2)	0.2 c (0–1)	5
Overall number captured	212	89	5	9	31

[a] In each line, treatments without letters in common differ significantly (LSD, 0.05 level). Overall differences among treatments: $F_{3,96} = 42.92$, $P \ll 0.0001$. Treatment × location interaction: $F_{18,96} = 2.25$, $P = 0.006$.

the geranic acid alone at six locations. Considering all location–treatment combinations, females accounted for 50–100% of the captured pepper weevils, with an overall average of 72% females.

DISCUSSION

Comparison of Pepper Weevil and Boll Weevil Pheromones. The total amount of pheromone released by male pepper weevils was ca. 15 μg/male/day and was higher than that reported for the boll weevil (ca. 4.2 μg/male/day) (Chang et al., 1989). The boll weevil (Tumlinson et al., 1969) and the pepper weevil have three pheromone components in common (i.e., **1**, **3**, and **4**). These three compounds are designated II, III, and IV, respectively in the boll weevil literature (Tumlinson et al., 1969). The relative percentages of the boll weevil pheromone as determined by collection of volatiles from boll weevils feeding on cotton (Chang et al., 1989) are shown in Table 1 for comparison. Compound **1** is the most abundant component of the pepper weevil pheromone and is the

TABLE 3. SPRING 1993 PHEROMONE EXPERIMENT COMPARING NCAUR FORMULATION AND COMMERCIAL HERCON FORMULATION USING OLSON STICKY TRAPS (EXPERIMENT 2)

Location (test period)	Mean trap catch (range)[a]			Replications (*N*)
	Pheromone (NCAUR)	Pheromone (Hercon)	Control	
Immokalee, Florida (3/12–5/14)	6.6 a (0–4)	11.0 a (0–8)	0.4 b (0–1)	5
Jupiter, Florida (3/17–5/17)	7.8 a (7–10)	9.0 a (5–12)	0.6 b (0–3)	5
Loxahatchee, Florida (3/22–4/4)	5.0 b (5–5)	17.5 a (16–19)	0.0 c	2
Bradenton, Florida (3/24–4/17)	3.5 b (0–7)	17.5 a (5–30)	19.0 a (7–31)	2
Immokalee, Florida (4/1–5/17)	20.4 a (6–37)	20.0 a (7–28)	8.0 b (5–16)	5
Weslaco, Texas (4/21–5/7)	17.8 a (4–45)	5.4 b (0–12)	0.4 c (0–1)	5
Overall number captured	280	297	85	24

[a] In each line, treatments without letters in common differ significantly (LSD, 0.05 level). Overall differences among treatments: $F_{2,57} = 28.02$, $P \ll 0.0001$. Treatment × location interaction: $F_{10,57} = 3.29$, $P = 0.002$.

second most abundant component of the boll weevil pheromone. In addition, compound **1** has been isolated from female pecan weevils, *Curculio caryae* (Horn) (Coleoptera: Curculionidae) (Hedin et al., 1979). Pepper weevils and boll weevils also have the aldehyde components (i.e., **3** and **4**) in common, although their relative percentages of the blend differ widely. Compounds **2, 5,** and **6** isolated from male pepper weevils have not been reported from the boll weevil and are apparently not part of its aggregation pheromone. Compound **5,** however, is reported to be part of the Nasonov pheromone of the honeybee, *Apis mellifera* (Pickett et al., 1980) and is attractive to foraging bees (Williams et al., 1981). A somewhat similar compound, (*E*)-3,7-dimethyl-2-octen-1,8-dioic acid (callosobrusic acid) has been isolated from female azuki bean weevils, *Callosobruchus chinensis* L. (Coleoptera: Bruchidae) (Mori et al., 1983). Compound **6** (geraniol) is not reported to be produced by boll weevils, although Tumlinson et al. (1970) suggested that geraniol was a possible precursor to the boll weevil pheromone components. Their reasoning can be applied to the production of all of the pepper weevil compounds, including compound **5** (i.e., geranic acid), which is the corresponding acid of geraniol. The most abundant

TABLE 4. SUMMER 1993 PHEROMONE EXPERIMENT COMPARING COMMERCIAL HERCON LURE WITH AND WITHOUT GERANIC ACID (NCAUR FORMULATION) USING OLSON STICKY TRAPS (EXPERIMENT 3)

Location (test period)	Mean trap catch (range)[a]				Replications (*N*)
	Hercon + Geranic acid	Hercon alone	Geranic acid alone	Control	
Immokalee, Florida (5/15–5/26)	3.8 a (1–6)	1.2 b (0–3)	0.8 b (0–2)	0.4 b (0–1)	5
Immokalee, Florida (5/17–5/25)	15.5 a (6–25)	15.0 a (6–24)	1.5 b (1–2)	0.5 b (0–1)	2
Bradenton, Florida (5/17–6/7)	12.8 a (11–16)	4.0 b (2–9)	5.5 b (0–10)	8.0 ab (3–18)	4
Weslaco, Texas (5/19–6/17)	26.0 a (12–39)	7.6 b (1–16)	4.6 b (2–7)	3.4 b (1–6)	5
Lantana, Florida (5/19–6/17)	27.2 a (21–34)	24.4 a (17–28)	22.2 a (19–26)	24.6 a (18–29)	5
Loxahatchee, Florida (5/25–6/2)	17.6 a (11–24)	4.8 b (1–7)	1.0 c (0–3)	0.8 c (0–2)	5
Las Cruces, New Mexico (6/28–10/14)	199.7 a (0–2828)	43.6 b (0–615)	26.4 b (0–357)	19.7 b (0–267)	15
Overall number captured	3440	890	437	474	41

[a] In each line, treatments without letters in common differ significantly (LSD, 0.05 level). Overall differences among treatments: $F_{3,134} = 36.28$, $P \ll 0.0001$. Treatment × cooperator interaction: $F_{12,134} = 4.40$, $P < 0.0001$.

component of the boll weevil pheromone (i.e., **7**) is apparently not produced by male pepper weevils.

Field Studies with Pepper Weevil Pheromone. Pheromone-baited sticky traps generally captured at least twice as many pepper weevils as did control traps, with several cases of over 20 times as many. This indicates that the synthetic pheromone is attractive to pepper weevils. Patrock et al. (1992) reported that male-baited boll weevil traps caught ca. three times as many pepper weevils as did control boll weevil traps. The three experiments reflect progressive improvement of pheromone baits. The ratio of overall trap catch by the best treatment to that by the sticky-trap control increased from 2.4:1 to 3.4:1 to 6.3:1 for experiments 1, 2, and 3, respectively. The trap captures were generally low, but it should be noted that most experiments were conducted in commercial fields where growers were applying insecticides to suppress pepper weevil populations.

The results of experiment 2 clearly indicate that the pheromone-baited

yellow sticky traps were more effective at capturing pepper weevils than either baited boll weevil traps or unbaited sticky traps. In all field experiments, unbaited control traps (especially yellow sticky cards) captured pepper weevils. This is probably a result of an attraction to the color of the traps. Previous research has shown that unbaited sticky traps (especially yellow and white) are attractive to pepper weevils (Segarra-Carmona and Pantoja, 1988; Riley, 1990). The yellow sticky cards and boll weevil traps used in these tests were found to have peak reflectances at 563 and 542 nm, respectively (unpublished data), and were very similar to the reflectance pattern of the yellow sticky cards used by Segarra-Carmona and Pantoja (1988). Segarra-Carmona and Pantoja (1988) reported that unbaited sticky traps were an effective monitoring technique for the pepper weevil and were superior to other sampling methods, including direct counting. The addition of the synthetic pheromone and resulting increased trap captures should make the pheromone–sticky card combination an even more effective monitoring technique.

Although both the NCAUR and Hercon formulations used in experiment 2 were attractive, the NCAUR formulation is made by hand and is impractical for large-scale production. The Hercon lure, on the other hand, is mass-produced. However, the Hercon lures used in experiment 2 released much less of compound **5** (geranic acid) than do male pepper weevils. In addition, the synthetic compound **5** used in experiments 1 and 2 contained a large amount of impurities with unknown effects. Although the lures used in experiments 1 and 2 were attractive, it is believed that a synthetic blend more closely approximating that released by males would be even more attractive. This difficulty in formulating compound **5** prompted the testing of whether or not it was an essential component of the synthetic blend (i.e., experiment 3). The blend of the synthetic boll weevil pheromone has a significant effect on its attractiveness. Tumlinson et al. (1969) reported that the individual components of the boll weevil pheromone were nearly inactive and that the two alcohols (i.e., **7** and **1**) were both required with at least one of the aldehydes (i.e., **3** or **4**) but response was highest to the complete blend. Our results of experiment 3 indicate that the pheromone blend is important for the maximum attractancy of the pepper weevil as well. Although geranic acid is inactive by itself, the six-component blend containing geranic acid is much more attractive than the five-component blend without geranic acid. Therefore either a new commercial lure that can properly release all six components must be developed or two separate formulations must be used to achieve this. Other preliminary data suggest the individual alcohols **1** and **2** are inactive by themselves and the two aldehydes together (i.e., **3** and **4**) are also inactive (unpublished). The effects of the individual components and blend optimization need to be investigated further.

In all three experiments, the treatment × location interaction was significant. We believe that much of this interaction was due to differences between

the locations in pepper phenologies and pepper weevil densities, which subsequently affected the response of weevils to the pheromone. The biologies of the pepper weevil and the boll weevil are very similar (Burke, 1976), and factors affecting one species are likely to affect the other species as well. The efficiency of traps baited with male boll weevils or synthetic pheromone is affected by both cotton phenology and boll weevil population dynamics (Hardee et al., 1970a; Ridgway et al., 1971). Traps baited with live male boll weevils only capture more boll weevils than control traps when cotton was in the pre-squaring stage. Midseason (i.e., after fruiting), control traps capture as many boll weevils as do male-baited traps, which elicit little or no response even though the field population is increasing. In addition, traps baited with male boll weevils capture the highest numbers at the end of the season. Several factors have been suggested to account for these observations. Early in the season, there is little competition from native males and a low availability of food/oviposition sites. Midseason, however, the attractiveness of the boll weevil pheromone decreases with the increased availability of food/oviposition sites, increased competition from native males, an increase in the percentage of mated females (which are no longer attracted to the pheromone) (Hardee et al., 1970b), and decreased movement from field to field. At the end of the season, the pheromone becomes attractive again as the boll weevils migrate out and as the cotton becomes unsuitable (i.e., a lack of oviposition sites).

Our data may reflect a similar situation for the pepper weevil. Early in the season (i.e., before fruiting) or when pepper weevil populations are low, the pheromone traps are more attractive than control traps, although relatively low numbers of weevils are captured. This was generally the case at most locations and all three experiments. As the peppers mature, competition from native males increases, the proportion of mated females increases, and the effect of the pheromone becomes less evident. This appeared to be the case at Lantana, Florida, where the pepper field had been abandoned and the pepper weevil population was very high (Table 4). At one location, (i.e., Bradenton, Florida), the pheromone-baited sticky traps never captured statistically more weevils than did control sticky traps (Tables 2–4). This location is a relatively small plot on an experiment station farm, and the results may be due to a population of pepper weevils on nightshade in the area adjacent to this plot. At the end of the season, when the pepper fields are destroyed by plowing or killed by frost, pheromone traps are again more attractive than control traps. After a pepper field was disked in Mexico, pheromone trap captures of over 200 pepper weevils per trap per day were recorded, compared to four per trap per day for control traps (Laborde, personal communication). In addition, after a frost killed the pepper plants in New Mexico, individual pheromone traps captured over 800 pepper weevils (virtually covering the sticky surface) over a three-day period, compared with ca. 20 on control traps.

The fact that male and female pepper weevils were captured in baited traps in both these experiments and in those of Patrock et al. (1992) suggests that the pepper weevil pheromone acts as an aggregation pheromone similar to that described for the boll weevil (McKibben et al., 1971). Although it has not been tested, the pepper weevil pheromone may act as an aggregation pheromone early in the season and more as a true sex pheromone (attracting primarily females) later in the season, as is true for the boll weevil pheromone (Tumlinson, 1985). Although the captured weevils from all locations were not available for examination, we were able to analyze for sex-by-treatment interactions for eight data sets (experiment–location combinations) (chi-square tests). Of these, four of the cases had significant interactions, and there was a tendency for traps with the complete pheromone to catch more females than control traps or traps baited with only the geranic acid. However, these latter traps captured relatively few insects and conclusions based on these results are tenuous.

Other Considerations. It may be possible to enhance the attractiveness of the pepper weevil pheromone by the addition of pepper compounds in a manner analogous to the enhanced attractiveness of grandlure by the addition of water extracts of cotton squares (Hardee et al., 1971), cotton essential oils (Dickens, 1986), or green leaf volatiles (Dickens, 1989). The effects of pepper compounds on the activity of the synthetic pepper weevil pheromone will be investigated in future studies.

The commercial availability of compounds **1–6** should expedite their use as attractants for pepper weevils. Three of these are available as boll weevil pheromone components (i.e., **1, 3,** and **4**), and **2** is also available as a "by-product" of the synthesis of **1.** Synthetic **5** (i.e., geranic acid) is also available commercially. Although the purity of compound **5** from commercial sources is low (ca. 60%), it can be purified relatively easily by recrystallization from acetone (70:30 mixture by volume, respectively) at −70°C. Compound **6** (i.e., geraniol) can be purchased in high purity.

We feel that the pepper weevil pheromone will be of greatest utility for the early detection of pepper weevil adults. In several instances during the course of this study, pepper weevils were captured on pheromone-baited traps in commercial pepper fields before they were detected by visual scouting (T.F.M., B.J., J.H.D.). If a strong correlation between pheromone trap captures and pepper weevil density or damage can be established, action thresholds based on pheromone trap captures may be more effective than those based on visual counts of adults (Andrews et al., 1986) or counts of damaged buds (Cartwright et al., 1990). In addition, it may be possible to control pepper weevils by using a system analogous to the bait-stick developed for control of the boll weevil (McKibben et al., 1991). The pepper weevil pheromone has great potential as part of a management program for the pepper weevil, just as the boll weevil

pheromone, grandlure, is an effective tool for the management of the boll weevil (Hardee et al., 1974).

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ROLE OF PHENOLIC COMPOUNDS IN THE ANTIALGAL ACTIVITY OF BARLEY STRAW

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Abstract—Barley straw decomposing in well-aerated water releases a substance(s) that inhibits algal growth. Phenolic compounds are toxic to algae but are unlikely to be present in sufficient quantities to account for the extended antialgal action of straw. However, straw is antialgal under conditions that may promote oxidation of phenolic hydroxyl groups to quinones; tannins are antialgal under similar conditions. The toxicity of authentic quinones towards *Microcystis* is confirmed; the quinones are some 10^3 times more antialgal than phenolic acids. The possibility that oxidized lignin derivatives may be involved in straw toxicity towards algae is discussed.

Key Words—Algal inhibition, phenolics, oxidized phenolics, quinones, barley straw, *Microcystis*, *Chlorella*.

INTRODUCTION

Barley straw rotting in water has been shown to control algal growth in both laboratory (Gibson et al., 1990) and field trials (Welch et al., 1990). The straw was active at very low concentrations against a range of algae in natural waters including unicellular and filamentous green algae and cyanobacteria (Ridge and Barrett, 1992); indeed, the current recommended dose rate of barley straw for practical algal control is 5 g/m^3 (Anon., 1991). The potential of this cheap and ecologically sound method of controlling the increasing problems of nuisance algae was confirmed by Newman and Barrett (1993a), who showed that rotted straw inhibited *Microcystis aeruginosa*, a cyanobacterium known to produce toxins.

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Liquor from straw rotted in the laboratory for several weeks was inhibitory to algal growth, and Gibson et al. (1990) suggested that production of an antialgal substance was associated with the straw decomposition. Pillinger et al. (1992) concluded that the production of an antialgal antibiotic by any of the fungi isolated from antialgal decaying straw was unlikely to explain the widespread antialgal effect shown by straw of differing sources, and differing mycoflora, rotting in differing water bodies throughout the U.K. and beyond. Likewise the phenolics, *p*-coumaric and ferulic acids, present as cell wall-bound components of barley straw (Chesson et al., 1982), while toxic towards *Chlorella vulgaris* (Dedonder and van Sumere, 1971) and *Microcystis aeruginosa* (Newman and Barrett, 1993b), are not present in sufficient amounts to account for the extended activity of straw. Gibson et al. (1990) suggested that another group of plant phenolics, tannins, which have the added ability to complex with proteinaceous substances, may account for algal control by barley straw. Plant-derived tannins have been implicated in antialgal activity (Janzen, 1974; Parks and Rice, 1969; Planas et al., 1981; Hussein Ayoub, 1982; Saito et al., 1989). Hussein Ayoub and Yankov (1985) reported that methylation (or acetylation) of phenolic hydroxy groups removed the antialgal activity of *Acacia* extracts, which they had attributed to the presence of tannins. The tannin status of barley straw is unknown (Mueller-Harvey, 1989), although barley grain does contain tannins (e.g., Brandon et al., 1982). We have, therefore, examined the antialgal activity of a known source of natural tannin (oak leaves) together with an authentic tannin, in order to compare inhibitory action with that of barley straw. Additionally, we have used a protein, namely hide powder, to remove tannin by classic protein–tannin precipitate formation, allowing the antialgal activity of tannin-free solutions to be determined.

Anecdotal evidence from field trials suggests that reduced levels of aeration were accompanied by a reduction in, or loss of, antialgal activity (Barrett, unpublished results). We have investigated the role of aeration on the antialgal effect of straw in laboratory tests. The significance of oxidation of phenolics in various situations has been stressed by Appel (1993) in an elegant review. Oxidized phenolic compounds are generally, but not always, more toxic than the phenols from which they are derived (Mason and Wasserman, 1987). Moreover, most phenolic materials are susceptible to autooxidation at pH values greater than 7 in the presence of air (Cason, 1967). Such conditions are always satisfied in the laboratory bioassays reported here and are also generally true in situations where straw has been shown to be active in large waterbodies. The antialgal activity of several oxidized phenolics, quinones, has been demonstrated (Dedonder and van Sumere, 1971) and some are active at very low dose rates (Fitzgerald et al., 1952). We have tested authentic quinones under the same bioassay conditions used to demonstrate antialgal activity of rotting barley straw,

and have compared the toxicity of a model phenol and quinone with similar substitution.

METHODS AND MATERIALS

Plant material and authentic chemicals were tested for antialgal potency against either a unicellular green alga, *Chlorella vulgaris* Beijerinck (CCAP 211/12) or a blue-green alga (cyanobacterium), *Microcystis aeruginosa* Kutz, emend Elenkin 1924 (CCAP 1450/6). In a typical bioassay, 250-ml Erlenmeyer flasks contained 50 ml Jaworski's (JM) algal culture medium (CCAP, 1988; amended Newman and Barrett, 1993a) supplemented with 20 mM HEPES to maintain the pH at 8 (± 0.5) throughout the experiment. Flasks containing autoclaved culture medium were amended as required with leaf material or straw, or 1 ml of an aqueous dilution of the chemical under investigation. Fresh barley straw, air-dried, was finely chopped and sieved (size range 0.1–2.0 mm). Liquor from tanks of decomposing straw was filtered and used in place of distilled water to prepare the algal culture medium. Treatments and controls, replicated five times, were inoculated with 1 ml of a 3- to 4-day-old culture (in exponential growth phase) of *Microcystis* or *Chlorella*, maintained as regularly cultured stock lines in JM medium. The bioassay flasks were incubated for either three or four days in a controlled environment cabinet, 20°C $\pm$ 1°C, with continuous illumination of mean photon flux density 375 μmol/m^2/sec (400- to 700-nm wavelength) and were agitated daily.

Barley straw (15 g) was decomposed submerged in 30 liter aged tap water with aeration (2500 cm^3/min) for ca. 3 months (Pillinger, 1993) by which time both the straw and the water are consistently inhibitory to the growth of algae in a bioassay. Subsamples of decomposing straw were subjected to about twofold aeration. Increased aeration during bioassays of straw, or water in which straw was rotting, was achieved by bubbling (unsterile) air via an aquarium pump into the flasks rather than relying on daily agitation of the flasks.

At the end of the experiment, the contents of each flask were filtered through a glass fiber filter (Whatman GF/C) and the filter containing algal residue extracted with 100% methanol (typically 10 ml). The amount of algal growth was quantified by measuring the absorbance of the methanolic extract due to chlorophyll *a* at 665 nm, less a background turbidity measurement at 750 nm. The results are expressed as percentage of growth compared to the control, which did not receive any amendment, thus allowing comparison between experiments conducted under slightly varying conditions, e.g., age of inoculum. The amount of chlorophyll *a* extracted from the controls typically gave an absorbance of 0.250–0.350 units, equivalent to 700–975 μg/liter. The maximum absorbance due to the inoculum was typically 3% of the control growth.

Tannic acid (Aldrich Chemical Company, Gillingham, U.K.) was tested for antialgal activity against both algae. The tannin-rich leaf material was finely ground, air-dried, naturally senesced leaves of *Quercus cerris*: the tannin content was estimated as tannic acid equivalent by the protein precipitation ring test method of Hagerman (1987). Hide powder (Sigma), the protein used to precipitate tannin from solution, was added at a ratio of 20:1 by weight of the estimated weight of tannin added, either as leaf material or tannic acid. The hide powder was well dispersed by shaking. In order to test the efficacy of hide powder at acidic pH, it was left in contact with the tannin for 1 hr and removed by retention on GF/C filters prior to addition of the alkaline bioassay nutrient solutions and buffer.

The antialgal activity of three authentic quinones (Aldrich) was assessed using *Microcystis*. Likewise, the algal toxicity of 2,6-dimethoxyphenol and the respective quinone, 2,6-dimethoxy-*p*-benzoquinone was compared. The integrity of 2,6-dimethoxyphenol dissolved in bioassay medium was monitored at intervals over the duration of the bioassay by UV spectrometry (UV Konicon, with quartz glass cells).

RESULTS

Antialgal Effect of Tannins. The effect of tannic acid on the growth of two algae is shown in Table 1. The concentration that was highly inhibitory to both algae is in the order of 10 mg/liter. The concentration of tannin liberated from oak leaves was estimated as tannic acid equivalent by the protein precipitation test and the leaf material was found to be inhibitory to *Chlorella* (Table 2).

TABLE 1. EFFECT OF TANNIC ACID ON ALGAL GROWTH

	Chlorella			*Microcystis*		
Tannic acid (mg/liter)	% control growth[a]	SE[b]	Significance[c]	% control growth	SE	Significance
17.0	20	1.8	***	9	1.2	***
8.5	33	4.2	***	ND[d]		
1.7	135	7.2	NS	105	8.0	NS
0.17	106	7.7	NS	97	6.3	NS

[a] In order to compare between experiments, the estimation of algal biomass (chlorophyll *a* content, $N = 5$) is normalized to the respective control ($N = 5$) and expressed as a percentage.
[b] Standard error of the mean of the normalized values ($N = 5$).
[c] Significant difference of treatment values ($N = 5$) from control values ($N = 5$) (unpaired *t* test); *** $P < 0.001$; NS, not significantly different.
[d] Not determined.

Thus the quantity of oak leaves estimated to contain 3.75 mg of tannin in the bioassay (equivalent to 75 mg/liter) was highly inhibitory, permitting only 5% of the growth of the control in the absence of any tannin source. At a dose of 15 mg/liter tannic acid equivalent, there was no significant difference between algal growth in the presence of oak leaves and in the control (Table 2). The results presented in Table 3 show that after tannic acid was in contact with hide powder at acidic pH values, the solution was not antialgal, but toxicity was retained when tannic acid and hide powder were combined under alkaline conditions. Likewise, the inhibitory action of oak leaves was not removed by hide powder at pH 8. Hide powder itself had no significant effect on algal growth.

Antialgal Effect of Quinones. Authentic quinones, used as models for oxidized phenolic compounds, were tested for antialgal activity against *Microcystis*. Three quinones were toxic at concentrations below 100 μg/liter (Figure 1a–c).

TABLE 2. EFFECT OF NATURAL TANNIN (OAK LEAVES) ON GROWTH OF *Chlorella*[a]

Weight of leaf (mg) 50 ml bioassay medium	Tannic acid equivalent (mg/liter)	% control growth	SE	significance
100	75.0	5	0.8	***
50	37.5	5	0.4	***
30	22.4	25	1.4	**
25	18.8	9	1.6	***
20	15.0	85	18.4	NS
10	7.5	141	20.3	NS

[a] See Table 1 footnotes.

TABLE 3. EFFECT OF TANNIC ACID IN PRESENCE OF HIDE POWDER (AT LOW AND HIGH pH) ON GROWTH OF *Microcystis*[a]

Tannic acid (mg/liter)	pH for hide-tannin complexation[b]	% control growth	SE	Significance
0 (control)	8.30	100		
17	8.23	9	1.2	***
	5.20	ND		
170	8.19	105	8.0	NS
	3.80	97	6.3	NS

[a] See Table 1 footnotes.
[b] At low pH, hide and tannin complex were removed before the pH was raised for the bioassay.

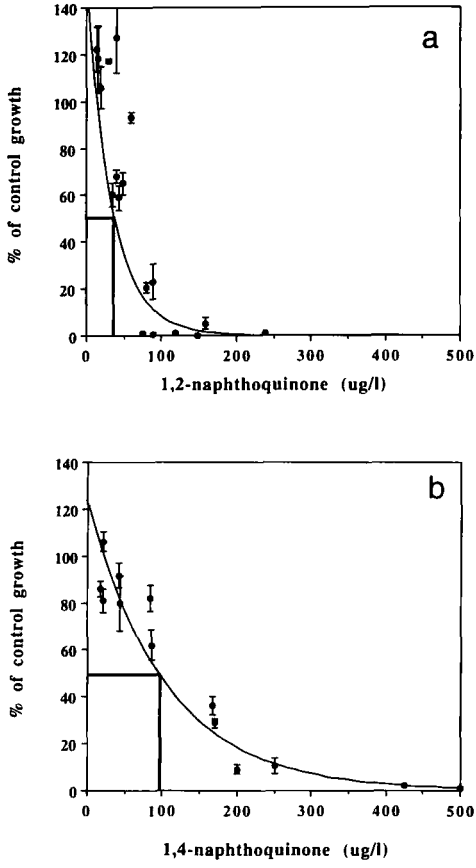

FIG. 1. The effect of authentic quinones on the growth of *Microcystis aeruginosa* in laboratory bioassays: (a) 1,2-naphthoquinone, (b) 1,4-naphthoquinone, and (c) 9,10-phenanthrenequinone. After four days, the amount of algal biomass in medium amended with the test quinone (five replicates) was quantified by measuring the absorbance of extracted chlorophyll *a*. The values were normalized to the mean of the respective controls (five replicates) and expressed as a percentage of control growth. Error bars represent the standard error of the normalized mean. For each quinone, the data were obtained from at least three separate experiments. Exponential curves were fitted to the data: (a) $R^2 = 0.621$, (b) $R^2 = 0.967$, (c) $R^2 = 0.778$.

The most active, 1,2-naphthoquinone and 9,10-phenanthrenequinone, inhibited algal growth by 50%, at concentrations of 40 and 25 μg/liter, respectively.

The antialgal activity of the 2,6-dimethoxy-substituted phenol and quinone was compared and found to be similar (Figure 2a and b), suggesting that the

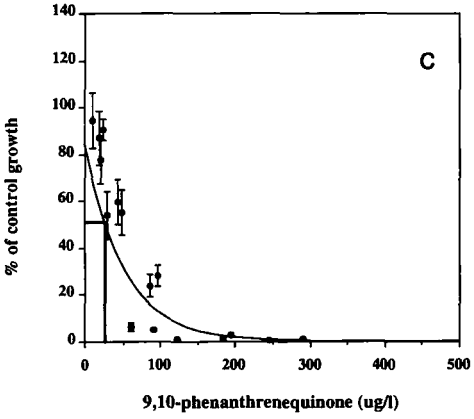

FIG. 1. Continued.

quinone functionality did not confer increased algal toxicity over that of the phenol. However, there is some evidence that the 2,6-dimethoxyphenol is chemically changed in the alkaline algal bioassay medium. The UV spectrum of the phenol dissolved in algal bioassay medium changed over the length of the bioassay, whereas the spectrum was unchanged if the phenol was dissolved in water (Figure 3a and b). A precipitate formed in the alkaline solution, and this had to be filtered off to measure the spectrum of the solution; thus the data shown are qualitative and not quantitative. The λ_{max} for the material remaining in alkaline solution increased with time but is not identical with the spectrum for the authentic 2,6-dimethoxy-*p*-benzoquinone (λ_{max} 287 nm).

Role of Aeration in Antialgal Activity. Increased aeration during the decomposition period of straw and during bioassays of rotted straw liquor and fresh, finely chopped, straw resulted in increased algal toxicity (Table 4). This supports the evidence noted from uncontrolled field experiments that oxidizing conditions are essential for straw to develop antialgal activity. The generation of an antialgal component from fresh, nondecomposed but finely chopped, straw lends support to our earlier report (Pillinger et al., 1992) that microbial decomposition is not essential for the production of the algal inhibitor.

DISCUSSION

Both authentic tannic acid and tannin-containing leaves (*Q. cerris*) were inhibitory to algal growth. Addition of a protein source, namely hide powder, which would be expected to complex with any tannin, negated the inhibitory action of tannic acid only when the two materials were in contact at low pH. The bioassays in which tannic acid and oak leaves were shown to be antialgal

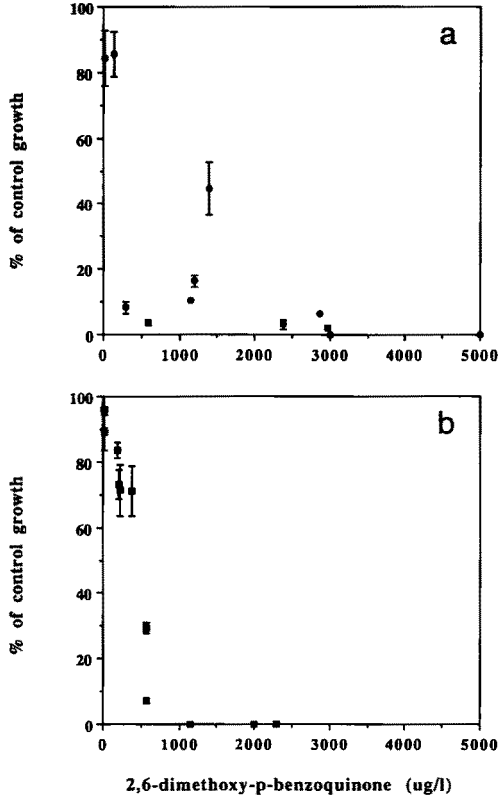

FIG. 2. The effect of 2,6-dimethoxyphenol and 2,6-dimethoxy-*p*-benzoquinone on the growth of *Microcystis aeruginosa* in laboratory bioassays: (a) 2,6-dimethoxyphenol and (b) 2,6-dimethoxy-*p*-benzoquinone. After four days, the amount of algal biomass in medium amended with the test chemical (five replicates) was quantified by measuring the absorbance of extracted chlorophyll *a*. The values were normalized to the mean of the respective controls (five replicates) and expressed as a percentage of control growth. Error bars represent the standard error of the normalized mean.

were, of necessity, conducted at high pH. The tests are restricted to the optimal algal growth range from pH 7 to 9 and comparisons of antialgal effects outside this range may be invalid due to pH effects. Thus, algal toxicity was demonstrated in basic culture medium, conditions under which protein precipitation by tannins would not occur. The data from the hide powder experiments (Table 3) suggest that while tannic acid is antialgal, it is not active by virtue of the protein precipitation capacity which defines the tannins as a distinctive type of polyphenolic material (e.g., Haslam, 1989).

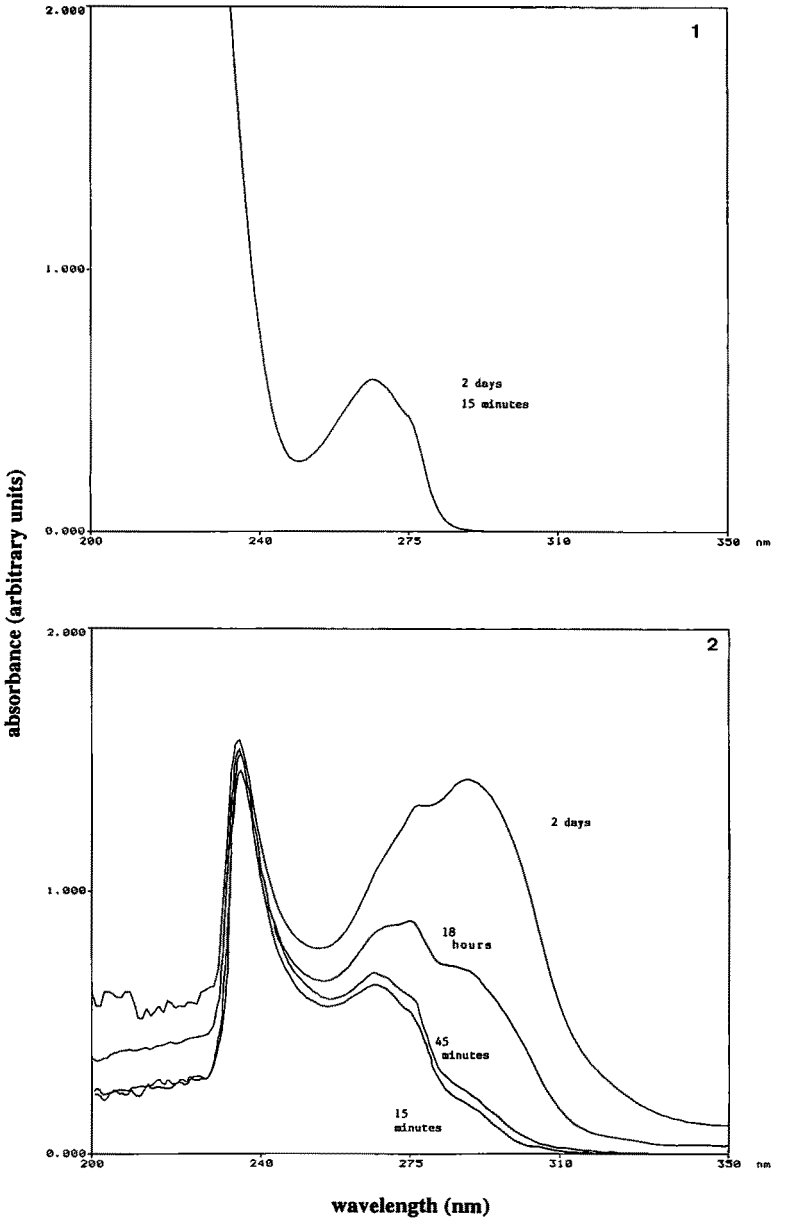

FIG. 3. Changes in the UV spectrum of 2,6-dimethoxyphenol dissolved in (1) water and (2) algal bioassay medium, with time.

TABLE 4. EFFECT OF AERATION ON POTENCY OF ALGAL INHIBITOR(S) FROM BARLEY STRAW[a]

	Absorbance[b]	Standard Error of mean ($N = 5$)	% of control growth[c]	
1. Liquor from straw undergoing decomposition				
Liquor after 28 days' decomposition	0.284	0.013	113	NS
Control	0.251[d]	0.022	100	
Liquor after 35 days' decomposition	0.302	0.013	85	NS
Control	0.353	0.018	100	
35 days' decomposition (with increased aeration and constant 20°C for previous 7 days)	0.116	0.008	30	***
Control	0.383	0.008	100	
2. Liquor from straw undergoing decomposition (35 days)				
Static during bioassay	0.302	0.013	85	NS
Static control during bioassay	0.353	0.018	100	
Aerated during bioassay	0.032	0.004	4	***
Aerated control during bioassay	0.924	0.121	100	
3. Fresh straw (500 mg/50 ml)				
Static during bioassay	0.161	0.013	84	NS
Static control during bioassay	0.191	0.008	100	
Aerated[e] during bioassay	0.085	0.004	29	***
Aerated control during bioassay	0.289	0.027	100	

[a] Three-day bioassay using *Chlorella*.
[b] Absorbance of methanolic extract of algal biomass at end of bioassay mesured at 665 nm, less background reading at 750 nm.
[c] Significant difference from control (t test);*** $P < 0.001$; NS, not significantly different.
[d] Absorbance of 0.251 appoximates to 700 μg chlorophyll a per liter.
[e] Forced aeration during bioassays was provided by unsterile air bubbled into each flask regulated *via* a manifold supplied with air at 2500 cm^3/min.

Tannic acid is reported to be readily oxidized, undergoing autooxidation when aerated and at alkaline pH; reduction of the pH to 5 stopped the reaction (Lyr, 1965). It is suggested, therefore, that the antialgal action of tannic acid, and of the oak leaf tannin, may be due to the action of oxidized tannin. Under field and bioassay conditions where plant material inhibits algal growth, any tannins that may be present could contribute to antialgal activity by virtue of oxidized phenolic groups.

The sample quinones tested herein, representing those relatively stable

examples that are available commercially, are very inhibitory to the growth of *Microcystis aeruginosa* (Figure 1). Thus, these examples of the quinone class of compounds are over 1000-fold more antialgal than *p*-coumaric and ferulic acids and 100 times more active than tannic acid. While most phenolic derivatives can be readily oxidized, the presence of free carboxylic acid groups may discourage autooxidation (Field and Lettinga, 1989). The ease of the reaction, and yield of quinone, is influenced by the nature and position of substituents in the ring (Fieser and Fieser, 1956). Continued oxidation can result in increasing polymerization to form the so-called humic acids. Thus, measured toxicity of the phenolic material is at first greatly increased during the quinone phase and then reduced as the material polymerizes (Lyr, 1965).

Another source of polyphenol material in straw is lignin, of which there is some 15% by dry weight in barley straw. Lignin is very readily oxidized under basic conditions in the presence of oxygen, and pathways involving ortho- or paraquinone intermediates have been postulated (Sarkanen and Ludwig, 1971; Kempf and Dence, 1975). We therefore suggest that lignin may be a major contributor to the potential source within straw of oxidizable phenolic material, which would be antialgal. Indeed, brown-rotted wood, which is enriched in lignin, is inhibitory towards both *Chlorella* and *Microcystis* (Pillinger et al., 1993) to a greater extent than lignin-depleted white-rotted wood.

Only a very low dose of straw rotting in natural waters is necessary for algal control; therefore, the inhibitor must be active at an exceedingly low concentration. It is possible that transient quinone formation from lignin could result in a more antialgal compound than those commercial quinones tested. Fitzgerald et al. (1952) reported that 2,3-dichloronaphthoquinone at concentrations as low as 2 μg/liter completely killed *Microcystis*, the same effect being achieved by only a 10-min exposure to a solution containing 10 μg/liter. Interestingly, Newman and Barrett (1993a) reported that *Microcystis* was controlled by a short exposure (24 hr) to decomposing straw. Furthermore, possible formation of unstable (ortho) quinones (Field and Lettinga, 1989) and oxygen radicals (Appel, 1993) makes bioassay-directed fractionation (Saito et al., 1989) an unsuitable approach for the isolation of the antialgal factor.

We have confirmed the control of algal growth by quinones in vitro and shown that antialgal activity of barley straw is increased with increased aeration. Moreover, we hypothesize that the activity of rotting barley straw could be due to oxidizable phenolic groups from the lignocellulosic material decaying under aerobic and basic conditions prevailing in laboratory bioassays and natural ecosystems. The chemical characterization of the algal inhibitory component from decaying straw is necessary in order to optimize the use of straw as a reliable method of algal control in recreational, navigable, industrial, and potable waters and to be sure that no toxic side effects are likely to build up with continued use. Our future work is aimed at identifying the source of potential oxidized

phenolics and, in particular, we are exploring lignin and its solubilization products to determine whether this major polyphenolic source can explain the algal toxicity of straw decaying in water. The toxicity of radical oxygen species formed during the oxidation of phenols (Appel, 1993), requires further study.

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NATURE AND ROLE OF SEXUAL PHEROMONES EMITTED BY MALES OF *Acrolepiopsis assectella* (LEP.)

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Abstract—Seven compounds that do not exist in the extracts from legs of males have been isolated in the hair-pencil extracts of male *Acrolepiopsis assectella*. By combining techniques of GC-MS and GC-FT-IR, six of these compounds have been identified. They are six *n*-alkanes: hexadecane (C_{16}), heptadecane (C_{17}), octadecane (C_{18}), nonadecane (C_{19}), eicosane (C_{20}), and heneicosane (C_{21}). Twelve *n*-alkanes of the homologous series, from the C_{14}–C_{25} compounds were presented to virgin females, mated females, and males. At the end of the scotophase, four of the *n*-alkanes (C_{16}, C_{17}, C_{19}, C_{21}) present in the hair-pencil extract induced the virgin females to adopt the acceptance posture after having induced the virgin females to remain stationary. The two other *n*-alkanes (C_{18} and C_{20}) present in the extract have less effect on the females similar to the *n*-alkanes not present in the males. The blends tested do not seem to indicate any synergy between the most active compounds. The three *n*-alkanes with an odd number of carbons and the C_{16} compound would thus be the principle components of the male pheromone of *A. assectella*. As well as their role of female aphrodisiac, they tend to make males and fertilized females flee.

Key Words—Male sex pheromone, *n*-alkanes, leek moth, Lepidoptera, Acrolepiidae, hair-pencils, gas chromatography–mass spectrometry, gas chromatography–FT-IR, female arrestant, acceptance posture.

INTRODUCTION

Recent articles (Birch and Hefetz, 1987; Birch et al., 1990; Boppré, 1984) have shown that several male pheromones have been identified, principally in the Noctuidae, Nymphalidae, Arctiidae, Pyralidae, and Hepialidae. Male phero-

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mones in other less studied families have started to attract attention, and it is interesting to compare the nature of the pheromones emitted by the males of these families with those of families that are more frequently the subject of study.

The male leek moth, *Acrolepiopsis assectella* (Acrolepiidae), possesses coremata or hair-pencils that are concealed when at rest in an abdominal pocket, which opens to the exterior laterally between the eighth and ninth abdominal segments (Thibout, 1972a). These organs are extruded during sexual behavior when the male, attracted by the sexual pheromones of calling females, approaches and flutters its wings a few millimeters from the female (Thibout, 1972b). Male pheromones emitted in this fashion provoke the adoption of the acceptance posture by the calling female and thus have an aphrodisiac role (Thibout, 1978).

Few or none of the male pheromones are known in the Acrolepiidae and more generally in the Hyponomeutoïdea, so the identification and role of those in the leek moth has been undertaken.

METHODS AND MATERIALS

Rearing. A. assectella was reared in the laboratory on the host plant, the leek *Allium porrum*. The stock was renewed annually by the harvesting of larvae in leek fields around Tours. The conditions of rearing were as follows: 16 hr of photophase at 25°C and 8 hr of scotophase at 15°C, relative humidity varying between 80% and 60%. Sexes were separated after the end of the fifth larval stage and the adults kept in the presence of damp cotton.

Extraction. Through pressure on the abdomen of virgin males 4–6 days old, the opening of the pocket containing the hair-pencil becomes visible. With fine forceps the hair-pencils were removed, still retracted within the epithelium of the pocket. Using this method, 300 hair-pencils were removed in the middle of the photophase and immediately placed in a glass tube at −80°C. Just before the analysis, 0.2 ml of ether was added. Five hundred hair-pencils were similarly taken either in the first 3 hr or in the last 3 hr of scotophase. This last period is that during which sexual behavior takes place (Thibout, 1976). These hair-pencils were immediately plunged in 0.5 ml hexane and kept at −20°C.

Two control extracts were made by plunging in 0.5 ml hexane either 50 legs of male leek moths or 150 female ovipositors composed of the eighth and ninth abdominal segments, which carry the pheromone glands (Thibout, 1972b). The latter were taken during photophase, i.e., in the absence of the synthesis of female sexual pheromone (Renou et al., 1981).

Chemical Identification. The extracts were first analyzed by gas chromatography on a Varian 3300 equipped with an on-column injector and a FID detector. Each injection corresponds approximately to the extract of two hair-pencils, i.e., a male equivalent.

The separations were made on a Hewlett-Packard capillary column (25 m × 0.22 mm) using a 0.33-μm methyl silicon HP1 film and of 99.999% helium as the carrier gas (Air Liquide N60). The temperature program was from 70 to 280°C at 2°C/min. The detector was maintained at 300°C.

The identification of the pheromones was obtained by the coupling of GC–mass spectrometry (GC-MS) and the coupling of GC–infrared spectrometry (GC-IR) at the same sensitivity levels. In fact, a GC-IR interface by direct deposition has been developed recently, which brings an increased sensitivity of one or two orders of magnitude in comparison with the classic "light-pipe" type interface. The principal used is the trapping, at 77K, on a mobile window transparent to IR, of separated products which will then enable their analysis in the solid state by microscopic IR-TF. The possibility of multiplying the subsequent analyses of the trapped products considerably improves the signal-to-noise ratio (Visser et al., 1993).

This method utilizes a mass spectrometer (Hewlett-Packard 5898 Mass Engine) with a HP UX work station and an infrared spectrometer (Biorad, Digilab FTS 45 A) equipped with a cryotrapping interface Tracer. These spectrometers are each coupled to a Hewlett-Packard 5890 II GC equipped with a split–splitless injector and a column identical to that used by the Varian 3300; the temperature program is the same. The transfer line GC-MS is maintained at 100°C; the temperature of the source and of the quadrupole are fixed at 200°C and 100°C, respectively. The transfer line GC-IR is maintained at 250°C and the "on-the-fly" spectrums are obtained by the coaddition of four interferogrammes, with a resolution of 8 cm^{-1}.

The combined use of these GC-MS and GC-IR enables us to put forward the structure of these pheromones without the risk of errors due to isomerism. For example, the structure of the alkanes first identified in MS by their molecular peak and their retention time was rapidly confirmed by using the relative intensities of the specific bands of CH_2 and CH_3 between 2800 and 3000 cm^{-1}, certain branched alkanes being able to show similar retention times to those of *n*-alkanes (Bourne et al., 1990).

The significance of the generalization of this type of combined analysis by GC-MS and GC-IR by direct deposition in chemical ecology will be subsequently shown.

Behavioral Observations. The six compounds identified in the male extracts as well as a certain number of analogs in the same series have been commercially obtained (Aldrich): their purity was always greater than 98%. To test their behavioral effects, 30 mg of each compound were dissolved in 1 ml of hexane. Three types of blend were prepared. Blends B3o and B3e, respectively, combined the three identified compounds in the extract with an odd number of carbons (C_{17}, C_{19}, and C_{21}), or with an even number of carbons (C_{16}, C_{18}, and C_{20}). These two blends contained 10 mg of each compound in 1 ml of hexane.

Blend B6 combined in 1 ml of hexane the six compounds identified in the male extracts a rate of 10 mg of compound with an odd number of carbons and of 1 mg of compound with an even number of carbons in order to correspond approximately with the natural proportions.

The male pheromones were active on calling females over a short distance (Thibout, 1972b), and the solutions previously mentioned as well as the hair-pencils extracted during the first 3 hr of scotophase were presented to the females by approaching them with a glass rod, 1.6 mm in diameter, which had previously been plunged in a solution. Virgin females between 4 and 6 days old were placed singly in cylindrical transparent boxes (6.2 cm high, 4 cm diameter). The behavioral observations took place in the last hour of the scotophase, when the proportion of calling females was likely to be greater than 50%. A weak illumination of 0.5 lux enabled us to follow the females. The cover of the box was removed, and the end of the glass rod was brought to within 1 or 2 mm of the female. In the absence of a reaction from the female, the rod, by analogy with the male's sexual behavior, was moved two or three times from one side of the female to other passing in front of the head, and then the wings of the female were touched on the side. This entire procedure lasted for about 10 sec and was repeated two or three times.

The principal characteristic female responses were as follows: They escaped by flying or by rapid walking as soon as the cover of the box was moved or the glass rod introduced. These females, which were seldom calling, were not taken into account. They escaped by flying or rapid walking when the rod approached them or made contact with their wings. They adopted the acceptance posture by the female. When the glass rod approached, the female lifted up her wings and showed the extremity of her abdomen, or when contact was made the female moved sideways a few millimeters to stop again in the calling or acceptance posture. They moved a few centimeters by walking forward, followed by stopping, either taking up the calling posture or not, when the rod approached or touched the wings. This movement was followed either by escape or adoption of the acceptance posture during a second or third approach sequence and contact with the rod.

Thirty isolated females were observed daily three times in succession at 20-min intervals with two different solutions or a control. This control was either a positive control—male hair-pencil extract—or one of the two negative controls—a clean glass rod or a glass rod plunged in hexane and used after 30 sec of evaporation. Hexane was also used as a clean chemical compound and not as a solvent; it was thus presented very quickly before total evaporation. Observations were made on three consecutive days, systematically changing the females and the order of the presentation of the solutions and the control. In certain cases a second series was made. Each solution or control was thus tested

on 90 or 180 females. The numbers of escape behavior, acceptance posture, and walking followed by escape or by acceptance were noted in those females that responded to the presence of the glass rod.

By way of comparison, the behavior of virgin males between 4 and 6 days old and of females of the same age mated the day before the observation was observed during the presentation of the hair-pencil extract or the evaporated hexane control. In the mated females, the B6 blend of six compounds identified in the hair-pencil extracts was also tested.

The results obtained were statistically compared using χ^2 contingency tests. They were compared one with the other, the positive control, or the negative control, i.e., to the whole of the comparable results obtained with the clean glass rod and with the evaporated hexane ($\chi^2 = 1.358$; ddl = 2; $\alpha = 0.51$). The classes were regrouped when the theoretical size of one of them was less than one (Snedecor and Cochran, 1957).

By using the difference in weight after plunging the glass rod 30 times into 1 ml of hexane, the quantity of compound deposited on the rod during an experiment was estimated at 45 μg. The quantity of male pheromone on the glass rod was estimated at about 1.5 males, i.e., three hair-pencils.

RESULTS

Other than abdominal hair-pencils, no other sexual scent apparatus were observed in the male leek moth, in particular on the abdomen or on the wings. Based on 15, the calculated average volume of a sheathed hair-pencil is 0.0244 $\pm$ 0.0020 mm^3.

Compounds Produced by Hair-Pencils. The gas chromatograms of the hair-pencil extracts obtained during the day and during the night showed, along with a certain number of peaks that are also present in the chromatogram of the leg extract (A–F), seven prominent peaks (1–7) (Figure 1). In contrast, the extracts from female glands did not contain peaks 1–7.

The spectra obtained by coupling GC-MS and GC-IR of the male extracts indicated a structure of saturated linear hydrocarbon for compounds 1, 2, 3, 4, 5, 7, which constitute the series hexadecane (C_{16}), heptadecane (C_{17}), octadecane (C_{18}), nonadecane (C_{19}), eicosane (C_{20}), and heneicosane (C_{21}) (Figure 2).

The corresponding retention times of commercial products are identical. Any doubt remaining as to the existence of ramified (branched) isomers is removed by the observation of the regular reduction of the ratio of intensity of the bands CH_3 and CH_2 of C_{16}–C_{21}, identical to those of the reference products. In contrast, compound 6, poorly separated from 7, has not yet been identified, and the studies in GC-IR by deconvolution of spectra are being carried out.

Per male-equivalent, the abundance of compounds is estimated as: C_{16} =

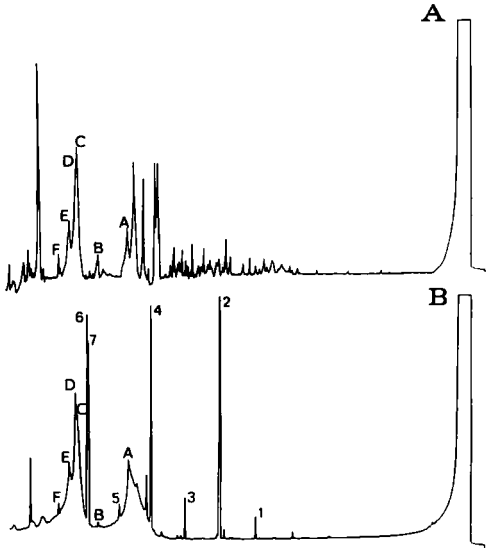

FIG. 1. Gas chromatograms of *A. assectella* male extracts: legs (A) and hair-pencils (B). 1: hexadecane; 2: heptadecane; 3: octadecane; 4: nonadecane; 5: eicosane; 7: heneicosane.

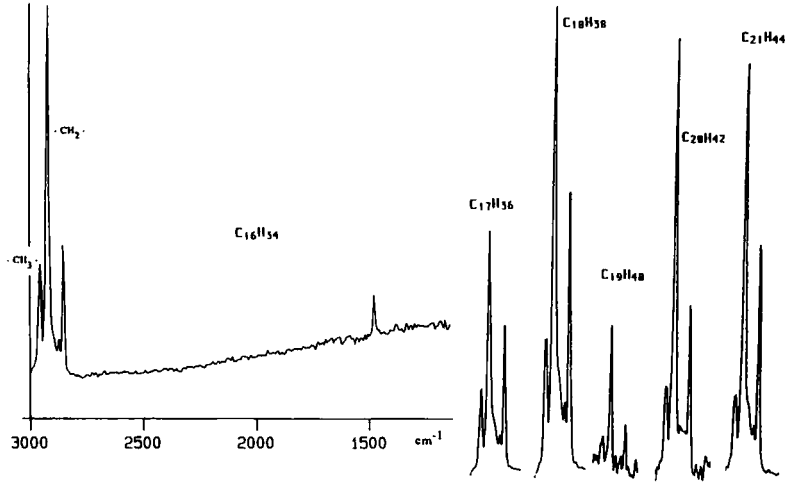

FIG. 2. IR spectra of compounds 1–5 and 7 only found in hair-pencil extracts. See Figure 1 for description of figure components.

4 ng; C_{17} = 400 ng; C_{18} = 10 ng; C_{19} = 200 ng; C_{20} = 4 ng; and C_{21} = 200 ng.

Pheromonal Role of Isolated n-*Alkanes.* Out of 90 adults, 40–50 responded to the approach of the glass rod and were studied.

The responses of virgin females to negative controls—the clean glass rod or one previously plunged in hexane provoked—as has been already shown, were comparable. Only a small percentage of these responded by adopting an acceptance posture and this always after walking. These two controls, having been replicated six times each (180 females), were regrouped, thus enabling us to obtain a total control on 188 virgin females having responded to the test (Figure 3).

Responses of virgin females to the hair-pencil extract and to the blends were as follows: In the presence of a rod plunged in the hair-pencil extract or in one of the three blends of the compounds isolated in these organs (Figure 3), the females responded in about 50% of cases by adopting the acceptance posture preceded or not by walking. Whether it was the tests with the hair-pencil extract, with the B6 blend with six *n*-alkanes (C_{16}, C_{17}, C_{18}, C_{19}, C_{20}, and C_{21}), or with the blend with three *n*-alkanes with an even number of carbons (C_{16}, C_{18}, and C_{20}) or an odd number of carbons (C_{17}, C_{19}, and C_{21}), there was no significant difference in the frequency of the various behavioral stages (χ^2 = 13.136; ddl = 9; α = 0.16).

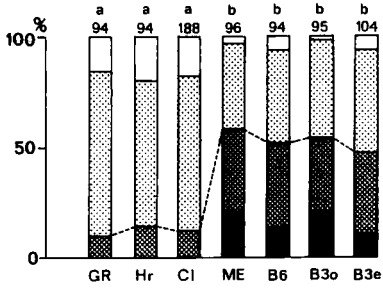

FIG. 3. Effects of various *n*-alkane blends, controls, and hair-pencil extract on the frequencies of the four behavioral responses (in percent) of *A. assectella* virgin females. Numbers above each column represent the total number of responding adults. Columns with the same letters are not significantly different according to χ^2 contengency tests ($P < 0.05$). White: escape; light grey: walk followed by escape; dark grey: walk followed by acceptation posture; black: acceptation posture. GR: Clean glass rod; Hn: evaporated hexane; Cl: total control (GR + Hn); ME: male hair-pencil extract; B6: blend of the six *n*-alkanes found in the hair-pencil extracts; B3o: blend of the three *n*-alkanes with an odd number of carbons found in the extracts. B3e: blend of the three *n*-alkanes with an even umber of carbons found in the extracts.

Responses of virgin females to *n*-alkanes taken singly were as follows: The behavioral activity of virgin females was analyzed in the presence of 13 *n*-alkanes taken in isolation [the complete series from tetradecane to pentacosane (C_{14}–C_{25}) as well as hexane (C_6)]. The frequency of escapes preceded or not with walking is significantly higher with hexane than those observed in all of the control females. Hexane thus appears to be repulsive (Figure 4).

The 12 other *n*-alkanes studied can be grouped into three categories: (1) Compounds C_{14}, C_{15}, C_{24}, and C_{25}. In this category the behavioral frequencies observed are not significantly different from those observed with the total control. (2) Compounds C_{18}, C_{20}, C_{22}, and C_{23}. The frequencies in this group differ significantly from the control frequencies and from the frequencies obtained with the hair-pencil extract (Figure 4). The frequencies of escape or acceptance posture preceded or not by walking are midway between those of the total of control females and those of the females in the presence of the hair-pencil extract. (3) Compounds C_{16}, C_{17}, C_{19}, and C_{21}. The behavioral frequencies do not differ from those obtained with the hair-pencil extract (Figure 4).

Responses of males and mated females were as follows: In the males neither behavior nor posture characteristic of reproduction were observed. Only the introduction of the glass rod or the presentation of the hair-pencil extract carried with it higher frequencies of escape with or without preliminary walking in the males (Figure 5). These frequencies are thus equal or greater than those observed in the females in the presence of hexane ($\chi^2 = 3.564$; ddl = 1; $\alpha = 0.06$ and $\chi^2 = 5.189$; ddl = 1; $\alpha = 0.02$).

The mated females were never observed calling and never responded to the introduction of the rod by taking the acceptance posture whether or not *n*-alkane or hair-pencil extract was present (Figure 5). The mated females responded like

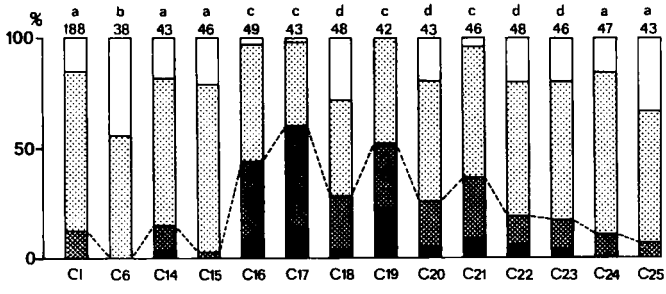

FIG. 4. Effects of various *n*-alkanes on the frequencies of the four behavioral responses (in percent) of *A. assectella* virgin females. Cl: total control; C_6 and C_{14}–C_{25}: *n*-alkanes with the corresponding number of carbons. See Figure 3 for description of figure components.

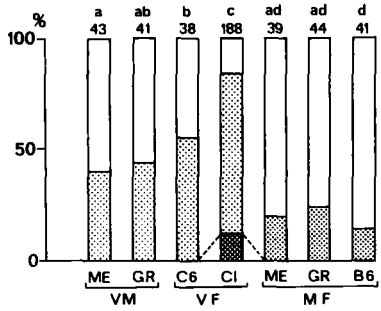

FIG. 5. Effects of various *n*-alkane blends, controls, and hair-pencil extract on the frequencies of the four behavioral responses (in percent) of *A. assectella* virgin males and mated females. Some previous virgin female data are added for comparison. VM: virgin males; VF: virgin females; MF: mated females. See Figure 3 for description of figure components.

the males, but in certain cases they fled even more rapidly than the males without any preliminary walking.

DISCUSSION

The six *n*-alkanes isolated uniquely in the hair-pencil extracts are the principle components of the male sex pheromone of *A. assectella*. A compound that has not yet been identified, peak 6 (Figure 1), could also be one of the pheromonal components. The behavior of adult *A. assectella* to the presentation of hair-pencil extract and certain homologs of the *n*-alkane series clearly shows that only virgin females show a response of a sexual nature. Among the compounds of this series only four *n*-alkanes of the six identified in the hair-pencil extracts provoked as many responses of a sexual nature in virgin females as did the extract. These are the three *n*-alkanes with odd numbers of carbons (C_{17}, C_{19}, and C_{21}), which are the most abundant, and also C_{16}, which is less abundant as are the other compounds with an even number of carbons. The two other *n*-alkanes identified in the extract, C_{18} and C_{20}, provoked the least response as did the *n*-alkanes absent from the extracts. The pheromonal activity of the *n*-alkanes thus follows fairly close their abundance in the hair-pencil extract.

The B6 blend of six *n*-alkanes and the B3o blend of three *n*-alkanes with an odd number of carbons have close concentrations of active product, comparable to the solutions of compounds C_{17}, C_{19}, or C_{21} taken in isolation. Moreover, their pheromonal activities are comparable. The B3e blend of three

n-alkanes with an even number of carbons, less concentrated in active products (only compound C_{16} has a strong activity on the females), is, in contrast, as active on the females as the two preceding blends. In the concentrations used, the diverse compounds do not seem to act in synergy, unless this has not yet been brought to light, the maximum response of a sexual nature having been obtained. The effect of weaker concentrations should thus be studied, but an absence of synergy between *n*-alkanes utilized as pheromones by insects has also been observed in the white marked tussock moth, *Orgyia leucostigma* (Grant et al., 1987).

The pheromonal compounds were isolated in the three hair-pencil extracts, whatever the time of extraction. Contrary to the female sexual pheromones of many moths, which are produced at the moment of calling, as in the leek moth (Renou et al., 1981), the male pheromone of *A. assectella* will be synthesized for the whole of the nycthemeron. This seems to be a frequent character in male Lepidoptera as is shown, for example, in *Pseudaletia unipuncta* (Fitzpatrick et al., 1985) or *Etiella zinckenella* (Hattori, 1987).

The *n*-alkanes are well known in diverse insects where they can play a role in intra- and/or interspecific chemical communication. In Lepidoptera they have, on several occasions, been identified in the Pieridae (Bergström and Lundgren, 1973; Grula et al., 1980; Hayashi et al., 1978), but their role as pheromones has rarely been shown. Nevertheless in *Orgyia leucostigma*, the scales of the female contain various *n*-alkanes, of which certain ones, such as compounds C_{24} and C_{25}, play a role as copulation releaser pheromone (Grant et al., 1987). In the males (other than in the case of *A. assectella* described here), there are no *n*-alkanes in which a pheromonal role has been clearly demonstrated.

The male pheromones of Lepidoptera could have different roles. Among the most important are: attraction of the female, sexual stimulation of the females, female arrestant, or sexual inhibition of competitive males (Bergström and Lundgren, 1973; Birch and Hefetz, 1987; Birch et al., 1990; Boppré, 1984; Fitzpatrick and McNeil, 1988). In the leek moth, in confined spaces, the male pheromones lessen the time necessary for mating and also lessen the length of wing-fluttering time for the males (Thibout, 1978). The hair-pencil extracts or the *n*-alkanes that they contain provoke in virgin females a lessening in the number of escapes and an accompanying rise in the numbers of acceptance postures. The male pheromones of *A. assectella* thus indeed have an aphrodisiac role on their females which cannot be dissociated from a previous role in their arrestment. These roles of male pheromones of *A. assectella* do not exclude other possibilities that should be researched.

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NATURAL AND SYNTHETIC OVIPOSITION STIMULANTS FOR *Catolaccus grandis* (BURKS) FEMALES[1]

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Abstract—Oviposition behavior was elicited from *Catolaccus grandis* (Burks) (Hymenoptera: Pteromalidae) females, an ectoparasitoid of the boll weevil, *Anthonomus grandis* Boheman (Coleoptera: Curculionidae), by smears of freshly cut cotton bolls or smears of extracts prepared with boll weevil damaged or undamaged cotton boll tissues. Oviposition behavior was also elicited from *C. grandis* females by smears made with *n*-pentane, *n*-hexane, *n*-heptane, and isooctane. This is the first report of oviposition behavior elicited for any parasitoid by these short-chain saturated hydrocarbons (alkanes), introducing a new concept on the chemical mediation of parasitoid behavior during host selection. Oviposition behavior was also elicited from *C. grandis* females by volatiles emanating from an artificial diet devoid of insect components that was specifically developed for the in vitro rearing of ectoparasitoids. The possible use of a synergistic combination of *n*-hexane and diet to optimize the mechanized production of noncontaminated eggs is also discussed.

Key Words—Ovipositional stimulants; *Catolaccus grandis*, Hymenoptera, Pteromalidae, short-chain hydrocarbons, artificial diet, kairomones, synomones.

INTRODUCTION

Recent research has shown that *Catolaccus grandis* (Burks), an ectoparasitoid, which was imported from southern Mexico, can parasitize a significant portion

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[1] Mention of a commercial or proprietary product does not constitute an endorsement by the USDA.

of boll weevil (*Anthonomus grandis* Boheman) larval populations when released in test cotton fields in the lower Rio Grande Valley of Texas (Morales-Ramos and King, 1991; Summy et al., 1993). These results suggest that augmentation releases of this parasitoid might be valuable as a component of our integrated pest management (IPM) program for the boll weevil. One important obstacle in devising such a program is the lack of an economically feasible rearing method for the mass production of this parasitoid. At present, *C. grandis* adults used in augmentation studies are being reared on third-instar boll weevil larvae using the Parafilm cell method described by Cate (1987). Because this method is dependent on the mass production of the host, it is very expensive to produce the numbers of parasitoids needed for an area-wide control program. Production costs might be reduced by developing an effective in vitro rearing system. To be feasible, an in vitro rearing system capable of large-scale production of *C. grandis* must include an adequate artificial diet devoid of insect components (Guerra and Robacker, 1991) and rearing techniques that can be completely mechanized (Guerra et al., 1993).

Guerra and Robacker (1991) and Guerra et al. (1993) reported the first successful in vitro rearing procedure that used larval artificial diets devoid of insect components for *C. grandis* and *Bracon mellitor* Say. Although effective, some basic problems related to rearing conditions and handling still need to be resolved before a completely automatic closed propagation system can be developed. Under natural conditions, the successful parasitization of an adequate host is dependent on the behavior elicited by a series of infochemicals produced by the herbivore, the plant used by the herbivore as food, or by synomones produced by the plant as a response to herbivore damage (Nordlund et al., 1981; Vinson, 1976, 1981; Dicke et al., 1990, 1993). These chemicals are involved in host habitat location, host location, and host acceptance (Doutt, 1959). The development of a mechanized in vitro rearing system for *C. grandis* will require stimulants that can induce probing and oviposition by the parasitoid on completely artificial sites. Currently, there are no reports in the literature concerning oviposition stimulants for *C. grandis*. Female boll weevils deposit their eggs inside cotton squares (flower buds) or bolls (fruit). Thus, it is most likely that *C. grandis* females must locate the third-instar larvae, its preferred host instar (Summy et al., unpublished data) via chemical cues emanating from the larval cuticule, other weevil damaged or uninfested cotton plant tissues, or volatile chemicals associated with feeding behavior or digestive processes. Henson et al. (1977) reported that chemical cues (long-chain fatty-acid esters) in the frass of boll weevil larvae elicited ovipositional behavior in females of *B. mellitor*. Since multiple analytical efforts to isolate the kairomone from third-instar boll weevil larvae have failed in the past few years, we decided to search for other semiochemicals that could be used to support the development of a mechanized in vitro mass propagation of *C. grandis*.

The objectives of this work, were to: (1) identify any cotton plant synomones produced in uninfested or infested (herbivore-induced synomones) plant materials, which can induce ovipositional activity in *C. grandis*, and (2) use these materials to develop an in vitro rearing system where egg and larval manipulation could be minimized or eliminated entirely.

METHODS AND MATERIALS

Biological Material

Catolaccus grandis adults were obtained from laboratory colonies maintained at the USDA-ARS Laboratory at Weslaco, Texas. *Catolaccus grandis* was reared in vivo on third-instar boll weevil larvae (preferred host) using the Parafilm cell method reported by Cate (1987) and rearing conditions reported by Guerra et al. (1993). After adult emergence, ca. 300 females were placed inside clear plastic cages (40 × 40 × 30 cm), and honey was provided daily by streaking the top of the cage with a fine-tip plastic syringe; distilled water was provided in small plastic jars stoppered with cotton rolls. On the third and fourth days after emergence, females were allowed to probe on the Parafilm pads containing boll weevil larvae. After this preconditioning period, females were ready for the bioassays.

Bioassay Materials

Several experiments were used to evaluate materials as oviposition stimulants that might facilitate the development of an automated closed rearing system requiring a minimum of manipulation. The basic test rearing unit consisted of a Multiwell tissue culture plate (24 wells) as described by Guerra et al. (1993) to rear *C. grandis* and *B. mellitor* on agar-retained (0.7% of final diet volume) artificial diets devoid of insect components.

The test units were prepared without eggs in the plate wells, with or without diet, and were covered with Parafilm held in place with a rubber band and smeared (using a cotton-tipped applicator) with the materials under evaluation. The treated units were then placed in the plastic cages containing gravid females, where they were held, except as indicated, for ca. 4 hr (10 AM to 2 PM, 4 hr after the onset of the light phase). Longer periods of time were used with female parasitoids that had been ovipositing for more than eight consecutive hours. The test units were then removed from the cages, and the eggs associated with each treatment were counted. Except as indicated, all tests and treatments were replicated 10 times and each rearing plate unit was considered a replicate.

Bioassays with Cotton Boll Parts and Weevil Larvae

Test 1. Infested and/or uninfested cotton bolls were cut in half and weevil immatures from damaged bolls were removed and saved for the bioassays. Exposed tissues from inside the infested or uninfested bolls and live weevil larvae previously removed from infested cotton bolls were individually rubbed on the Parafilm covering the plates. The treated plates were then placed for 15 hr (5 PM to 8 AM, 5 hr photophase) inside the parasitoid cages, and the number of eggs oviposited were counted to evaluate ovipositional behavior in each treatment. Untreated Parafilm covers were used as controls.

Test 2. Smears made with weevil-damaged cotton boll parts from a half cotton boll (after removal of weevil larvae), that included inner boll peel surfaces, carpel septa between seeds, and lint-covered seeds, were evaluated as indicated in test 1.

Bioassays with Chemical Extracts of Cotton Bolls.

Because preliminary observations indicated that smears made with infested cotton boll tissues induced ovipositional behavior in *C. grandis* females, cotton carpels obtained from infested cotton bolls were chemically extracted for further evaluation as follows:

Test 3. Cotton boll carpels, obtained from single medium-sized infested bolls, were rinsed for different time intervals (5, 10, 15, and 20 min) with 20 ml of a mixture of chloroform–methanol (2:1, v/v) to extract the active components. Extracts were evaporated under nitrogen to 1-ml volume samples, and aliquots were smeared around the edge of the Parafilm rearing chamber covers in straight vertical and horizontal lines in such a pattern that no materials were smeared directly above the Parafilm-covered wells. These chambers were exposed to caged *C. grandis* females, and the number of eggs oviposited in a 4-hr period were counted. This test was conducted using rearing plates with and without the parasitoid diet in the plate wells, and rearing chamber plates covered with untreated Parafilm were used as control treatments.

Test 4. Cotton boll carpels obtained from single medium-sized infested and uninfested bolls were extracted for 15 min with *n*-hexane. Extracts were then evaporated under nitrogen to 1-ml volume samples, and aliquots were smeared on Parafilm-covered rearing plates that contained larval diet in the wells.

Plates that had the Parafilm cover smeared with *n*-hexane (no diet) and plates covered with untreated Parafilm (no diet) were used as controls. Test rearing chambers were exposed to ca. 300 *C. grandis* gravid females for 4 hr, and the number of eggs oviposited was recorded. Treatments in this test were also replicated 10 times.

Bioassays with Saturated Hydrocarbons (Alkanes)

Test 5. Because smears with the solvent *n*-hexane control in test 4 induced a strong oviposition of *C. grandis* females, further tests were conducted to evaluate the direct ovipositional stimulant activity of this chemical and other short-chain hydrocarbons (alkanes), including *n*-pentane, *n*-hexane, *n*-heptane, and isooctane. Smears with individual hydrocarbons were applied on the Parafilm covering the rearing plates (without artificial diet in the well). Treated rearing plates were exposed to *C. grandis* females overnight (5 PM to 8 AM, 5-hr photophase) because females had been ovipositing for at least eight consecutive hours before this test.

To determine if the *n*-alkanes were acting as contact chemicals or as volatile materials when inducing parasitoid oviposition, *n*-pentane, *n*-hexane, *n*-heptane, and isooctane were impregnated in 1-cm circular pieces of cotton fabric and placed on the bottom of the tissue culture plate wells. In these tests, there was no contact by the parasitoid's ovipositor (ca. 2 mm long) with the test material in the wells (2 cm deep) when they probed through the Parafilm cover. Empty rearing plates with untreated Parafilm covers were used as untreated controls.

Utilization of a Model Propagation System

Test 6. An effort was made to develop a modified rearing chamber where chemical oviposition stimulants could be used to induce parasitoid probing and oviposition through a Parafilm membrane directly into the diet container. For this test, *n*-hexane was evaluated to determine its potential as an ovipositional stimulant when placed on Parafilm covering the tissue culture plates containing small amounts of diet (0.1 ml) in the plate wells. In these tests, we compared the oviposition stimulant efficiency of *n*-hexane and diet combinations to determine possible synergistic effects on egg production in a brief period of time. Treatments were exposed to female parasitoid for 4 hr as indicated previously.

Experimental data were analyzed wit the GLM procedure on PC-SAS (SAS Institute, 1990) and means were compared using the Waller-Duncan multiple-range test ($P = 0.05$). Data from tests 5 and 6 were normalized using a logarithmic transformation [$\ln(X + 0.5)$], and then evaluated with a one-way ANOVA and associated multiple-range test (SYSTAT, 1989).

RESULTS

Results of tests 1 and 4 demonstrated that smears made directly with weevil-damaged or uninfested cotton boll tissues or their chemical extracts induced *C. grandis* females to oviposit on artificial surfaces. However, smears made with live boll weevil larvae did not stimulate oviposition behavior.

Test 1. Results of exposing *C. grandis* females to residues of infested or uninfested cotton boll halves that were smeared over the surface of Parafilm covering the rearing plate units indicated significantly greater numbers of eggs ($X \pm SD = 48.0 \pm 3.71$) were oviposited when parasitoids were stimulated by smears made with weevil-damaged cotton bolls, as compared to the number of eggs deposited on Parafilm smeared with uninfested cotton boll halves ($X \pm SD = 24 \pm 3.09$), the number of eggs oviposited on Parafilm smeared with live third-instar boll weevil larvae ($X = o$), or the number of eggs deposited on untreated (control) Parafilm coverings ($X \pm SD = 1 \pm 0.82$).

Test 2. When the ovipositional activity of smears made with weevil-damaged cotton boll parts on Parafilm covering the rearing plates was evaluated in *C. grandis* females, significantly greater number of eggs were elicited by inner peel surfaces ($X \pm SD = 234.5 \pm 19.45$) followed in order of importance by carpel tissues ($X \pm SD = 140.0 \pm 0.48$). Ovipositional means for all treatments including the control (untreated Parafilm) ($X \pm SD = 2.0 \pm 1.6$) were statistically different (minimum SD = 2.31).

Test 3. Aliquots of chloroform–methanol extracts (5, 10, 15, or 20 min) of weevil-damaged cotton boll carpels smeared on Parafilm covering the plates containing diet elicited a strong ovipositional stimulant activity in *C. grandis* females. A significantly greater number of eggs was elicited by smears made with the 10-min extract ($X \pm SD = 79 \pm 9.2$) when compared to those laid in response to smears made with the 5-min extract ($X \pm SD = 68.0 \pm 5.1$). Number of eggs oviposited by females stimulated by smears made with the 15-min extract ($X \pm SD = 23.0 \pm 3.5$) and the 20-min extract ($X \pm SD = 21.0 \pm 2.0$) were not significantly different among themselves, but they were significantly different to those induced by the 5- and 10-min extracts. The number of eggs deposited on untreated (control) Parafilm ($X \pm SD = 2.0 \pm 1.2$) was significantly different than those elicited by all extracts.

When the same test was conducted without including diet in the rearing plate units, significantly greater numbers of eggs were elicited by the 10-min extract ($X \pm SD = 42.0 \pm 5.1$) when compared to egg depositions elicited in *C. grandis* by the 5-min ($X \pm SD = 35.0 \pm 3.8$) and 15-min ($X \pm SD = 38.0 \pm 1.8$) extracts. Egg depositions elicited by smears with the 20-min extract were not significantly different than those of the control (untreated Parafilm) shown above.

Test 4. When *n*-hexane 15-min extracts of weevil-damaged and uninfested cotton boll carpels were smeared on Parafilm covering the plate units containing diet, significantly greater numbers of eggs were deposited by females stimulated by the damaged carpels extract ($X \pm SD = 118 \pm 19.6$) as compared to egg depositions stimulated by the undamaged carpels extract ($X \pm SD = 77 \pm 6.3$). There were no significant differences between egg depositions elicited by the

undamaged carpel extract and the control ($X \pm SD = 83.0 \pm 7.6$) treatment (Parafilm cover smeared with *n*-hexane).

Oviposition Stimulant Activity of Hydrocarbons

Test 5. Results of tests where short-chain saturated hydrocarbons standards were smeared individually on Parafilm covered rearing plates without diet in their wells (Table 1) indicated that *n*-hexane (C_6H_{14}), *n*-heptane (C_7H_{16}), and isooctane (C_8H_{18}) induced strong oviposition behavior among caged *C. grandis* females. Greater mean egg depositions were induced by isooctane; however, the mean numbers of eggs oviposited as a response to stimuli from isooctane (75.2), *n*-hexane (70.4), and *n*-heptane (61.4) were not significantly different. The mean number of eggs per plate deposited on Parafilm covers as a response to *n*-pentane smears (4.2), which was the most volatile hydrocarbon tested, was not significantly different from the mean number of eggs (1.8) oviposited on untreated Parafilm.

Results of test 5, where the same short-chain saturated hydrocarbons evaluated in test 5 were impregnated on 1-cm cotton fabric circular pads (Table 1) and placed on the bottom of tissue culture plates covered with untreated Parafilm to prevent contact with the parasitoids' ovipositor, indicated there were no significant differences among the numbers of eggs oviposited in 15 hr as a response to *n*-pentane (44.4), *n*-hexane (65.2), *n*-heptane (50.0), and isooctane (50.0)

TABLE 1. MEAN NUMBER OF EGGS OVIPOSITED BY 300 (10- to 12-day-old) *Catolaccus grandis* FEMALES IN 15 HOURS AT INDICATED SITES AS RESPONSE TO SEVERAL SATURATED HYDROCARBONS[a]

Treatments	Materials Applied	
	As smears on parafilm[b]	Impregnated on cotton fabric pieces[c]
Control[d]	1.8 b[e]	1.8 b[e]
n-Pentane	4.2 b	44.4 a
n-Hexane	70.4 a	65.2 a
n-Heptane	61.4 a	50.0 a
Isooctane	75.2 a	55.0 a

[a] Three tests, five replicates/test, one rearing plate/replicate. Mean number of eggs oviposited between 5:00 PM and 8:00 AM (5-hr photophase).
[b] Parafilm-covered rearing tissue culture plate (24 wells).
[c] Treated 1 cm (diam.) cotton fabric circles placed at bottom of each plate well (24 wells).
[d] Untreated Parafilm covering the rearing plates.
[e] Means with the same letter are not significantly different at $P = 0.05$ by Tukey's test. Test actually conducted on transformed data [ln (X + 0.5)].

volatiles. The numbers of eggs oviposited on untreated (control) Parafilm were significantly different ($P = 0.05$) from those recorded for each of the saturated hydrocarbons tested. These results suggested that short-chain hydrocarbons do not act as contact chemicals, since the approximate length of the female ovipositor is ca. 2 mm, and the distance between the Parafilm and the bottom of the plate wells was ca. 2 cm. *n*-Pentane, the most volatile of the liquid *n*-alkanes tested, was a better ovipositional stimulant when it was impregnated in cotton circles placed in the bottom of the plate wells covered with Parafilm than it was when smeared on an exposed Parafilm surface.

Test 6. Results of tests, where the combined effects of *n*-hexane and the parasitoid larval diet were tested (Table 2), indicated that relatively large numbers of eggs (mean = 67.9) were oviposited (10 AM to 2 PM, lighted phase, 4 hr after the onset of photophase) by *C. grandis* females in response to stimuli induced by volatile chemicals in the diet alone. However, significantly more eggs (mean = 141.9) were oviposited by females exposed to the combination of *n*-hexane smears on Parafilm covering the rearing plates and 0.1 ml of diet dispensed at the bottom of each of the 24 wells. A mean of 2.0 eggs were oviposited by females on untreated control (Parafilm plate without diet).

These results are important for the development of a feasible augmentation program because inducement of the oviposition of larger number of eggs in shorter time periods would result in a more economical and efficient rearing system. In addition, inducement of the oviposition of larger numbers of eggs would allow us to dispense the desired number of properly surface-sterilized eggs per rearing chamber in a mechanized system.

TABLE 2. MEAN NUMBER OF EGGS OVIPOSITED BY 300 (10- to 12-Day-Old) *Catolaccus grandis* FEMALES IN 4 HOURS AT INDICATED SITES[a]

Treatments	Mean[b] ± SD
n-Hexane + diet[c]	141.90 a ± 36.7
Diet alone[d]	67.90 b ± 32.8
Control[e]	2.00 c ± 3.0

[a] Three tests, five replicates/test, 1 rearing plate/replicate). Mean number of eggs oviposited between 10:00 AM and 2:00 PM (lighted phase).

[b] Means with the same letter are not significantly different at $P = 0.05$ by the Waller-Duncan *K*-ratio *t* test.

[c] *n*-Hexane smeared on parafilm covering the rearing plate + 0.1 ml parasitoid diet dispensed at each plate well (24 wells).

[d] Untreated Parafilm-covered rearing plates/without diet.

[e] Untreated Parafilm covering 0.1 ml parasitoid diet dispensed at each plate well.

DISCUSSION

This study demonstrated the strong influence that plant synomones have on some of the key steps involved in the host selection processes of *C. grandis*.

Evidence from our first attempt to discover an ovipositional stimulant for this parasitoid species indicated egg deposition could be elicited in vitro with cotton boll synomones. After years of research, isolation and characterization of natural kairomones from boll weevil larvae have been elusive. In this study, we were not able to induce oviposition in *C. grandis* females with direct smears of live third-instar weevil larvae (the preferred developmental stage) or smears with *n*-hexane or chloroform–methanol larval rinses. Most probably boll weevil larval kairomones are highly volatile, and they must be produced continuously in vivo to stimulate female probing and/or oviposition.

In our studies with cotton boll tissues, parasitoids were stimulated to probe and oviposit on Parafilm covers smeared directly with tissues from undamaged and weevil-damaged cotton bolls whose host larvae and their frass had been removed. We know that Parafilm (a mixture of high-molecular-weight paraffins) by itself provides a chemical cue needed for probing (piercing); therefore it is possible the parasitoids also responded to other plant synomones that stimulated egg deposition because untreated Parafilm (control) elicited very heavy parasitoid probing but very rarely induced oviposition. The few eggs laid on untreated Parafilm were probably due to the highly stressed conditions of captivity observed by other authors (Thompson et al., 1983; Clausen, 1940).

Of interest during this work was the fact that we were able to elicit strong ovipositional behavior in *C. grandis* females with direct plant part smears or smears made with extracts of uninfested cotton boll tissues on Parafilm covers. In this case, the female parasitoids were probably induced to oviposit by the exclusive action of plant synomones produced and emitted by clean cotton bolls tissues. Recent research by Steinberg et al. (1993) indicated that the braconid *Cotesia glomerata* was more attracted by infochemicals produced and emitted by artificially damaged and herbivore-damaged cabbage than those emitted by its host *Pieris brassicae*. Based on this information, it is possible that during our work cotton boll synomones could have also been produced and emitted as a response to mechanical tissue damage caused when the clean cotton boll tissues were cut and dissected before bioassays.

In the present work, direct plant part smears or extracts of weevil-damaged cotton boll tissues (weevil larvae and frass removed) always elicited significantly greater egg depositions by *C. grandis* females than those induced by uninfested (clean) cotton boll tissues. When smears were made with herbivore-damaged cotton boll tissues, the oviposition response they elicited may have been induced by host-related cues such as long-chain hydrocarbons found in frass or sterol esters (Vinson et al., 1975; Nettles and Burks, 1975) that contaminated cotton

boll tissues or by infochemicals coming from sugars (sucrose and fructose) that are commonly produced in herbivore-damaged plant tissues (Hassel, 1968) or by microorganisms developing within them. The significantly greater ovipositional responses we induced in *C. grandis* females with weevil-damaged cotton materials have also been reported for other parasitoids in recent studies (Steinberg et al., 1993; Dicke et al., 1990, 1993). Therefore, it is likely that the great efficiency of the weevil-damaged cotton boll smears shown in our tests was due to synomones produced by the cotton bolls as a response to weevil damage.

Although the chemical nature, production sites, and exact role of these cotton boll synomones are still unknown, it appears these kinds of stimulants could be very useful as part of an in vitro mass propagation system for *C. grandis* parasitoids.

It was also very interesting to determine that the parasitoids' diet was capable of eliciting a strong oviposition behavior. Since the diet was not in direct contact with the females, it is possible that diet odors emanating from some of the key nutrients in the formulation, such as amino acids, minerals, vitamins, fatty acids, etc., could have been responsible for stimulating *C. grandis*. Similar results have been reported by Arthur et al. (1972), with the ichneumonid parasitoid *Itoplectis conquisitor* and by Nettles et al. (1983) with *Trichogramma pretiosum*. In any case, the synergistic effect created by combining the diet and *n*-hexane to induce greater parasitoid ovipositions could be very valuable in the development of a massive mechanized egg production and collection system.

Some very promising results that came out of this work were the discovery of a strong oviposition stimulant activity elicited by short-chain hydrocarbons in *C. grandis* females. To the best of our knowledge, this is the first time these saturated hydrocarbons (*n*-alkanes) have been reported as oviposition stimulants for any known parasitoid.

Because there is no detailed information available on the chemical nature of weevil kairomones or the cotton synomones discussed here for *C. grandis*, we believe that commercially available short-chain alkanes *n*-hexane, *n*-heptane, or isooctane could be used very effectively as oviposition stimulants for the in vitro propagation of this parasitoid. In such a system, parasitoids would be induced to deposit sterile eggs through Parafilm into separate diet compartments (wells), where they would hatch and complete their biological cycle with less risk of diet contamination and without human manipulation.

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REPELLENT PROPERTIES OF THE HOST COMPOUND 4-ALLYLANISOLE TO THE SOUTHERN PINE BEETLE[1]

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Abstract—The phenylpropanoid 4-allylanisole is a compound produced by loblolly pines (*Pinus taeda* L.), an abundant species in southern pine forests and a preferred host of southern pine beetle (*Dendroctonus frontalis* Zimmermann). Repellency of individual beetles was demonstrated in laboratory behavioral assays of *D. frontalis* and other scolytids. Inhibition was demonstrated in natural populations of *D. frontalis* using baited traps. In both tests, response to the inhibitory pheromone verbenone was used for comparison. In the laboratory, a higher proportion of newly emerged and reemerged *D. frontalis* responded negatively to 4-allylanisole than to verbenone. However, fewer reemergent than newly emerged individuals responded to either compound. In all field trials, the response of *D. frontalis* to its attractant pheromone in funnel traps was significantly reduced by simultaneous release of 4-allylanisole. In most trials total reduction did not differ from verbenone; however, unlike verbenone, 4-allylanisole reduced male and female catches proportionally. Both compounds together did not significantly further reduce trap catch. The response of a major predator, *Thanasimus dubius* (F.), to the attractant pheromone of *D. frontalis*, did not differ with the simultaneous release of either verbenone or 4-allylanisole. The results of preliminary field applications are presented and discussed.

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Key Words—Coleoptera, Scolytidae, *Dendroctonus frontalis*, *Pinus*, host compound, 4-allylanisole, repellent, semiochemical, verbenone, inhibitor.

INTRODUCTION

Compounds originating in host trees are known to produce a wide range of behavioral responses in scolytids. Due to their importance in tree defense and insect olfaction, volatile components of the host's pre-formed resin system have received particular attention in research with aggressive, tree-killing bark beetles. Most studies have examined either pesticidal (e.g., Smith, 1965; Coyne and Lott, 1976; Bridges, 1987; Himejima et al., 1992) or semiochemical (see reviews by Wood, 1982; Raffa et al., 1993) effects of monoterpenes on beetles and their associated organisms. Studies usually focus on their role in mass-attack: either as primary attractants (e.g., Heikkenen and Hrutfiord, 1965; Rudinsky, 1966a,b; Werner, 1972a,b; Moeck et al., 1981; Moeck and Simmons, 1991) or as precursors (e.g., Byers and Wood, 1981; Francke and Vité, 1983; Hunt et al., 1989) or synergists (e.g., Renwick and Vité, 1969, 1970) in secondary attraction. In the southern pine beetle (*Dendroctonus frontalis* Zimmermann)–southern yellow pine (*Pinus taeda* L., *P. palustris* Mill., *P. elliotti* Engelm. var. *elliottii*, *P. echinata* Mill., *P. virginiana* Mill.) system, primary attraction has not been demonstrated, but α-pinene, a major host monoterpene in all of these pines, is well known to act synergistically with frontalin, the primary component of the aggregation pheromone, to stimulate mass attack by *D. frontalis* (Renwick and Vité, 1969, 1970).

Anti-aggregation pheromones are important components in terminating the attack process of many aggressive scolytids (Wood, 1982; Raffa et al., 1993), but less is known about the potential of host compounds as inhibitors or repellents. The host compound myrcene was shown to interrupt the response of *Dendroctonus brevicomis* LeConte to its pheromone blend, which also contains myrcene (Tilden et al., 1981). Both limonene, with *Scolytus ventralis* LeConte (Bordasch and Berryman, 1977), and β-pinene, with *D. pseudotsugae* Hopkins (Heikkenen and Hrutfiord, 1965), have repellent properties in walking olfactory assays. *D. pseudotsugae* is also repelled by volatile components of its host resin while walking (Rudinsky, 1966b; Jantz and Rudinsky, 1965), but these same compounds are attractants during flight (Rudinsky, 1966a). Nonhost, green-leaf volatiles are the only phytochemicals known to inhibit aggregation of *D. frontalis*, and these effects are mild compared to those of inhibitors produced by conspecifics (Dickens et al., 1992). Attempts to apply pine oil, a mixture of various host and other compounds (Nijholt, 1980), to unattacked pines as a repellent for *D. frontalis* have met with limited success (Berisford et al., 1986; O'Donnell et al., 1986). Presumably host compounds contained in the mixture

are responsible for its repellent properties, but further work is needed to identify active compounds and verify this assertion.

The phenylpropanoid, 4-allylanisole (also commonly known as methyl chavicol or estragole) is known from numerous pine and other conifer species (Mirov, 1961; Drew and Pylant, 1966). Although common, this compound usually exists in relatively minor quantities of the volatile component of the resin (Sutherland and Wells, 1956; Mirov, 1961; Drew and Pylant, 1966; Hayes et al., 1994); however, foliar quantities may be higher (Zavarin et al., 1971; Cobb et al., 1972). In the southern yellow pines, 4-allylanisole is found in roughly comparable amounts among species, usually making up 1–5% of the turpentine yields (Sutherland and Wells, 1956; Mirov, 1961; Drew and Pylant, 1966). While receiving less emphasis than individual monoterpenes, 4-allylanisole appears to have potentially important biological effects. A saturated 4-allylanisole environment retards the growth of fungi associated with *D. frontalis* including the blue-staining fungus *Ophiostoma minus* (Hedgc.) H. & P. Sydow and two mycangial fungi (Bridges, 1987). Although they erroneously claim to have been the first to identify 4-allylanisole from loblolly pine (a claim cited again by Nebeker et al., 1993), Gambliel et al. (1985) found higher levels in phloem inoculated with *O. minus*. In addition to a higher level of 4-allylanisole produced as part of a localized wound response to fungi, this compound has also been shown to increase more globally in *Pinus ponderosa* Laws. leaves exposed to high levels of air pollution (Cobb et al., 1972).

The potential semiochemical role of 4-allylanisole for scolytids has received little attention. Testing of 4-allylanisole as a semiochemical for *D. frontalis* has been limited to a single study in which synergistic activity with frontalin was not found (Renwick and Vité, 1969, 1970). In walking olfactory assays, using terpenoid compounds isolated from *P. taeda*, Werner (1972a,b) found that 4-allylanisole was attractive to (especially male, the pioneering sex) *Ips grandicollis* (Eichoff) at a 1% concentration. In a survey of volatiles associated with *D. frontalis* brood trees, Salom et al. (1991, 1992a) determined that 4-allylanisole elicited little in the way of electroantennogram responses of two *D. frontalis* parasitoids. Cobb et al. (1972) speculate that foliar 4-allylanisole levels of *P. ponderosa*, especially as influenced by photochemical air pollution, may influence attack behavior of *D. brevicomis* and *D. ponderosae* Hopkins, but the idea was not directly tested. The potential role of 4-allylanisole as an antiaggregant or repellent for *D. frontalis* and other scolytids is unknown.

In this paper, we describe and report the results of laboratory and field assays of the repellent properties of the host compound 4-allylanisole against *D. frontalis*, related bark beetles, and associated beetles, compared with the *D. frontalis* inhibitory pheromone, verbenone. Preliminary field applications of this potential repellent on lightning-struck trees are also described.

METHODS AND MATERIALS

Laboratory Assay. Individual beetle response to 4-allylanisole, versus verbenone, was determined in a simple assay. A strip (5 mm wide) of 4-allylanisole (Aldrich Chemical Co., Inc. Milwaukee, Wisconsin) or verbenone (34% + :66% −, Borregaard, Inc., Sarpsborg, Norway) was "painted" (neat) with a camel's-hair brush in a circle (≈ 17 cm diam) on a piece of uncoated (tablet backing) cardboard (28 × 21.5 cm). For comparison (controls), we observed the behavior of *D. frontalis* released in untreated circles (outlined in pencil) and in circles treated with the attractant frontalure (2 : 1 α-pinene–frontalin; Aldrich and BASF, Limbergerhoff, Germany, respectively). Three minutes after treatment, which allowed the materials to be absorbed into the cardboard, beetles (two to five individuals) were released in the center of the treated circle. Responses (≤ 30 sec exposure) were recorded as not repelled or repelled; scored beetles were then placed in separate containers by response and later sexed. A beetle was designated *not repelled* if it walked through the treated circle or stopped but proceeded across the circle within 30 sec of exposure. A beetle was designated *repelled* if it moved toward the circle but stopped abruptly and raised its antennae (some "rear-up" on hind legs), stood motionless, and/or moved away from the circle (some move abruptly in the opposite direction) without crossing out of the circle.

Testing was conducted at room temperature (22–25°C) with light supplied from an adjoining room. An object was used to cast a shadow over the test circle (< 1 lumen/m^2). Beetles were refrigerated briefly before testing to reduce their tendency to fly. Only beetles that were capable of walking up the sides of a collection container were used for this experiment. In each trial, including controls, 50 apparently healthy beetles were selected at random and tested. Another 50 beetles, previously unexposed, were then selected and tested with the other compound. The order of compound use was also random.

Trials were conducted with:

1. Newly emerged *D. frontalis* in three trials (N = 50 beetles per compound per trial) were collected on three different dates from the same source population (Colfax, Louisiana). Beetles were obtained from two infested loblolly pines (≈ 24 cm dbh), which were felled with brood in the pupal or callow adult stage. Four bolts (45 cm long) were cut from each tree and placed in two separate (by tree) rearing cans to collect adults as they emerged (Browne, 1972). Assays were conducted on the day of emergence; beetles tested on March 12, 1992, originated from one tree and those tested on April 3 originated from the other tree.

2. Newly emerged and reemerged *D. frontalis* from the same generation and source population (Colfax, Louisiana) were used. Newly emerged beetles (N = 100) were obtained as described above from an infested loblolly at the

front of an active infestation with brood in the callow adult stage. A nearby freshly attacked tree was felled in order to obtain parent adults ($N = 100$), which emerged $\approx$20 days later. Bolts from each tree were placed in rearing cans for collection.

3. Trials were also conducted with a clerid beetle, *Thanasimus dubius* (F.) and five other scolytid species. *T. dubius* ($N = 50$) for this study were obtained over a three-day period from five traps baited with *D. frontalis* attractant (frontalin + turpentine) (Catahoula Ranger District, Kisatchie National Forest, Louisiana). Newly emerged *Ips avulsus* (Eichhoff) ($N = 50$) and *I. calligraphus* (Germar) ($N = 9$), typical associates of *D. frontalis* were obtained from rearing cans containing bolts of a single loblolly tree removed from an infestation containing both *D. frontalis* and *Ips*. Mountain pine beetles ($N = 50$), *D. ponderosae*, a univoltine western species, were extracted prior to emergence from bolts of lodgepole pines (*P. contorta* Dougl. ex. Loud.) from a site ($\approx$ 16 km north of LaPine, Oregon) in central Oregon and mailed overnight on ice to the Alexandria Forestry Center (AFC), Pineville, Louisiana, for testing. Similarly, western pine beetles ($N = 50$), *D. brevicomis*, another western species ecologically similar to *D. frontalis* were extracted prior to emergence from ponderosa pine (*P. ponderosa*) bolts from a site near MiWuk Village, California, in the Sierra foothills. These insects were sexed and then mailed overnight on ice to AFC for testing. Newly emerged *Ips pini* (Say), a transcontinental northern species, were obtained from a laboratory colony maintained on red pine (*Pinus resinosa* Ait.), housed at the University of Wisconsin, which was originated from Sauk County, Wisconsin, and replenished annually; specimens were mailed overnight to AFC. Newly emerged spruce beetles, *Dendroctonus rufipennis* (Kirby), from sitka spruce [*Picea sitchenis* (Bong.) Carr.] were mailed to AFC from Fairbanks, Alaska. In all cases, only apparently healthy beetles were used.

Field Assay. A test of the response of local beetle populations to 4-allylanisole and verbenone (vs. the attractancy of frontalure) was conducted using baited multiple-funnel traps (Lindgren, 1983). Traps (16-unit; Phero Tech, Inc., Delta, British Columbia, Canada) were placed in active *D. frontalis* infestations in the spring (April–June) (six replicates in six sites) and fall (September) (seven replicates in four sites); site, elution device, and inclusive dates for each trial are given in Table 1. Treatments (two traps each) consisted of frontalure, frontalure + verbenone, and frontalure + 4-allylanisole. Traps were placed no less than 10 m from each other, from green trees, or from infested trees with emerging brood. Placement of treatments was initially randomly assigned and then changed daily in a sequential order for six days. In the spring replications, baits were moved daily among stationary traps, whereas in the fall whole traps with baits were moved. Although no contamination effects were detected, the protocol was changed to avoid the possibility of contaminating traps while mov-

TABLE 1. SOUTHERN PINE BEETLE INFESTATION LOCATION, ELUTION DEVICE, AND INCLUSIVE DATES OF EACH FIELD ASSAY WITH SEMIOCHEMICAL-BAITED TRAPS

Site	Location	Elution device	Dates
A. Spring			
GT1	Winn RD	bark	4/14–4/22
GT2	Winn RD	sponge	4/23–5/04
HUNT	Catahoula RD	sponge	5/03–5/20
EV2306	Evangeline RD	sponge	5/15–6/20
EV2273	Evangeline RD	sponge	5/31–6/07
EV2277	Evangeline RD	sponge	5/30–6/07
B. Fall			
EV2402	Evangeline RD	wick	9/12–9/17
EV2402	Evangeline RD	sponge	9/22–9/28
EV2403	Evangeline RD	wick	9/12–9/17
EV2403	Evangeline RD	sponge	9/22–9/28
CT	Catahoula RD	wick	9/12–9/18
CT	Catahoula RD	sponge	9/21–9/27
Winn	Winn RD	sponge	9/25–9/30
C. 4-Allylanisole + verbenone combination trial			
Winn	Winn RD	(4-allyl) wick (verb) sponge	10/08–10/15
D. Dose response (one, two, or four 4-allylanisole elution devices)			
GT2	Winn RD	sponge	5/09–5/17
EV2277	Evangeline RD	sponge	6/16–6/21
CT	Catahoula RD	wick	10/03–10/15
EV2402	Evangeline RD	wick	10/03–10/15
EV2403	Evangeline RD	wick	10/03–10/07
E. Dose–response (1:0, 3:1, 1:1, 1:3, 0:1 turpentine–4-allylanisole)			
GT	Winn RD	pipet	4/24–5/18

ing baits. To kill collected beetles, collection cups contained a 5.5 × 2.0-cm piece of 2,2-dichlorovinyldimethylphosphate (Pest Strip, Loveland Industries, Inc., Greeley, Colorado). *Dendroctonus frontalis* and *T. dubius* were collected and the number recorded daily.

Bait preparation differed slightly between seasons (Table 1): In spring, frontalure (≈3.5 ml) was eluted from a single polyethylene transfer pipette (Samco, St.-Amand Mfg. Co., Inc., San Fernando, California); verbenone (5 ml; 66% − : 34% +) was eluted from a 2.5 × 1.75 × 0.375-cm cellulose sponge in a 0.7-mil white plastic bag (low density polyethylene; United Plastic Films, Inc., Carterville, Georgia); and 4-allylanisole (5 ml) was prepared in bags the same way as verbenone. In fall, frontalure was unchanged; verbenone (10 ml; 66% − : 34% +) was eluted from a bag containing two cellulose sponges (Phero

Tech); and in four sites 4-allylanisole was eluted from a 20-ml polyethylene scintillation vial (Kimble Glass Inc., Vineland, New Jersey) with cotton wick (Fisher Scientific, Pittsburgh, Pennsylvania) in one trial, followed by a second trial in three of the four sites using the bag devise employed in the spring replicates.

Elution rates were determined gravimetrically at 24-hr intervals under typical field conditions in the spring and in the laboratory during the fall (Table 2).

To determine if there was a difference between sexes in response to the various baits, sex ratios were obtained from one spring replicate (GT1, see Table 1) based on subsampling up to 50 *D. frontalis* per trap from daily collections. Similarly, as part of another study, sex ratios were obtained for *D. frontalis* trapped per day in an assay conducted July 3–9, 1993, in an active infestation on the Catahoula RD (CA3034). In this test, traps were baited with frontalure alone and simultaneously with verbenone, 4-allylanisole, limonene, or 4-allylanisole + limonene (only the sex ratio data will be reported here, study results will be reported elsewhere). These data were analyzed for fit to 1 : 1 by chi-square and contingency-table analysis (Statistix 4.0; Analytical Software, 1992).

The dose–response of *D. frontalis* to 4-allylanisole was tested using two methods: (1) Response was tested to frontalure given an increasing number of 4-allylanisole elution devices (five replicates, May–October 1992; Table 1). Traps were baited (two traps per treatment) with frontalure alone or frontalure + one, two, or four, 4-allylanisole elution devices. Treatment position was initially randomly assigned and then changed daily in a sequential order for five to eight days. (2) Response was tested to frontalin given an increasing percentage of 4-allylanisole when mixed with turpentine (one replicate, one site; Table 1). Each trap was baited with one pipet containing frontalin and one with either

TABLE 2. GRAVIMETRICALLY DETERMINED ELUTION RATES ($\bar{X}$ ± SE) FOR VARIOUS SEMIOCHEMICALS AND DEVICES USED IN BAITED-TRAP FIELD ASSAYS

Test	Compound	Device	*N*	T (°C)	Location	Elution rate (mg/24 hr)
Spring	frontalure	3.5 ml pipet	17	17–32	field	54.7 ± 2.4
Spring	4-allylanisole	2.0 ml bark	2	17–32	field	729.7 ± 59.9
	4-allylanisole	5.0 ml sponge	2	17–29	field	1149.7 ± 4.7
Spring	verbenone	5.0 ml sponge	4	17–32	field	94.0 ± 5.4
Fall	frontalure	3.5 ml pipette	3	22–27	lab	79.9 ± 5.5
Fall	4-allylanisole	5.0 ml sponge	3	22–27	lab	1236.6 ± 81.1
	4-allylanisole	20.0 ml wick	3	22–27	lab	159.5 ± 5.8
Fall	verbenone	10.0 ml sponge	3	22–27	lab	148.1 ± 8.4

100% turpentine, 75% turpentine and 25% 4-allylanisole, 50% turpentine and 50% 4-allylanisole, 25% turpentine and 75% 4-allylanisole, or 100% 4-allylanisole. In both dose–response experiments, the numbers of *D. frontalis* and *T. dubius* were recorded daily as described above.

In a single trial (Table 1), response of *D. frontalis* to 4-allylanisole in combination with verbenone was tested. Treatments (two traps per treatment) consisted of frontalure alone, frontalure + 4-allylanisole (two wicked 20 ml vials), frontalure + verbenone (two 10 ml bags), and frontalure + 4-allylanisole (one vial) + verbenone (one bag).

Data Analysis. In all field assays, mean values of *D. frontalis* and *T. dubius* were tested for normality, transformed by ln(Y + 1), and analyzed by analysis of variance (ANOVA) with separation of the transformed means performed by protected LSD and least-square mean where appropriate (PROC GLM; SAS Institute, 1988). Dose–response test data were analyzed by ANOVA and regression analysis (PROC GLM and PROC REG; SAS Institute, 1988). Mean values for dose–response data were transformed by ln(Y + 0.001) and separation of the transformed means was performed by protected LSD.

Preliminary Field Application. Two loblolly pines (*Pinus taeda*), one struck by lightning on June 1 and another on June 28, 1992, were treated with 4-allylanisole within two days of being struck and before attack by *D. frontalis*. A longleaf pine (*P. palustris*), struck by lightning on July 1 was treated with 4-allylanisole on July 2, 1992. The treatment consisted of placing nine 20-ml polyethylene vials with cotton wicks, evenly spaced from the ground to 8 m up the tree bole on the damaged side. Nearby pines of the same species, struck by lightning in the same storms, were located and served as controls. At day 30, numbers of *D. frontalis* attacks were counted in a 15.2-cm-wide band around the tree circumference at 2 and 4 m up the bole, and the general condition of the trees recorded.

RESULTS

Laboratory Assay. Male and female *D. frontalis*, newly emerged and to a lesser extent reemerged, were repelled when exposed to 4-allylanisole in laboratory assays. If the cardboard was untreated, all beetles walked immediately (<20 sec) out of the circle in all directions (i.e., 0% repelled). When placed in a frontalure-treated circle, 0% of the tested beetles were repelled: some beetles walked immediately (<20 sec) across the painted strip to the surrounding area out of the circle; others would stop at the strip, walk around, in, or back and forth across the painted strip before moving out to the surrounding area (<30 sec). Although the percentage varied from trial to trial, higher percentages of *D. frontalis* in all trials were repelled by 4-allylanisole than verbenone using

the same assay method, and generally beetles that were "repelled" by 4-allylanisole demonstrated a higher degree of alarm and more abrupt behavior than beetles "repelled" by verbenone. In trial 1, regardless of sex, 84% of the newly emerged beetles exposed to 4-allylanisole were repelled, while 11% of those exposed to verbenone were repelled (Table 3). In trial 2, regardless of sex, higher percentages of the newly or reemerged beetles exposed to 4-allylanisole were repelled than those exposed to verbenone (46 and 52% higher, respectively)

TABLE 3. RESPONSE OF NEWLY EMERGED *D. frontalis* TO 4-ALLYLANISOLE AND VERBENONE IN LABORATORY ASSAYS[a]

		4-Allylanisole		Verbenone	
Date	Sex	N	Repelled (%)	*N*	Repelled (%)
4/12/92	M	27	78	20	20
	F	23	78	30	7
	Subtotal	50	78	50	12
5/2/92	M	27	85	25	12
	F	23	83	25	16
	Subtotal	50	84	50	14
5/3/92	M	22	91	27	7
	F	28	89	23	4
	Subtotal	50	90	50	6
	Total	150	84	150	11

[a]Controls consisted of: untreated ($N = 50$; 0% repelled) and frontalure ($N = 50$; 0% repelled).

TABLE 4. RESPONSE OF NEWLY AND REEMERGED *D. frontalis* TO 4-ALLYLANISOLE AND VERBENONE IN LABORATORY ASSAYS

	4-Allylanisole		Verbenone	
Sex	*N*	Repelled (%)	*N*	Repelled (%)
Newly emerged adults (10/1/92)				
M	63	95	58	47
F	37	95	42	52
Total	100	95	100	49
Reemerged parent adults (10/22/92)				
M	57	65	61	5
F	43	60	39	21
Total	100	63	100	11

(Table 4). In both treatments, fewer reemerged beetles were repelled (32% less for 4-allylanisole and 38% less for verbenone).

The *D. frontalis* predator, *T. dubius*, showed no repellent response when exposed to 4-allylanisole or verbenone in laboratory assays. Other scolytids tested, including local (*I. avulsus* and *I. calligraphis*) and nonresident (*I. pini*, *D. rufipennis*, *D. brevicomis*, and *D. ponderosae*) species, were also repelled when exposed to 4-allylanisole in laboratory assays (Table 5). Response to verbenone was assayed in species where verbenone is known to serve an inhibitory function; in all cases, equal or higher percentages were repelled by 4-allylanisole than verbenone.

Field Assay. In the spring trials, 4-allylanisole-baited traps captured significantly fewer *D. frontalis* than traps baited with frontalure alone or with verbenone ($P < 0.0001$ and $P < 0.0004$, respectively, ANOVA and LSD of transformed data) (Figure 1). The mean number of *D. frontalis* captured in frontalure alone and verbenone-baited traps did not differ ($P < 0.2179$). *T. dubius* captures did not differ significantly between traps baited with 4-allylanisole and frontalure alone, but both captured significantly more *T. dubius* than verbenone-baited traps ($P < 0.0391$ and $P < 0.0231$, respectively). Although statistically significant, the differences between treatments for *T. dubius* were small and were not apparent in any other trial.

In the fall trials, verbenone and 4-allylanisole-baited traps captured significantly fewer *D. frontalis* than traps baited with frontalure alone ($P < 0.0001$, ANOVA and LSD of transformed data) (Figure 2). There were no significant differences between *D. frontalis* captured in 4-allylanisole- and verbenone-baited traps. *T. dubius* captures did not differ significantly among treatments.

TABLE 5. RESPONSE OF OTHER INSECT SPECIES TO 4-ALLYLANISOLE AND VERBENONE IN LABORATORY ASSAYS

Insects	4-Allylanisole		Verbenone	
	N	Repelled (%)	*N*	Repelled (%)
T. dubius	50	0	50	0
Ips spp.[a]	59	88	50	70
I. pini	193	97		
D. brevicomis				
Males	16	88	16	75
Females	17	82	15	87
D. ponderosae	30	97	23	78
D. rufipennis	58	81		

[a] Comprised of *Ips* species from the southern pine bark beetle guild: *I. avulsus* ($N = 50$) and *I. calligraphus* ($N = 9$).

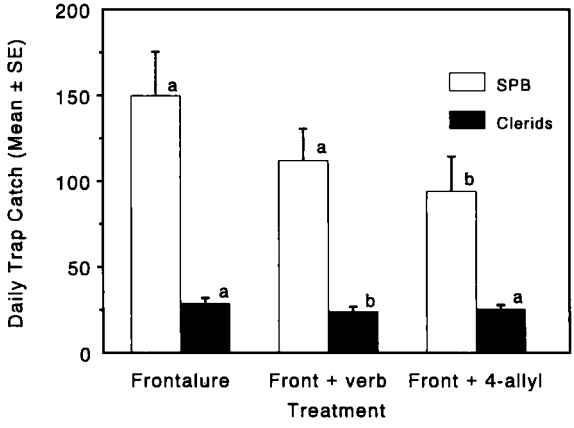

FIG. 1. Mean (± SE) daily capture in the spring of *D. frontalis* (SPB) and its predator, *T. dubius* (clerids), in traps (two per treatment) baited with the SPB attractant frontalure alone and in combination with the SPB antiaggregation pheromone, verbenone, or with the host compound, 4-allylanisole. Trapping was carried out in active SPB infestations ($N = 6$) in the spring and trap positions rotated daily for six days. For each species, bars with the same letter are not significantly different (based on ANOVA and LSD of transformed data).

Taken altogether, significantly fewer *D. frontalis* were captured in the spring and fall in traps baited with 4-allylanisole + frontalure than frontalure alone; trap captures differed between 4-allylanisole- and verbenone-baited traps in the spring but not in the fall trials (Figures 1 and 2; Table 6). No trial × treatment interaction was found with analysis of variance in either spring or fall trials ($F = 0.36$; $df = 5,2$; $P < 0.9614$ and $F = 0.19$; $df = 6,2$; $P < 0.9984$, respectively). However, that trap captures vary day to day, presumably influenced by weather conditions, is evident in significant results of a model in which day is treated as a nested component of trial (Table 6). *T. dubius* attraction was apparently unaffected by the addition of 4-allylanisole; however, in the spring trials significantly fewer *T. dubius* were captured in traps baited with verbenone + frontalure than 4-allylanisole + frontalure of frontalure alone. Although statistically different, it is not clear whether the difference is biologically meaningful. Based on mean captures in frontalure-alone traps, both *D. frontalis* and *T. dubius* showed seasonal differences in abundance, with significantly more *D. frontalis* captured in the fall than in the spring (245.4 ± 40.3 vs. 149.9 ± 25.6), in contrast significantly more *T. dubius* were captured in the spring than in the fall (28.5 ± 3.2 vs. 4.8 ± 0.6).

Sex ratios of *D. frontalis* captured in traps baited with aggregation pheromone alone were, on average, significantly male-biased (57% GT1 and 74%

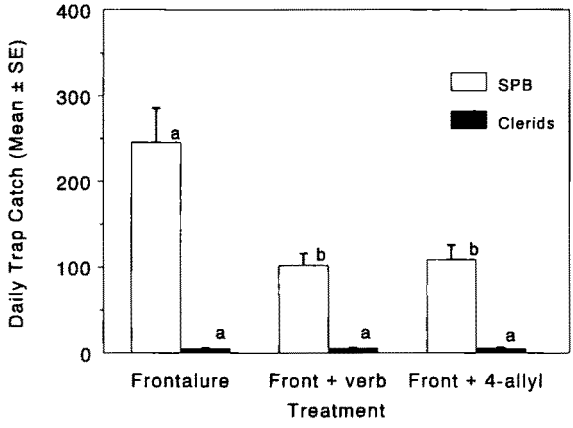

FIG. 2. Mean (± SE) daily capture in the fall of *D. frontalis* (SPB) and its predator, *T. dubius* (clerids), in traps (two per treatment) baited with the SPB attractant frontalure alone and in combination with the SPB antiaggregation pheromone, verbenone, or with the host compound, 4-allylanisole. Trapping was carried out in active SPB infestation ($N = 7$) in the fall and trap positions altered daily for six days. For each species, bars with the same letter are not significantly different (based on ANOVA and LSD of transformed data)

CA3034), as has been previously reported (e.g., Kinzer et al., 1969; Payne et al., 1978). The mean sex ratio (55% male GT1 and 71% CA3034) for *D. frontalis* captured in traps with frontalure + 4-allylanisole did not differ significantly from frontalure alone, whereas captures in traps baited with frontalure + verbenone differed significantly in sex ratio from frontalure alone (Pearson's chi square = 102.46, $P < 0.0001$ GT1; chi square = 23.94, $P < 0.0001$ CA3034). A significant female bias (66% female; chi square = 33.06, $P < 0.0236$) was observed in verbenone-baited traps at GT1 and a slight but not significant female bias was observed at CA3034 (52% female), suggesting that female *D. frontalis* are less inhibited by verbenone than males. A similar biased response was also apparent in trapping results presented by Salom et al. (1992b).

In a single trial in which 4-allylanisole, verbenone, and the combination were added to frontalure-baited traps, all three captured significantly fewer *D. frontalis* than frontalure alone ($P < 0.0004$; $P < 0.0011$; $P < 0.0016$; ANOVA and LSD of transformed data, respectively) but did not differ from each other. In rank order, 4-allylanisole caught the least (16.75 ± 4.02; $\bar{X}$ ± SE) followed by 4-allylanisole + verbenone (19.19 ± 4.37), verbenone (21.19 ± 5.95), and frontalure alone (45.88 ± 11.02). The mean number of *T. dubius* captured did not differ among treatments.

The repellent effect of 4-allylanisole on *D. frontalis* was not significantly

TABLE 6. ANALYSES OF VARIANCE OF FIELD ASSAYS WITH *D. frontalis* AND *T. dubius* USING BAITED TRAPS

Insect	Model	*df*	MS	*F*	*P*	RSQ
Spring trials						
D. frontalis	trial	5	27.88	20.34	0.0001	0.85
	date (trial)	36	1.37	3.28	0.0001	
	treatment	2	5.44	13.00	0.0001	
	error	82	0.42			
T. dubius	trial	5	3.22	1.90	0.1179	0.81
	date (trial)	36	1.69	7.43	0.0001	
	treatment	2	0.75	3.27	0.0431	
	error	82	0.23			
Fall trials						
D. frontalis	trial	6	8.39	3.83	0.0049	0.85
	date (trial)	35	2.19	6.58	0.0001	
	treatment	2	12.91	38.79	0.0001	
	error	82	0.33			
T. dubius	trial	6	0.88	1.19	0.3336	0.62
	date (trial)	35	0.74	3.15	0.0001	
	treatment	2	0.0008	0.00	0.9965	
	error	82	0.23			

enhanced by the addition of more than one 20-ml wicked elution device (Table 7, a). The dose–response of *D. frontalis* was defined by $Y = 26\ X^{-0.16}$ ($R^2 = 0.895$; $N = 5$; $P < 0.0001$) (Figure 3). Frontalure-baited traps captured significantly more *D. frontalis* than traps baited with frontalure + one, two, or four 4-allylanisole elution devices ($\bar{X} = 79.8 \pm 7.98$, $P < 0.0001$, ANOVA and LSD of transformed data). However, there were no differences in mean captures between the different number of devices ($\bar{X} \pm$ SE $= 29.4 \pm 0.87$; 21.6 ± 0.75; 20.8 ± 2.76) (Figure 3). The addition of one or more 4-allylanisole elution device did not impact *T. dubius* attraction to frontalure (10.9 ± 0.98, 9.8 ± 0.80, 11.3 ± 0.89); however, all three captured significantly fewer clerids than frontalure alone (17.6 ± 1.61, $P < 0.01$ ANOVA and LSD of transformed data).

Differing ratios of turpentine–4-allylanisole that contain more than 25% 4-allylanisole did not significantly influence the repellent effect of 4-allylanisole on *D. frontalis* (Table 7, b). Traps baited with frontalin + turpentine (in separate elution devices) captured significantly more *D. frontalis* than traps baited with frontalin (in one elution device) + 3:1, 1:1, 1:3, or 0:1 turpentine:4-allylanisole (in a separate elution device) ($\bar{X} = 87.6 \pm 21.0$; $P < 0.0001$, ANOVA and LSD of transformed data). There was little difference between 25, 50, or

TABLE 7. ANALYSES OF VARIANCE OF DOSE-RESPONSE TO 4-ALLYLANISOLE BY *D. frontalis* AND *T. dubius* IN FIELD ASSAYS

Insect	Model	*df*	MS	*F*	*P*	RSQ
a. Dose–response trials (one, two, and four 4-allylanisole elution devices)						
D. frontalis	site	4	39.33	0.36	0.8293	0.90
	treatment	3	3976.60	36.86	0.0001	
	error	12	107.89			
T. dubius	site	4	2.17	0.29	0.8788	0.68
	treatment	3	61.54	8.21	0.0031	
	error	12	7.49			
b. Dose–response trial (1:0, 3:1, 1:1, 1:3, 0:1 turpentine–4-allylanisole)						
D. frontalis	date	21	4.47	3.72	0.0001	0.58
	treatment	4	9.36	7.80	0.0001	
	error	78	1.20			
T. dubius	date	21	2.28	2.63	0.0011	0.42
	treatment	4	0.44	0.51	0.7285	
	error	78	0.87			

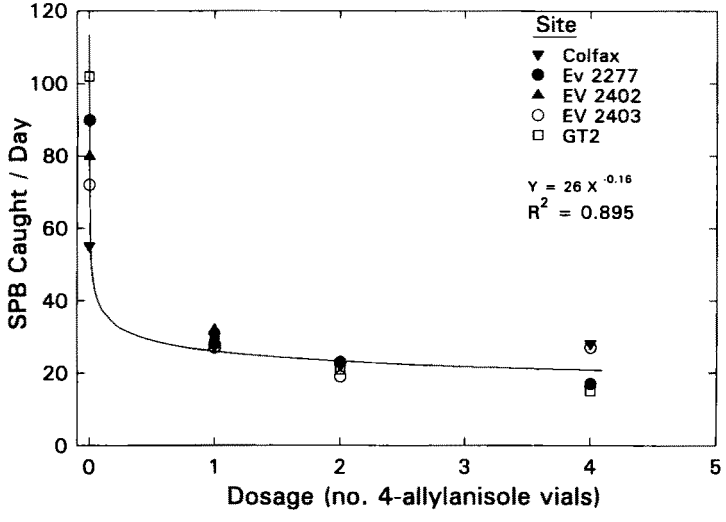

FIG. 3. Dose–response of *D. frontalis* (SPB) [mean daily trap catch transformed by ln(Y + 0.001)] to increasing numbers of 4-allylanisole elution devices (0–4) per trap baited with frontalure (trap captures were normalized across sites; see text for detailed methodological description).

TABLE 8. PAIRED TREATMENTS WITH 4-ALLYLANISOLE OF LIGHTNING-STRUCK TREES

Lightning strike date	Pine spp.	dbh (cm)	Treatment type	Attacks (N/m^2) 2 m	Attacks (N/m^2) 4 m	Tree fate
6/1/92	loblolly	49.3	treated	0	0	alive
6/1/92	loblolly	40.9	control	38.8	86.1	dead
6/28/92	loblolly	45.7	treated	0	0	alive
6/28/92	loblolly	53.3	control	86.1	150.7	dead
7/1/92	longleaf	51.3	treated	16.1	6.9	alive
7/1/92	longleaf	43.2	control	96.9	148.5	dead

75% 4-allylanisole, but 100% 4-allylanisole caught significantly fewer *D. frontalis* than 25 and 50% ($P < 0.0347$ and $P < 0.0439$) ($\bar{X} = 29.5 \pm 8.5$; 22.2 ± 4.9; 39.4 ± 11.8; 15.0 ± 6.0). The addition of 4-allylanisole in differing concentrations with turpentine did not impact *T. dubius* attraction to frontalin ($\bar{X} = 34.1 \pm 5.5$; 33.5 ± 7.8; 42.4 ± 10.5; 28.1 ± 5.8; 39.5 ± 8.9).

Preliminary Field Application. Individual tree attributes and results of 30-day treatment of lightning-struck pairs of pines are given in Table 8. The 4-allylanisole-treated member of each lightning-struck pair suffered considerably fewer attacks than the untreated control and were apparently still alive after the test period, while controls were obviously dead. In two other noteworthy instances, large lightning-struck loblolly pines in residential settings were treated within 48 hr as described above. In one case the tree was protected even though a few pitch tubes were evident and an adjacent (untreated) lightning-struck tree was attacked by *D. frontalis* and removed. In the other case, the tree was protected from significant attack even though a few pitch tubes were present at the time of treatment. After treatment was stopped at day 30, the tree was attacked by *Ips* spp. and *D. frontalis*.

DISCUSSION

4-Allylanisole was identified in loblolly pine as early as 1956 (Sutherland and Wells, 1956) and reported as a component of needle oils (Zavarin et al., 1971; von Rudloff, 1975; Adams and Edmunds, 1989; von Rudloff and Lapp, 1992) and resins from many species since (Mirov, 1961; Drew and Pylant, 1966; Renwick and Vité, 1969, 1970; Werner, 1972a; Gambliel et al., 1985; Pierce et al., 1987). 4-Allylanisole also has been recognized and used as an attractant in other insect–host systems, e.g., western corn rootworm (*Diabrotica v. virgifera* LeConte (Coleoptera: Chrysomelidae) (Lampman et al., 1987).

Speculations about the role(s) of this phenylpropanoid compound in pine–bark beetle interactions have varied from parasitoid (Salom et al., 1991, 1992a) and bark beetle attractant (Renwick and Vité, 1969, 1970; Werner, 1972a,b) to microbial growth inhibitor (Gambliel et al., 1985; Bridges, 1987). Bridges (1987) demonstrated reduced fungal growth, although others have dismissed 4-allylanisole as unimportant (Himejima et al., 1992). Cobb et al. (1972) hypothesized but did not directly test a connection between a drop in 4-allylanisole in leaves of *P. ponderosa*, air pollution, and susceptibility to bark beetles.

In previous publications, 4-allylanisole has been identified from *D. frontalis*-infested tree parts and female frass (R.M. Silverstein and J.R. West, personal communication, as cited in Thatcher et al., 1980), and although no reference is given, 4-allylanisole is cited by Salom et al. (1991, 1992a) as being one of numerous volatiles arising from trees containing heavily parasitized *D. frontalis* brood. In surveys of potential semiochemicals and electroantennogram (EAG) responses of two *D. frontalis* parasitoids, Salom et al. (1991, 1992a), found that 4-allylanisole elicited little activity at the sensory level of either of these natural enemies. Pierce et al. (1987) found 4-allylanisole in *P. ponderosa* phloem oil, but not in the abdominal or frass volatiles of *D. ponderosae*.

Our laboratory bioassay appears to provide a simple, reliable method for screening compounds that elicit repellent or inhibitory responses from bark beetles. The results of exposure to 4-allylanisole are consistent with our baited-trap experiments, which is the most commonly used field technique. The relative difference in repellency of 4-allylanisole and verbenone evident in the laboratory assays was not consistently apparent in the baited-trap experiments, but may reflect the difference between the beetle's response to a single stimulus versus the multiple stimuli (both visual and chemical) emanating from a trap. In contrast to our laboratory assay results, in which we found no differential response between the sexes, McCarty et al. (1980) reported that only males responded to verbenone using a walking olfactometer. Our trap results are in agreement with findings of other researchers (Renwick and Vité, 1969; Salom et al., 1992b), who found that the presence of verbenone in traps significantly reduces total catch but has little or no influence on female *D. frontalis*. With the exception of the spring trials, our results are in agreement with Richerson and Payne (1979), Billings and Cameron (1984), and Salom et al. (1992b), who found that the addition of *D. frontalis* inhibitor to attractant did not influence the response of *T. dubius*. Generally, there were no differences between the number of *T. dubius* captured in traps baited with frontalure alone versus 4-allylanisole. The exception was the first dose–response assay, and we have no explanation for this apparent deviation.

Our laboratory assays suggest that 4-allylanisole would be an effective repellent of *D. frontalis*, its associated scolytids (e.g., *Ips* spp.), and in other

scolytid–conifer systems. The strong reactions to verbenone of the species tested is consistent with other reports in which this inhibitor is known to play a role. Specific antiaggregation pheromones have not been identified from *Ips* spp. (Borden, 1986); however, a negative reaction to the *D. frontalis* inhibitor is in agreement with the findings of others (Richerson and Payne, 1979; Byers and Wood, 1980, 1981). Both *D. brevicomis* and *D. ponderosae* are known to produce and be responsive to verbenone as an inhibitory pheromone, but apparently in slightly different enantiomeric blends and concentrations than *D. frontalis* (Amman et al., 1989; Paine and Hanlon, 1991; Shea et al., 1992). Renwick and Vité (1970) found *D. brevicomis'* reaction to verbenone was more pronounced than that of *D. frontalis*.

The results of the laboratory and field assays indicate the consistent repellent properties of 4-allylanisole to *D. frontalis* throughout the times of year when dispersal is considered highest (Thatcher and Pickard, 1964; Turchin and Thoeny, 1993). Additionally, unlike verbenone, which elicits a male-biased response, there is no apparent differential response between the sexes to this host compound; females and males are repelled proportional to their attraction to frontalure, a desirable finding given that the female is considered the pioneering sex (i.e., the sex that initiates attack on a new host) in *D. frontalis*. The combination of the two semiochemicals does not appear to further suppress beetle response than either alone. The differential response we observed between newly emerged and reemerged beetles to 4-allylanisole and verbenone should be further explored and may be a contributing factor in the inconsistencies seen with verbenone studies (e.g., Payne and Billings, 1989; Payne et al., 1992).

Among compounds that are inhibitory or repellent to scolytids, those originating from the host are especially important because of their potential olfactory role in host selection by pioneering individuals. Due to a variety of evidence, Smith (1977) speculated that *P. ponderosa* high in limonene may be resistant to *D. brevicomis*. A considerable amount of research has been devoted to finding primary attractants of tree-killing bark beetles (Tunset et al., 1993, and references therein) and the predominant hypotheses are that beetles either land randomly on potential hosts or that host selection is the result of chemical and/or visual cues. The latter hypothesis implies that beetles are able to detect a suitable host in flight, but does not preclude the possibility that the information perceived is alternatively about the unsuitability of a potential host. Although additional replicates are necessary, protection of lightning-struck trees, highly susceptible hosts for *D. frontalis* (Hodges and Pickard, 1971), suggests that in contrast to (or in addition to) the primary attractant hypothesis, host selection may be dependent on a lack of repellency (see similar discussions by Heikkenen and Hrutfiord, 1965; Cobb et al., 1968). The attractancy or synergistic natures of many oleoresin compounds (e.g., α-pinene) have been well documented, but

the repellent or inhibitory effects of others, such as 4-allylanisole, have been largely overlooked and warrant further exploration.

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INFLUENCE OF AGGREGATION INHIBITORS (VERBENONE AND IPSDIENOL) ON LANDING AND ATTACK BEHAVIOR OF *Dendroctonus brevicomis* (COLEOPTERA: SCOLYTIDAE)

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Abstract—The influence of the aggregation inhibitors verbenone and ipsdienol on the response of western pine beetle, *Dendroctonus brevicomis*, to attractive host trees was investigated. Paired ponderosa pine trees (*Pinus ponderosa*) were baited with aggregation semiochemicals to stimulate mass attack. One tree in each pair received an inhibitor treatment consisting of five sets of two verbenone and two ipsdienol dispensers spaced 1 m apart vertically along the tree bole. Beetle landing was monitored with sticky traps on the tree bole, and attack density was assessed from bark samples removed four or seven days after baiting. The inhibitor treatment resulted in a significant reduction of both the numbers of beetles landing on trees and the density of attacking beetles compared to control trees (without inhibitors). The ratios of beetle landing density to attacking density were not different between inhibitor-treated and control trees, nor were the vertical distributions of beetles landing or attacking, suggesting that beetle behavior was primarily influenced at a longer range, prior to landing on the tree. Although the application of verbenone and ipsdienol did not prevent *D. brevicomis* from attacking baited trees, our results suggest that when applied to unattacked (and unbaited) trees, their effectiveness at reducing the attack pressure might allow trees having a certain amount of resistance to survive attack by pioneer beetles.

Key Words—*Dendroctonus brevicomis*, Coleoptera, Scolytidae, *Pinus ponderosa*, aggregation, verbenone, ipsdienol, pheromone, inhibitors, tree protection.

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INTRODUCTION

As much as 2 billion board-feet per year of ponderosa pine (*Pinus ponderosa* Laws.) have been killed during outbreaks of the western pine beetle, *Dendroctonus brevicomis* LeConte (Miller and Keen, 1960; Smith, 1990). Aggregation by *D. brevicomis* on host trees is mediated by attractive and inhibitory semiochemicals released by beetles and host trees (Wood, 1982; Byers et al., 1984). The principal aggregation pheromones of *D. brevicomis* are (+)-*exo*-brevicomin, produced by females (Silverstein et al., 1958; Stewart et al., 1977), and (−)-frontalin, produced by males (Kinzer et al., 1969; Vité and Pitman, 1969; Stewart et al., 1977). Volatile resin components, primarily myrcene, released from the host tree during colonization by the beetles, synergize the beetle-produced attractants (Bedard et al., 1969).

Semiochemicals that inhibit the response of bark beetles to sources of aggregation pheromones have been investigated for use in preventing bark beetle attacks on individual trees. Verbenone has been the focus of a great deal of research because it inhibits attraction to aggregation semiochemicals in several species of *Dendroctonus*, including *D. brevicomis* (Renwick and Vité, 1970; Bedard et al., 1980; Tilden and Bedard, 1988; Paine and Hanlon, 1991), *Dendroctonus ponderosae* Hopkins (Borden et al., 1987), and *Dendroctonus frontalis* Zimmermann (Payne et al., 1978). Verbenone released from dispensers attached to the boles of ponderosa pines reduced the number of *D. brevicomis* landing on the trees and prevented attack during one year of a study, but when the study was repeated the following year all treated trees were attacked (Bedard et al., 1980). Verbenone treatment of individual lodgepole pine (*Pinus contorta* var. *latifolia* Engelmann) trees did not reduce the number of trees attacked by *D. ponderosae* or the attack density compared to unbaited control trees (Shore et al., 1992). Attack by *D. frontalis* was not prevented, nor was the vertical distribution of beetles landing on sticky traps influenced, when verbenone was released from the boles of loblolly pines (*Pinus taeda* L.) (Richerson and Payne, 1979). However, verbenone applied in a liquid polymer formulation to trees in the path of advancing *D. frontalis* infestations significantly reduced the number of new trees attacked, while the combination of felling infested trees and treating uninfested trees with verbenone significantly reduced infestation growth in most treated infestations (Payne et al., 1992).

In addition to verbenone, racemic ipsdienol has also been shown to inhibit the response of *D. brevicomis* to synthetic attractants in the field (Byers, 1982; Paine and Hanlon, 1991). Furthermore, releasing a combination of 14% (+)-/86% (−)-verbenone and racemic ipsdienol appeared to have an additive inhibitory effect, reducing to very low levels the number of *D. brevicomis* trapped at a source of synthetic attractants (Paine and Hanlon, 1991). The objective of our study was to determine the effects of a combination of synthetic verbenone

and ipsdienol on the landing and attack behavior of *D. brevicomis* as an initial evaluation of these inhibitors as a potential treatment to reduce attack on standing trees.

METHODS AND MATERIALS

Twelve pairs of ponderosa pine trees were selected within an approximately 30-year-old plantation at 1460 to 1580 m elevation in the Wright's Creek drainage near Sonora, Tuolumne County, California. The distance between trees in each pair was at least 25 m, and the distance between tree pairs was at least 100 m. To minimize within-pair differences in vigor and resistance to beetle attack, each tree pair was selected for uniformity in size (difference in dbh $\leq$ 1 cm), age, crown class, and site characteristics. It was assumed that the distance of 25 m or more between trees in a pair was adequate to minimize interaction between the semiochemicals, but close enough to minimize differences within pairs with respect to beetle abundance and site characteristics. The diameters of the trees at 1.5 m above the ground (diameter at breast height) ranged from 28 to 36 cm.

Trees were limbed to the height of 6 m to permit the use of climbing ladders and to clear a path for the sticky trap pulley system. One tree in each pair received the inhibitor treatment, while the other tree in each pair served as an unprotected control. The inhibitor treatment consisted of two verbenone and two ipsdienol bubble capsules stapled equidistant around the bole at each a 1-m interval to a height of 5 m (10 each/tree), with the two different inhibitors arranged so that they alternated both around the bole and vertically up the bole. Synthetic aggregation semiochemicals (attractants) were placed on all trees to stimulate attack and thereby challenge the inhibitor treatment. Attractant baits were stapled to the north side of trees at a height of 2 m. Release rates for the attractants and inhibitors were: racemic *exo*-brevicomin 2.6 mg/24 hr (at 24°C), racemic frontalin 2.4 mg/24 hr (at 20°C), myrcene 95 mg/24 hr (at 25°C), 14% (+)-/86% (−)-verbenone 100 mg/24 hr total per tree (each bubble capsule 10 mg/24 hr at 24°C), and racemic ipsdienol 2 mg/24 hr total per tree (each bubble capsule 0.2 mg/24 hr at 24°C). All compounds and release devices were obtained from Phero Tech Inc., Delta, British Columbia, Canada (chemical purity >98%).

Beetle Arrival. The arrival of beetles at trees was monitored with flat, 0.64-cm mesh, 15 × 30-cm metal screen traps coated on both sides with Stikem Special (Seabright Laboratories, Emeryville, California). Two lines of five traps spaced 1 m apart were suspended with nylon cord from screw-hooks fastened to the tree on opposite sides of the bole (Stephen and Dahlsten, 1976). This

pulley system allowed the traps to be raised and lowered without climbing the tree. The individual traps were spaced 1 m apart, with one line of traps positioned at 1, 2, 3, 4, and 5 m above ground, and the traps on the opposite side at 1.5, 2.5, 3.5, 4.5, and 5.5 m above ground. This arrangement of traps allowed for the comparison of numbers of beetles landing on the tree adjacent to (level with) inhibitor release devices and between (0.5 m away from) inhibitor devices. The sticky traps were collected daily and replaced with fresh ones, and the collected traps were wrapped in wax paper and returned to the laboratory for removal and counting of trapped beetles.

To assess the density and distribution of beetle attacks over time, trees were sampled four and seven days after baiting with attractants. One set of six pairs of trees was used for each time interval (four or seven days) because of the destructive nature of the sampling procedure. Because setup and sampling procedures were labor intensive, the two sets of trees were run at different times rather than at once. Six pairs of trees were baited initially and beetle arrival was monitored for seven days (August 18–24, 1992), after which the trees were felled for bark sample removal. Six additional pairs were then baited, and arrival was monitored for four days (September 1–4, 1992) before the trees were felled for bark sampling.

Beetle Attacks. Circular bark disks approximately 1 dm^2 in area were removed from opposite sides of the bole at 0.5-m intervals from 1- to 5-m heights. Sampling was done without regard to aspect using a gasoline-powered drill fitted with a 11.5-cm-diameter hole saw (Berryman et al., 1970). The sampling procedure for attack density was modified from that of Stephen and Dahlsten (1976) in that samples were taken at shorter height intervals and two disks, rather than four, were removed at each sample height. DeMars (1970) found that the number of bark samples could be reduced from four to two per sampling height without a loss in precision when sampling for broods of *D. brevicomis*.

The samples were returned to the laboratory and stored at 6°C until dissected. An attack site was indicated by a pitch tube and/or resin- and frass-filled tunnel in a bark sample (Stephen and Taha, 1976; Linit and Stephen, 1978), or by the presence of a beetle in a bark crevice along with boring dust, but no gallery construction. The latter was assumed to be a newly initiated attack site rather than a ventilation hole due to the absence of a gallery directly underneath the hole.

Data Analysis. Sticky trap catch data from trees monitored for seven days were transformed by $\log_{10}$ prior to analysis of variance to correct for unequal variances (Steel and Torrie, 1980). Trap catch data from trees that were monitored for four days were omitted from the analysis due to their redundancy with data from the first four days of monitoring the seven-day trees. To test for differences in the number of beetles landing on trees between the two treatments,

daily totals of beetles caught per tree were analyzed as a split-block (repeated measure) design. An analysis of variance was performed using the blocks × treatment term as the *F*-test denominator to test the inhibitor–treatment main effect (Steel and Torrie, 1980).

To test the hypothesis that beetles not prevented from landing on trees would avoid the point sources of inhibitors as they landed, the mean number of beetles (per square decimeter) landing on sticky traps was compared for traps positioned between inhibitor dispensers and adjacent to the inhibitor dispensers. We assumed that any effect of aspect on the numbers of beetles landing on "adjacent" or "between" traps due to their being positioned on opposite sides of the tree would be canceled out by the random positioning of trap lines among trees with regard to aspect. An analysis of variance for split-block designs was again used, wherein the main effects of trap position was tested using the tree × position term as the *F*-test denominator (Steel and Torrie, 1980).

Vertical distributions of numbers of beetles landing by trap height were compared between inhibitor and control trees on the first and seventh days after baiting using the Kolmogorov-Smirnov two-sample test on the proportion of the total numbers of beetles landing on that day at each trap height (Sokal and Rohlf, 1981).

Attack site data were transformed by $\log_{10}(x + 1)$ prior to analysis to correct for unequal variances (Steel and Torrie, 1980). The mean attack density (attacks per square decimeter) for control and treatment trees sampled at four or seven days after baiting were compared with a two-way analysis of variance. To test the hypothesis that beetles would avoid the point sources of inhibitors as they searched the bark for sites to initiate galleries, attack density (attacks per square decimeter) was compared from samples taken between the inhibitor dispensers and adjacent to the inhibitor dispensers. To determine if the inhibitor treatment caused a greater number of beetles to leave the tree without initiating attacks, the cumulative density of beetles landing on sticky traps (beetles per square decimeter of trap area) over the four or seven days after baiting was divided by the attack density (attacks per square decimeter of bark) to obtain the ratios of landing to attacking beetles for each tree. These ratios were compared for inhibitor-treated and control trees with a two-way analysis of variance. All ANOVAs were performed using the Minitab statistical software package for mainframe computers (Minitab, Inc., 1992).

RESULTS

Beetle Arrival. The inhibitors verbenone and ipsdienol significantly reduced the number of *D. brevicomis* caught on sticky traps on the boles of attractant-baited trees monitored for seven days after baiting ($F = 141.5$; $df = 1, 5$; P

< 0.001) (Figure 1). There was no significant difference between the numbers of beetles caught per day on traps positioned adjacent to inhibitor dispensers and on traps positioned between [1.8 $\pm$ 0.1 and 1.4 $\pm$ 0.10, (mean $\pm$ SE); respectively; $F = 1.3$; $df = 1, 5$; $P = 0.311$], suggesting that those beetles that were not inhibited from landing on the trees did not avoid the point sources of inhibitors as they landed. There was no significant difference between inhibitors and control trees in the proportion of total number beetles landing at each trap height on days 1 or 7 after baiting for the trees monitored for seven days; landing densities were highest around attractant baits for both inhibitor and control trees (Figure 2). This suggests that, while the inhibitors reduced the quantities of beetles landing on trees, they did not influence the vertical distribution of the beetles that did land.

Beetle Attacks. Mean attack density was significantly lower on inhibitor-treated trees than on control trees at four days after baiting with attractants (0.07 $\pm$ 0.03 and 0.57 $\pm$ 0.14, respectively; $F = 17.92$; $df = 1, 5$; $P = 0.008$), as well as at seven days after baiting (0.34 $\pm$ 0.09 and 1.25 $\pm$ 0.34, respectively; $F = 11.90$; $df = 1, 5$; $P = 0.018$). Four days after baiting, the distribution of attacks by height on the bole was concentrated around 2 m, level with the attractant bait, with mean attack densities at that height of 0.17 and 1.42 for treated and control trees, respectively (Figure 3). Seven days after baiting, mean density remained highest around the attractant bait, but had increased at the

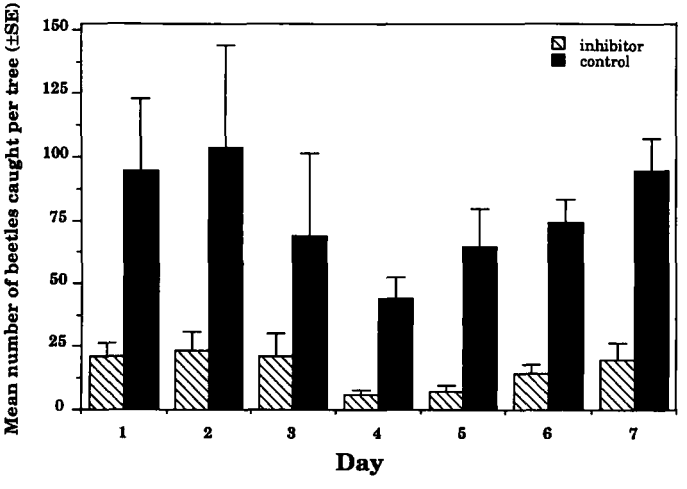

FIG. 1. Mean number of *D. brevicomis* caught per tree on sticky traps over a seven-day period on ponderosa pine trees baited with attractant and either a combination of the inhibitors verbenone and ipsdienol (inhibitor) or no inhibitors (control).

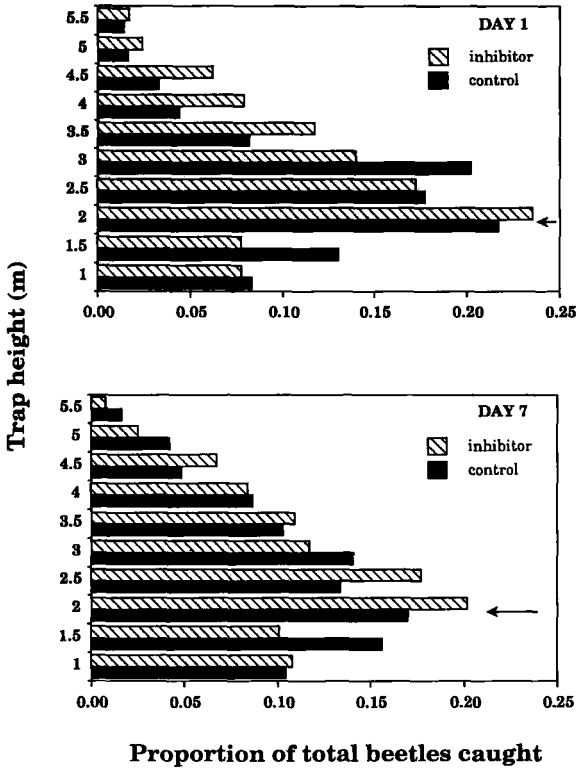

FIG. 2. Comparison of the distribution of *D. brevicomis* landing on sticky traps on the boles of inhibitor-treated and control trees one and seven days after baiting with attractants. Arrows indicate position of attractant bait. Distributions within days not significantly different, Kolmogorov-Smirnov two-sample test, $\alpha = 0.05$.

2-m height to 0.75 for the treated trees, and 2.42 for the control trees (Figure 3). Attack density between inhibitor dispensers was not significantly different from the attack density adjacent to inhibitor dispensers after four days (0.32 ± 0.02 and 0.07 ± 0.01, respectively; $N = 60$; $F = 0.01$; $df = 1, 47$; $P = 0.94$) or seven days (0.38 ± 0.02 and 0.06 ± 0.01, respectively; $N = 54$; $F = 0.08$; $df = 1, 47$; $P = 0.79$). The mean ratio of cumulative landing density/attack density was not different for treated and control trees at four days (5.7 ± 3.9 and 11.5 ± 2.2, respectively; $F = 5.65$; $df = 1, 5$; $P = 0.06$) or seven days after baiting (11.7 ± 4.2 and 10.6 ± 1.1, respectively; $F = 0.24$; $df = 1, 5$; $P = 0.64$).

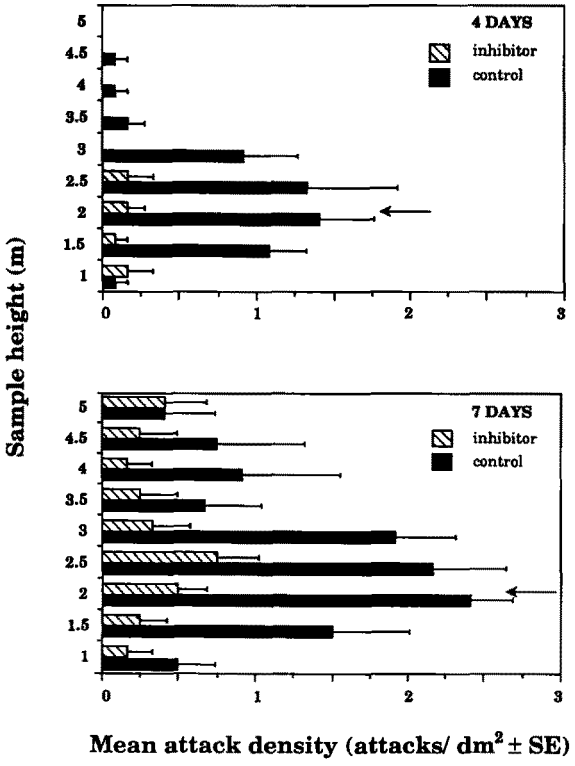

FIG. 3. Mean density of *D. brevicomis* attacks per sampling height, counted from two 1-dm^2 bark samples taken from opposite sides of the tree bole on trees baited with attractant and either a combination of the inhibitors verbenone and ipsdienol (inhibitor) or no inhibitors (control). Samples were taken four or seven days after baiting with attractants. Arrows indicate position of attractant bait. Mean attack density was significantly lower on inhibitor-treated trees than on control trees at both four days and seven days after baiting.

DISCUSSION

Verbenone and ipsdienol released from the bole significantly lowered the numbers of beetles landing on attractant-baited trees compared to trees baited with attractants alone. Nevertheless, all trees were attacked. Attack density was also significantly lower on trees treated with verbenone and ipsdienol, compared to trees baited with attractants alone. Seven days after baiting, mean attack density on the inhibitor-treated trees was 0.34 attacks/dm^2 (compared to 1.25 for control trees). Hodges et al. (1979) estimated that a threshold attack density

above which a loblolly pine of average susceptibility would be killed by *D. frontalis* is ≈ 1 attack/dm^2. This threshold would vary for individual trees, depending on tree size and inherent resistance characteristics (Hodges et al., 1979). If a similar threshold applied for *D. brevicomis* attacking ponderosa pine, our inhibitor treatment held the density of attacking beetles to below lethal limits for an average tree. We acknowledge that this comparison may not be entirely valid because of the differences between the species, and furthermore, that our estimated attack densities may have been additionally reduced slightly by the interception by sticky traps of some colonizing beetles as they landed. Nevertheless, the mean attack density on inhibitor-treated trees in our study seven days after baiting was considerably lower than the range of densities observed on successfully attacked trees for *D. brevicomis* by Dudley (1971).

Our comparison of cumulative landing and attack densities suggests that roughly 10 beetles land on trees for every attack initiated. This is in general agreement with the results of Anderbrandt et al. (1988), who reported that 80% of *Ips typographus* (L.) landing on trees under attack left the tree after landing, and Coster et al. (1977), who found that cumulative landing densities of *D. frontalis* were "considerably higher" than attack densities reported by other workers for that species.

Because verbenone and ipsdienol have been detected in hindgut samples of *D. brevicomis* in early stages of colonization, the compounds were postulated to function as close-range inhibitors, regulating attack density, rather than as long-range inhibitors indicating a fully colonized resource (Byers, 1982; Byers and Wood, 1980; Byers et al., 1984). These hypotheses suggested that, as a landing or crawling beetle neared a concentrated source of verbenone or ipsdienol, contact with the inhibitor plume would evoke an avoidance of the areas adjacent to the dispensers, resulting in a higher concentration of beetles landing and attacking in areas further removed from the inhibitor sources. Our results do not suggest that the release of synthetic verbenone and ipsdienol from point sources on the tree bole influenced the distribution of *D. brevicomis* landing or initiating attacks on treated trees. However, because the quantities of compounds and the method of their release in our study cannot be considered to be similar to the natural release of these compounds by the beetles and trees, the above hypotheses are not disproven by our results. It is also possible that verbenone and/or ipsdienol released from attacking beetles confounded the effects of the strong point sources of synthetic inhibitors. These results, as well as the apparent lack of influence by the release of verbenone and ipsdienol on cumulative landing density/attack density ratios, suggest that the behavior of *D. brevicomis* is primarily being influenced by these synthetic inhibitors prior to landing on the tree.

Host trees are able to resist being killed by bark beetle attack through mechanisms that include a preformed resin system and an induced biochemical response (Berryman, 1972). Vigorously growing trees may have sufficient quan-

tities of resin in their tissues to prevent successful mass attack by killing pioneer beetles directly or, possibly, by preventing them from releasing pheromones (Raffa and Berryman, 1983). Induced defenses in conifers are manifested in resin-soaked, necrotic lesions within which attacking beetles and their associated fungi are isolated from surrounding tissue and toxins are synthesized that may reduce beetle fitness (Berryman, 1972; Paine, 1984; Paine and Stephen, 1988). Induction of defenses in response to beetle attacks is followed by a delay (≈ 60 hr in loblolly pine) before lesions form, so that rapid colonization by beetles may preempt this defense mechanism (Paine et al., 1988). The inhibitor treatment investigated here significantly lowered the numbers of beetles arriving at trees and the density of attacking beetles over a seven-day period in the presence of a highly attractive pheromone source. Since the ability of a tree to resist attack is inversely related to the number of beetles attacking (Raffa and Berryman, 1983), it appears that this inhibitor treatment has the potential to prevent a mass attack from progressing past the initial stages by reducing the insect pressure and allowing the tree's resistance mechanisms to respond to the invasion. As long as the number of attacking beetles is held below a lethal threshold level that varies with tree condition, the risk of tree death is minimized. The amount of *exo*-brevicomin released from the attractant baits used in this study is equivalent to what would be released by approximately 300 female beetles feeding in a tree (Browne et al., 1979), and represents what might be released from a tree during the concentration phase of colonization (Wood, 1972). Thus, treatment with verbenone and ipsdienol was effective in reducing the response of *D. brevicomis* to highly attractive host trees. However, in operational use, the inhibitor treatment would be placed on unattacked trees; therefore, the crucial time for the treatment to be effective is when only a few pioneer beetles are producing pheromones and trees are considerably less attractive. Our sample size was small, and the destructive sampling procedures did not allow us to determine whether our study trees would have survived the attacks they sustained. Additional studies on a larger scale are required to evaluate the efficacy of this inhibitor treatment on unbaited trees and when only a few pioneer beetles are producing aggregation pheromones.

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DIFFERENTIAL TOXICITY OF JUGLONE (5-HYDROXY-1,4-NAPHTHOQUINONE) AND RELATED NAPHTHOQUINONES TO SATURNIID MOTHS

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Abstract—The preferred hosts of the saturniid moth *Actias luna* include members of the Juglandaceae, whose foliage contain the toxin juglone (5-hydroxy-1,4-naphthoquinone). The performance of *Actias luna* and *Callosamia promethea* was compared when fourth-instar larvae of each were fed birch foliage, a mutually acceptable food plant, or birth supplemented with 0.05% (w/w) juglone. *A. luna* fed juglone exhibited no changes in developmental time or mortality compared to a diet without juglone. In contrast, juglone-supplemented diets, when fed to *C. promethea*, caused negative growth rate, and a 3.6-fold decrease in consumption rate. The performance of *A. luna* also was compared on birch and walnut; larvae developed and grew more rapidly on an all-walnut vs. an all-birch diet. To examine the effect of 1,4-naphthoquinone structure on *A. luna* survival, first instars were fed on birch supplemented with varying concentrations of juglone (J), menadione (M), plumbagin (P), or lawsone (L). In diets supplemented at 0.05% (w/w), none of the compounds produced effects significantly different from controls. In diets supplemented at 0.5% (w/w), the treatments produced significant toxic effects in the order P > M = L > J for mortality, and P > L > M = J for increased developmental time. Late-instar *A. luna* are clearly resistant to juglone compared to *C. promethea*, and early-instar *A. luna* are resistant to several related 1,4-naphthoquinones. These results suggest a chemical basis for host choice among saturniids. In addition, the luna–walnut system may be a valuable model for studying quinone detoxication.

Key Words—1,4-Naphthoquinone, allelochemical toxicity, detoxification,

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plant–insect interaction, Lepidoptera, Saturniidae, *Actias luna*, *Callosamia promethea*, juglone, plumbagin, menadione, lawsone, quinones.

INTRODUCTION

Quinones are ubiquitous in plant and animal cells, where they function in respiration, photosynthesis, and as defensive toxins (O'Brien, 1991). The toxicity of quinones is a consequence of their ability to redox cycle. During redox cycling, quinones undergo a one-electron reduction to the corresponding semiquinone radical. The oxidative reversion of semiquinone to quinone correspondingly reduces oxygen to superoxide radical. Excess superoxide, and its further metabolism to other reactive oxygen species such as H_2O_2 and $\cdot OH$, results in oxidative cell damage. Cell toxicity from quinones may also result from covalent adduct formation between the semiquinone species and cell macromolecules and from the depletion of glutathione (Sies, 1988; Makawiti et al., 1990; O'Brien, 1991).

The metabolism of naphthoquinones has been extensively studied in vertebrates, particularly through the use of cultured rat hepatocytes. A primary detoxication route in that system involves a two-electron reduction of the quinone to the corresponding hydroquinone. This is catalyzed by NAD(P)H-dependent quinone oxidoreductase or DT-diaphorase (Ernster et al., 1962). Detoxication may also follow from conjugation of quinone or its reduced products with glucuronide, sulfate, or glutathione (O'Brien, 1991; Öllinger and Brunmark, 1991).

In contrast to mammals, relatively little is known about quinone detoxication in insects. Interest in quinone detoxication in insects has increased relatively recently (Ahmad, 1992). Many herbivorous insects are exposed to quinones either directly, through consumption of quinone-containing foliage, or indirectly, via the metabolic oxidation of dietary phenols (Felton et al., 1992a,b). Extrapolating from studies in mammalian systems, dietary naphthoquinones also probably potentiate oxidative stress in insects. Thus, insects that tolerate high dietary doses of naphthoquinones should offer insights into the mechanism of naphthoquinone metabolism, the bases of interspecific variation in quinone toxicity, and the evolutionary role of naphthoquinones in shaping plant–insect associations.

Luna (*Actias luna*) and promethea (*Callosamia promethea*) moths are two saturniid species whose larval host ranges differ in terms of preference of the former for members of the Juglandaceae (Tietz, 1972). Foliage of members of this plant family contains 5-hydroxy-1,4-naphthoquinone (juglone) as well as juglone's hydroquinone form (Rietveld, 1983). Luna develops successfully on foliage of black walnut, one of the Juglandacae, whereas promethea cannot survive on this plant. Here we report results from two sets of experiments

investigating the tolerance of luna larvae to juglone-containing diets. We quantitated the growth and developmental performance of fourth-instar luna and promethea reared on mutually acceptable paper birch foliage, supplemented with juglone. We also compared the toxicity of juglone and structurally related 1,4-naphthoquinones to first-instar luna larvae.

METHODS AND MATERIALS

Materials. Juglone (5-hydroxy-1,4-naphthoquinone), menadione (2-methyl-1,4-naphthoquinone), lawsone, (2-hydroxy-1,4-naphthoquinone), and plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone) (Figure 1) were obtained from Sigma Chemical Co. (St. Louis, Missouri). Paper birch (*Betula papyrifera*) and black walnut (*Juglans nigra*) foliage was obtained from trees in the University of Wisconsin-Madison Arboretum. Insect eggs were obtained from Larry Kopp, Klingerston, Pennsylvania.

Juglone Feeding Trial. This experiment was conducted to assess differences in responses of luna and promethea to juglone, as well as to determine differences in performance between luna fed birch or walnut diets. Accordingly, we had five treatment groups, consisting of luna and promethea each fed birch foliage with or without supplemental juglone, plus luna fed walnut foliage. Birch foliage was collected from several adjacently growing and similarly aged trees. We used mature, medium-size leaves 100–300 mg in weight. It was necessary to collect fresh foliage several times during the course of the feeding trial. To account for possible differences in percent dry weight in the leaves from each collection batch, 30 leaves from each collection were weighed fresh and following oven drying. A wet-to-dry conversion factor was calculated from each collection batch for use in calculating the wet weight of food consumed from dried,

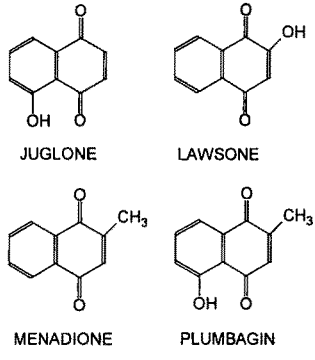

FIG. 1. Structure of juglone and related 1,4-naphthoquinones.

partially eaten foliage. Only minor variations was seen in the conversion factor from each collection batch.

Prior to conducting this fourth-instar feeding trial, both promethea larvae and luna larvae were reared on birch (25°C, 15L:9D photoperiod). We had observed that individual luna showed signs of gut trauma upon being switched from a birch to a walnut foliage diet (mortality with fluid excrement). Therefore, walnut-treated luna compared in this feeding trial were maintained on walnut foliage prior to the feeding trial, also at 25°C, 15L:9D. Upon molting into the fourth instar, individual larvae (10 replicates per treatment) were assigned to a test diet and maintained under the same environmental conditions. Juglone was dissolved in acetone and painted onto leaves at concentrations of 0.05% (leaf wet weight), a level within the range of naturally occurring juglone concentrations in walnut foliage (Cline and Neely, 1984). Control birch and walnut leaves received acetone only. The leaf petioles were placed in small water tubes, and the leaves were replaced every 24–48 hr as needed.

The feeding trial was completed when each insect either molted into the fifth stadium or died. Nearly all promethea died during the study; among control larvae this was attributable to disease characterized by languishing development late in the stadium, and death either just before or during the molt. In contrast, promethea caged on birch trees in the field were successfully reared to the fifth instar. Nonetheless, performance of promethea prior to death reflected strong treatment effects, as will be described in terms of relative growth rate, absolute weight gain, and relative consumption rate.

Upon completion of the fourth stadium, insects were frozen, dried (60°C, one week), and weighed. Frass and residual leaf matter were also dried and weighed. Nutritional indices were calculated using standard formulas (Waldbaur, 1968; Scriber, 1977) for relative growth rate (RGR), relative consumption rate (RCR), approximate digestibility (AD), efficiency of conversion of digested foods (ECD), and efficiency of conversion of ingested food (ECI).

The performance of luna on the three test diets was analyzed with the SAS general linear models procedure, followed by multiple comparison with the Student-Newman-Kuels test. The performance of promethea on two test diets was analyzed using one-way analysis of variance (SAS Institute, 1985). The developmental interval used for *A. luna* was time-to-molt. Since most of the *C. promethea* died prior to completing the molt, the interval used was time-to-death. Due to differences in the end points used, we did not attempt to make interspecies comparisons.

Structure–Activity Relationship of Juglone and Structurally Related 1,4-Naphthoquinones. Luna larvae were treated with several different 1,4-naphthoquinones in order to compare structure–activity relationships with respect to survival and developmental time. Newly eclosed first-instar luna were placed on 1,4-naphthoquinone-treated foliage in ventilated plastic Petri dishes, 10 insects

per dish, five dishes per treatment. Treatment foliage was prepared by painting leaves with 0, 0.01, 0.025, 0.05, 0.10, 0.25, and 0.50% (w/w) of each 1,4-naphthoquinone dissolved in acetone. This dose range included the range of juglone naturally occurring in walnut foliage (Cline and Neely, 1984). The leaf petioles were placed in small water tubes, and the leaves were replaced every 24–48 hr as needed. At 12-hr intervals, the number of insects surviving or that had molted was recorded. Each trial was concluded when all insects in a given cage had either died or molted. Average time-to-molt or time-to-death (mean $\pm$ SE) were calculated.

RESULTS

Juglone Feeding Trial. The most striking result of the feeding trial was that juglone supplements resulted in substantially lower weight gain, relative growth rate (RGR), and relative consumption rate (RCR) in promethea (Table 1). In contrast, the effect of 0.05% (w/w) juglone on fourth-instar luna was marginal. Larvae grew 15% less on the juglone-supplemented diet in terms of weight gain, efficiency of conversion of ingested food (ECI), and relative growth rate (RGR). However, the juglone supplement did not affect developmental time, approximate digestibility (AD), efficiency of conversion of digested food (ECD), or relative consumption rate (RCR) (Table 1). Nine of 10 juglone-treated and eight of 10 control luna successfully molted to the fifth stadia. These survival rates are typical for laboratory-reared luna (personal observations), which are quite sensitive to handling and disease. Surprisingly, luna reared on walnut foliage grew much better than did the birch-reared control in terms of developmental time (1.2-fold faster), RGR (1.8-fold higher), RCR (1.9-fold higher), AD (1.6-fold higher), and ECI (1.7-fold higher) (Table 1).

Toxicity of Juglone and Analogs. The mortality of first-instar luna was affected little by lawsone, juglone, plumbagin, or menadione at doses lower than 0.1% (w/w) (not shown). At doses above 0.1% (w/w), plumbagin became increasingly lethal after two to three days. No insects survived longer than four days on plumbagin (Figure 2). In contrast, the maximum dose of juglone, menadione, and lawsone, 0.5% (w/w), produced toxic effects only slightly greater than controls (Figure 3). The order of effect on mortality at the maximum dose was plumbagin $>$ menadione $=$ lawsone $>$ juglone (Student's t distribution, $P < 0.05$).

Developmental time of first-instar luna was more sensitive to 1,4-naphthoquinone dose than was mortality and was dose-responsive to all of the 1,4-naphthoquinones tested (Figure 4). At the maximum dose administered, 0.5% (w/w), the treatments increased developmental time in the order plumbagin $>$ lawsone $>$ menadione $=$ juglone (Student's t distribution, $P < 0.05$).

TABLE 1. PERFORMANCE INDICES OF FOURTH-INSTAR LUNA AND PROMETHEA LARVEA TREATED WITH AND WITHOUT JUGLONE (MEAN ± SE)[a]

Diet[b]	Duration (days)	Weight gain (mg)	RGR (mg/mg/day)	RCR (mg/mg/day)	AD (%)	ECD (%)	ECI (%)
Luna							
Birch control	6.10 ± 0.2[a]	113.7 ± 5.4[a]	0.41 ± 0.01[b]	5.45 ± 0.24[b]	28.5 ± 1.1[b]	26.6 ± 1.9[a]	7.5 ± 0.3[a]
Birch ± juglone	6.33 ± 0.3[a]	96.4 ± 4.6[b]	0.35 ± 0.02[c]	5.41 ± 0.20[b]	32.5 ± 4.7[b]	22.4 ± 2.4[a]	6.5 ± 0.2[b]
Walnut control	5.00 ± 0.3[b]	121.8 ± 5.0[a]	0.75 ± 0.03[a]	10.10 ± 0.33[a]	46.8 ± 1.4[a]	16.0 ± 0.7[b]	7.5 ± 0.3[a]
Promethea							
Birch control	8.26 ± 0.6[a]	153.8 ± 33.6[a]	0.28 ± 0.04[a]	3.98 ± 0.38[a]	ND	ND	ND
Birch + juglone	6.99 ± 0.7[a]	−3.85 ± 2.7[b]	−0.013 ± 0.01[b]	1.10 ± 0.21[b]	ND	ND	ND

[a] Rate indices were calculated using initial larval weights. Comparisons were made within but not between species (see text) using the Student-Newman-Keuls test for luna, and one-way ANOVA for promethea. Within each species column, means bearing the same superscript are not significantly different ($P < 0.05$). Number of insects used in the analysis: luna/birch/control, 8; luna/birch/juglone, 9; luna/walnut, 9; promethea/birch/control, 6; promethea/birch/juglone, 8. Duration: days to complete developmental stage; weight gain: weight gain over developmental stage; 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. ND: not determined.

[b] Diets: Birch control = larvae reared on birch foliage diet, then assayed on birch foliage painted with vehicle only. Birch + juglone = larvae reared on birch foliage diet, then assayed on birch foliage painted with vehicle/juglone, 0.05% w/w juglone/leaf. Walnut control = larvae reared on walnut foliage diet, then assayed on walnut foliage painted with vehicle.

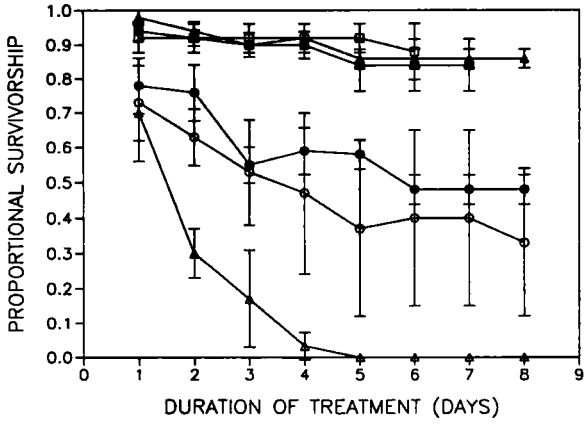

FIG. 2. Effects of various concentrations of plumbagin (2-Me-5-hydroxy-1,4-naphthoquinone) on survivorship of first-instar luna larvae. Each point represents the mean (±1 SE) of five treatment replicates. Mortality of control larvae (shown in Figure 3) was not significantly different from lowest dose. ▲: 0.01%; ■: 0.025%; □: 0.05%; ●: 0.01%; ○: 0.25%; △: 0.5%

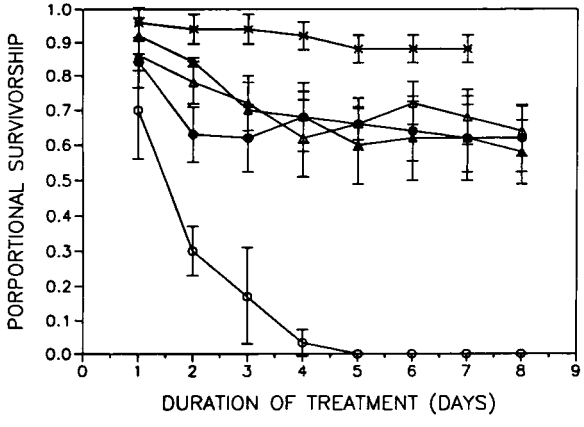

FIG. 3. Daily mortality of first instar luna vs 0.5% w/w 1,4-naphthoquinone. Each point represents the mean (±1 SE) of five treatment replicates. ×: control; ▲: lawsone; △: juglone; ●: menadione; ○: plumbagin. All surviving control animals had molted by day 7.

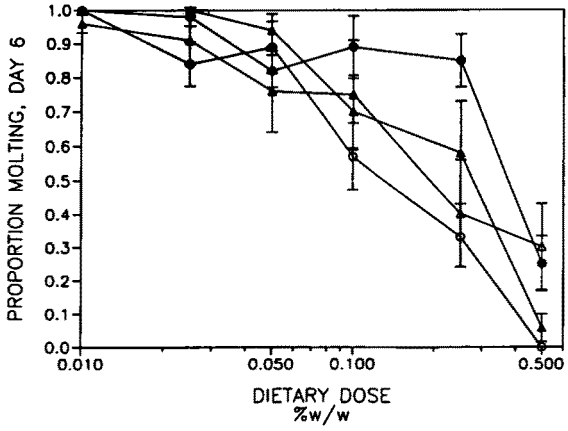

FIG. 4. Development, day 6, of first-instar luna vs w/w naphthoquinone dose. Development is the proportion of insects completing molt, mean of five treatment replicates ± 1 SE. Control = 0.98 ± 0.02. ▲: lawsone; △: juglone; ●: menadione; ○: plumbagin.

DISCUSSION

The major findings of this study were that luna was much more tolerant to juglone than was promethea, that the performance of luna is superior on a walnut diet than on a birch diet, and that the pattern of mortality caused by plumbagin is distinct from that of lawsone, juglone, and menadione. The comparative performance of the two saturniid moth species was consistent with the fact that luna but not promethea naturally feeds on juglone-containing diets.

The biochemical and/or physiological basis of the differential toxicity of juglone to luna and promethea is unknown at this time. The relative tolerance of luna to juglone may be partially explained by differences in midgut biochemistry, particularly that relating to glutathione. In vertebrates, it is known that cellular glutathione plays a key role in protection against reactive oxygen species generated during redox cycling of quinones. For example, both glutathione depletion and inhibition of glutathione reductase enhance menadione toxicity in cultured hepatocytes (O'Brien, 1991; Öllinger and Brunmark, 1991). Liu et al. (1993), in comparing menadione-susceptible and -resistant mouse cell lines, concluded that resistance to menadione is largely due to differences in cellular GSH levels. Finally, several 1,4-naphthoquinones react readily with glutathione (Buffington et al., 1989) and may be glutathione *S*-transferase substrates. Although it is too early to assign a role for GSTs in quinone detoxication in luna, Lindroth (1989) measured increased total glutathione *S*-transferase activity in larvae reared on several Juglandaceae (walnut, butternut, shagbark hickory)

compared to those reared on birch. In addition, a preliminary comparison of glutathione *S*-transferase subunit profiles from luna and promethea reveal qualitative differences in the glutathione *S*-transferase isoenzyme makeup of luna and promethea (Thiboldeaux and Tracy, unpublished data).

Differences in midgut physiology offer possible explanations for the differential toxicity of juglone to luna and promethea. Both species have alkaline midgut environments (H. Apple, personal communication), which would favor redox cycling of hydronaphthoquinones. It is not presently known if the midgut redox environments of these species are sufficiently dissimilar on a birch diet to affect the relative toxicity of juglone. Other physiological differences among species such as feeding rates, peritrophic membrane permeability (Barbehenn and Martin, 1992), gut detergency (Felton and Duffey, 1991), and gut motility also may influence net exposure to dietary 1,4-naphthoquinones.

The order of toxicity previously observed in hepatocytes parallels the order of increasing redox potential in these compounds (Table 2) (O'Brien, 1991). The propensity of 1,4-naphthoquinones to redox cycle is dependent upon the relative electrophilicity of the semiquinone radical, which is in turn influenced by the structure and position of ring substituents (Öllinger and Brunmark, 1991).

In the present 1,4-naphthoquinone structure–activity study, we observed mortality to luna larvae in the order plumbagin > menadione = lawsone > juglone. The order of naphthoquinone toxicity seen in luna was comparable to that reported for the nematodes *Haemonchus contortus* and *Ascaris suum* (Fetterer and Fleming, 1991). Fetterer and Fleming also report that plumbagin and 1,2-naphthoquinone, but no other tested naphthoquinones, caused delayed

TABLE 2. COMPARISON OF REDOX POTENTIAL, HEPATOCYTOTOXICITY, AND RESPIRATION-INDEPENDENT OXYGEN UPTAKE OF SELECTED 1,4-NAPHTHOQUINONES (ADAPTED FROM O'BRIEN, 1991)

Compound	Redox potential (mV)	Cytotoxicity, ED_{50} (nmol compound $\cdot 10^{-1}$ cells)	Respiration-independent O_2 uptake (nmol/10^6 cells/5 min) (conc. compound)
Juglone (5-OH-1,4-NQ)[a]	−93	20 ± 5	145 ± 15 (10 μM)
Plumbagin (5-OH-2-Me-1,4-NQ)	−156	25 ± 5	135 ± 15 (10 μM)
Menadione (2-Me-1,4-NQ)	−203	150 ± 15	185 ± 20 (10 μM)
Lawsone (2-OH-1,4-NQ)	−415	400 ± 50	1.5 ± 0.3 (100 μM)

[a] NQ: naphthoquinone.

embryogenesis in *Ascaris* eggs. Both Fetterer and Fleming's and the presently reported results contradicted the order of toxicity (juglone > plumbagin > menadione > lawsone) observed in rat hepatocyte studies (Cohen and Stubberfield, 1990; Doherty et al., 1987; Miller et al., 1986).

All of the compounds tested affected developmental time in a dose-responsive manner. We report the significance levels of the order of toxicity at the 0.5% (w/w) dose. However, the order of increase in developmental time varied with each dose level, making it difficult to clearly distinguish the overall order of the dose–response curve. Although each of the 1,4-naphthoquinones caused a similar pattern of delayed development, plumbagin produced mortality sharply higher than juglone, lawsone, and menadione.

Several authors have observed altered development, molting, and ecdysis caused by plumbagin and other 1,4-naphthoquinones. Plumbagin caused molting failure to another saturniid, *Bombyx mori*. Mitchell and Smith (1988) and Mitchell et al. (1993) report that both juglone and plumbagin inhibit ecdysone 20-monoxygenase activity in protein extracts from larval *Aedes aegypti*, *Drosophila melanogaster*, and *Manduca sexta*. Although it is still unclear whether naphthoquinones influence ecdysis sufficiently to account for the toxicity patterns seen in the present study, the fact that the relative toxicity of 1,4-naphthoquinones to luna does not follow relative redox rates suggests that redox cycling alone cannot fully explain their mode of action.

The species differences in tolerance to juglone noted in this study are significant in view of the nonpreference by promethea for Juglandaceae. Further studies will explore species differences to juglone toxicity on a mechanistic level.

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IDENTIFICATION AND FIELD EVALUATION OF *Anomala octiescostata* (COLEOPTERA: SCARABAEIDAE) SEX PHEROMONE[1]

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Abstract—Using GC-EAD, the sex pheromone of the scarab beetle *Anomala octiescostata* was identified to be a 8:2 binary mixture of (*R*,*Z*)-5-(−)-(oct-1-enyl)oxacyclopentan-2-one and (*R*,*Z*)-5-(−)-(dec-1-enyl)oxacyclopentan-2-one. These semiochemicals have been also reported as sex pheromone constituents of other *Anomala* species, either geographically or seasonally isolated from *A. octiescostata*. Synthetic sex pheromone was highly attractive in the field; 0.1 mg captured significantly more males than two virgin females. Buried traps were significantly more attractive than those positioned at 30, 90, and 150 cm above the ground. In a dose–response test (0.1–100 mg), no saturation due to overdose of pheromone was observed, but in most cases, two dosages differing by 10-fold were not significantly different. Response of males to traps baited with different ratios of the two components was tested in two experiments with randomized blocks and Latin-square designs. Deviation from the natural ratio (8:2) of sex pheromone did not significantly diminish the response of males. Peak flight activity of beetle was recorded at 9:00–10:00 AM JST on sunny days in the end of April 1993.

Key Words—Coleoptera, Scarabaeidae, *Anomala octiescostata*, (*R*,*Z*)-5-(−)-(oct-1-enyl)oxacyclopentan-2-one. (*R*,*Z*)-5-(−)-(dec-1-enyl)oxacyclopentan-

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2-one, GC-EAD, *Anomala cuprea*, *Anomala albopilosa sakishimana*, flight activity, Latin square.

INTRODUCTION

The scarab beetle *Anomala octiescostata* Burmeister (Japanese name: hirata-aokogane) is native to Japan and was originally recorded only in Kyushu Island in the south of the country. The adults appear in grass fields on sunny mornings during the months of April and May and have short seasonal flight activity. Due to the lack of a monitoring system, heavy outbreaks of the beetle may be overlooked.

Although *A. octiescostata* was not previously recorded in the Kanto district—Gumma, Tochigi, Saitama, Ibaraki, Tokyo, Chiba, and Kanagawa (probably because it never reached pest status)—heavy infestations were recently detected in Chiba and Ibaraki prefectures. The insecticides registered for scarabs in Japan were not effective in controlling the beetle, and it reached large populations in a few years, showing the pattern of an exotic species, devoid of natural enemies, exploiting new habitats.

As part of our project aimed at investigating the possibility of applying semiochemicals to the integrated pest management (IPM) of scarab beetles, we have recently successfully identified the sex pheromones of some economically important scarab pests in Japan (Leal et al., 1994), some of which are promising in mass trapping (Hasegawa et al., 1993). The alarming level of the *A. octiescostata* population in Chiba and Ibaraki prefectures prompted us to investigate the sex pheromone of *A. octiescostata* and evaluate its potential for field application. We report here on the identification and field testing of the sex pheromone.

METHODS AND MATERIALS

Insects. Beetles were first collected at a golf course, Chiba Springs Country Club, in Chiba Prefecture at the end of the flight season (May) in 1992. In order to start a laboratory colony, eggs laid by the field-captured females were collected daily and transferred to wet sand in ice cream cups, which were kept at 25°C. After hatching, grubs were individually transferred to ice cream cups filled with humus and supplied with slices of sweet potato or carrot. These cups were kept at 25°C and the foodstuff was renewed weekly. Diapause termination of adults was achieved by chilling at 10°C for three to four months and then raising the temperature to 25°C. After emerging from diapause, adults were fed on cherry (*Prunus donarium*) leaves or on an artificial diet for wild silk moth (Nihon Chlorella Co.) at 25°C, 70% relative humidity, and 14L:10D photoperiod.

Pheromone Collection. The airborne volatiles from 97 field-collected female *A. octiescostata* fed on cherry leaves (or supplied only with syrup on pieces of cotton) were collected according to the method of Leal et al. (1992). The volatiles were extracted with hexane and, after concentration, the extracts were stored at −40°C. Headspace volatiles of laboratory-raised beetles, in diapause or not, were also collected. Whole-body extracts of laboratory-raised virgin female or male beetles were obtained by washing in dichloromethane for 5 min.

Isolation of Pheromone. Volatiles collected from the headspace of female beetles were separated on a silica gel column (Wako C-200) by successive elution with hexane–ether mixtures: 100:0, 95:5, 90:10; 80:20, 50:50, and 0:100.

Chromatographic and Mass Spectral (MS) Analyses. GC analyses were performed on Hewlett-Packard 5890, and mass spectra were recorded on a Hewlett-Packard 5891 mass selective detector equipped either with an HP-1 column (12 m × 0.2 mm; 0.33 μm) or a DB-wax column (30 m × 0.25 mm; 0.25 μm), operated in splitless mode at 50°C for 1 min, programmed at 4°C/min, held at this temperature for 1 min, programmed again at 10°C/min to 230°C and held at this temperature for 20 min [i.e., 50(1)–180(1)/4–230(20)/10].

Enantiomeric resolution of buibuilactone was achieved on a Chiraldex GTA column (20 m × 0.25 mm; 0.125 mm, Astec), operated in split mode at 115°C, using helium as carrier gas at 2 kg/cm^2 head pressure (flow rate of 4.4 ml/min).

Gas Chromatography–Electroantennographic Detector (GC-EAD). The responses of *A. octiescostata* antennae were recorded with a previously described GC-EAD system (Leal et al., 1992, 1994). Antennae of beetles in diapause or not were used as the sensing elements.

Synthesis. (*R*,*Z*)-5-(−)-(Oct-1-enyl)oxacyclopentan-2-one (buibuilactone) was synthesized as previously reported (Leal, 1991) and (*R*,*Z*)-5-(−)-(dec-1-enyl)oxacyclopentan-2-one was prepared starting from protected D-ribose (Koseki et al., 1993). (*S*,*Z*)-5-(−)-(Oct-1-enyl)oxacyclopentan-2-one was synthesized by the method of Doolittle et al. (1980), starting from (*S*)-(+)-5-oxo-2-tetrahydrofurancarboxylic acid (Aldrich Chemical Company, Inc.). The optical purity of all synthetic pheromones was determined to be >97% ee by chiral chromatography (Leal, 1991).

Wind Tunnel. The attractancy of the pheromone was preliminarily tested in a wind tunnel (2 m long, 30 cm ID) internally covered with wire mesh. Experiments were carried out from 9:00 AM to 4:00 PM in a clear room at 23°C and 60% relative humidity. Blank or synthetic sex pheromone containing filter paper was set 1.5 m away from groups of five insects placed in the downwind end of the tunnel and observed for 15 min at an airflow of 40 cm/sec.

Field Evaluation. Tests on synthetic sex pheromone were conducted in two golf clubs, Chiba Springs Country Club (Chiba) and Edosaki Country Club

(Ibaraki) from April to May 1993. A survey for the occurrence of beetles in the autumn was done in Edosaki in 1993. Funnel JT traps (Japan Tobacco Inc.) were baited with synthetic sex pheromone incorporated into pellets (4–5 mm in diameter) made of a polyethylene–vinyl acetate copolymer. These were placed inside a pellet holder (Fuji Flavor Co.) and set 2 cm above the trap lip. The pheromone dosages in Ibaraki and Chiba were 1 and 10 mg of a 8:2 blend, respectively, unless otherwise mentioned.

Experimental Design and Statistical Analysis. The experiments were done using two different designs: the candidate lures were replicated in randomized blocks in the Chiba and in Latin square in the Ibaraki experiments. Traps were set with an intertrap distance of 10 m and the pheromone dispenser set at 30 cm above the ground, unless otherwise mentioned. Capture data were transformed to log $(x + 1)$ and differences between means were tested for significance by ANOVA using JMP software (Version 2) (SAS Institute, 1989). In this paper, treatments followed by the same letters are not significantly different at the 5% level in the Tukey-Kramer honestly significant difference test.

RESULTS AND DISCUSSION

Identification of Pheromone. Volatiles collected from field-captured female beetles were stored until the emergence of the first laboratory-raised group. As *A. octiescostata* enters diapause as soon as the adult eclose from pupal stage, freshly eclosed male beetles could not be used in laboratory bioassay for monitoring the isolation of the sex pheromone because they did not display any sexual behavior. However, the antennae of diapausing males could be used in GC-EAD analyses. Two EAD-active peaks (ratio 8:2) were found in the female extract, the major one appeared at 33.62 and 24.77 min whereas the minor one gave R_t at 37.07 and 30.57 min on DB-wax and HP-1 columns, respectively. The same EAD-active peaks were also detected in the volatiles collected from laboratory-raised virgin females after they were brought out of diapause. No trace of these two peaks was found in the volatiles collected either from females in diapause or males. The mass spectra and retention times in the two capillary columns of the two EAD-active peaks were identical to those of buibuilactone [(*R*,*Z*)-5-(−)-(oct-1-enyl)oxacyclopentan-2-one or (*R*,*Z*)-5-dodecen-4-olide] and japonilure [(*R*,*Z*)-5-(−)-(dec-1-enyl)oxacyclopentan-2-one or (*R*,*Z*)-5-tetradecen-4-olide].

The stereochemistry of the major component sex pheromone was determined by chiral chromatography (Leal, 1991). (*R*)-buibuilactone appeared at R_t 65.54 min ($k' = 81.03$), whereas the *S* enantiomer appeared at 67.15 min ($k' = 83.04$, $\alpha = 1.02$). The natural product from *A. octiescostata* was first separated by silica acid column in a hexane–ether (80:20) fraction before inject-

ing in the capillary column. It gave the same R_t as synthetic (*R*)-buibuilactone, and no trace of its enantiomer was detected. Therefore, the major component sex pheromone was fully identified as (*R*,*Z*)-5-(−)-(oct-1-enyl)oxacyclopentan-2-one. Nevertheless, it was not possible to determine analytically the absolute configuration of the minor component due to the small amount of natural japonilure.

Buibuilactone and japonilure have been identified as constituents of the sex pheromone system of other scarab beetles, *A. cuprea* (Leal et al., 1993a) and *A. albopilosa sakishimana* (Leal et al., 1994). However, these species are geographically and/or seasonally isolated. *A. cuprea* and *A. octiescostata* have been recorded on the main island (Honshu) of the Japanese archipelago, but their flight activity is seasonally isolated. The former is active in summer, whereas the emergence of the latter occurs mainly in early spring. On the other hand, *A. albopilosa sakishimana* is geographically restricted to Okinawa.

GC-EAD using *A. octiescostata* antenna as the sensing element gave the best performance of all scarab beetles investigated thus far (reviewed in Leal et al., 1994). The same antennae could be used for as long as 12 hr, and the response to the major sex response showed a signal-to-noise (S/N) ratio of 12. In the range tested, the same S/N ratio was recorded regardless of the amount of pheromone. Amounts below the detection limit of our flame ionization detector (FID) system (<0.1 ng) gave nearly the same response as that to 500 ng of synthetic sex pheromone. Whole-body extracts of female *A. octiescostata* generated such a small amount of the sex pheromone that they were not detected by FID; however their occurrence in the extracts was confirmed by EAD.

Although the reproducibility of the response to the minor component was far better than that obtained with male *A. cuprea* antenna as the sensing element (Leal et al., 1993a), a few times the signal of the minor (but not the major constituent) was missed, although both compounds were detected by FID.

Bioassay. The attractancy of the synthetic sex pheromone was first demonstrated in a wind tunnel. Males responded to 100 ng of a synthetic blend (8:2) by walking to and gathering near the pheromone source (89.6 ± 7.9%; $N = 5$). No response was observed to control (0%, $N = 8$) or to filter paper loaded only with buibuilactone (0%, $N = 2$).

Evaluation of Synthetic Sex Pheromone. Preliminary field experiments explored the possibility of using sticky traps because with them it would be easier to rerandomize the blocks. However, the fact that they become saturated with beetles within 30 min (Figure 1) prevented their use. Even JT funnel traps had to be emptied twice a day because they were completely loaded (>6000 beetles/trap) within 3 hr (8:00–11:00 AM) (Figure 1B).

Height of Traps. On sunny days, *A. octiescostata* males emerge from the soil and appear on the ground, where they crawl and fly at low levels searching for females. Later they are also found feeding in the high part of plants around

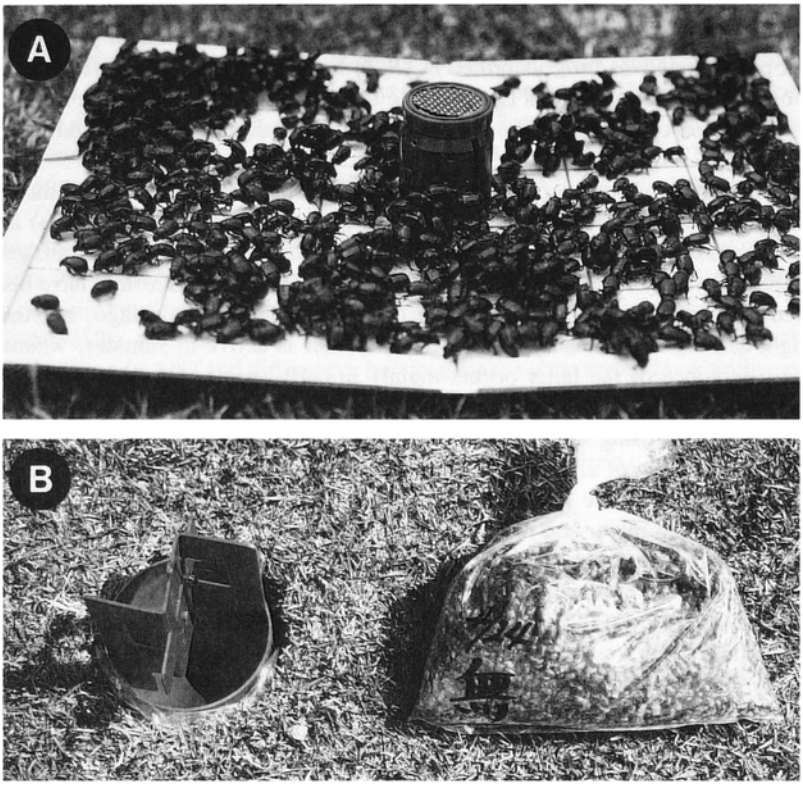

FIG. 1. Response of *A. octiescostata* to synthetic sex pheromone. Beetles captured in 30 min in a sticky plate (A) and in 3 hr in a buried funnel trap (B).

golf courses. Therefore, we first investigated the effect of height on captures of the beetle. These experiments were carried out in Chiba (April 24–27) and Ibaraki (April 27–May 6). Catches in traps at 30 cm (Chiba) were significantly higher than those at 90 and 150 cm. On the other hand, experiments at Ibaraki demonstrated that buried traps (0 cm) captured significantly more beetles than those at 30 cm (Figure 2). By contrast, traps baited with virgin female *Popillia japonica* captured significantly more beetles when positioned at 28 cm than at 0, 56, or 112 cm (Klein et al., 1972). Catches of *A. schonfeldti* in synthetic sex pheromone-baited traps at 0 and 30 cm were not significantly different (Hasegawa et al., 1993). Interestingly, these three species display a very similar flight behavior (they all search for conspecific females at low levels); however, their patterns of response to sex pheromone-baited traps are different.

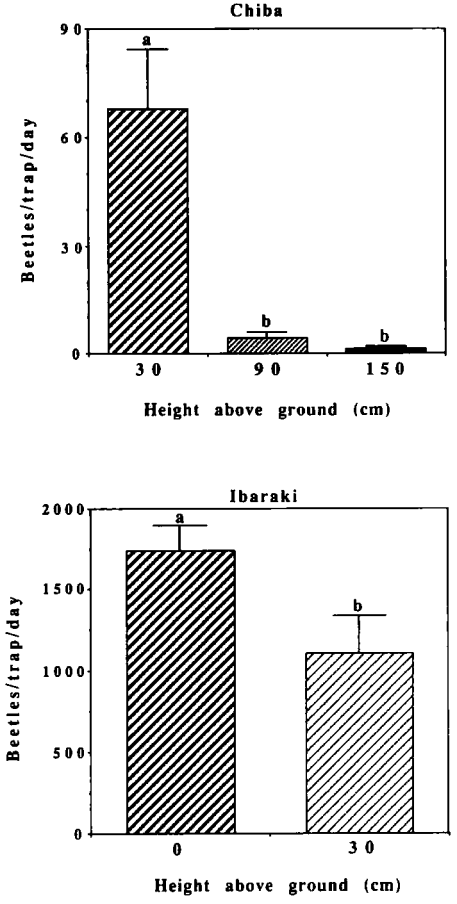

FIG. 2. Effect of the height of the traps on catches of *A. octiescostata*.

In practical applications, burying a trap would require more labor and would not always be possible. However, the fact that the lower the trap is positioned the better is the response of *A. octiescostata* to synthetic sex pheromone should be taken into consideration for the design of new "user-friendly" traps.

Effect of Dosage. Experiments were carried out in Chiba (May 5–8) and Ibaraki (May 1–6) to determine the optimal dosage for capture of *A. octiescostata* with synthetic pheromone. A 10-fold increase in the amount of pheromone caused significantly better catches only from 0.1 to 1 mg (Chiba and Ibaraki). However, captures with 10 mg were not significantly different from 1 mg. Traps baited with 100 mg of the synthetic lure (Chiba) captured significantly more

beetles than those with 1 mg (Figure 3). In the range tested, there was no saturation due to the overdose of sex pheromone.

Attractancy of the synthetic sex pheromone was compared with that of two virgin female *A. octiescostata* (Chiba, May 3–13). Even 0.1 mg of the sex pheromone was a significantly better lure than two virgin female beetles. Catches were 18 times higher in traps having 1 mg of the synthetic sex pheromone than those baited with two virgin female beetles (Figure 4). We therefore recommend the use of 1 mg of synthetic sex pheromone in monitoring this species.

Effect of Ratio of Two Components on Catches. The optimal ratio of two

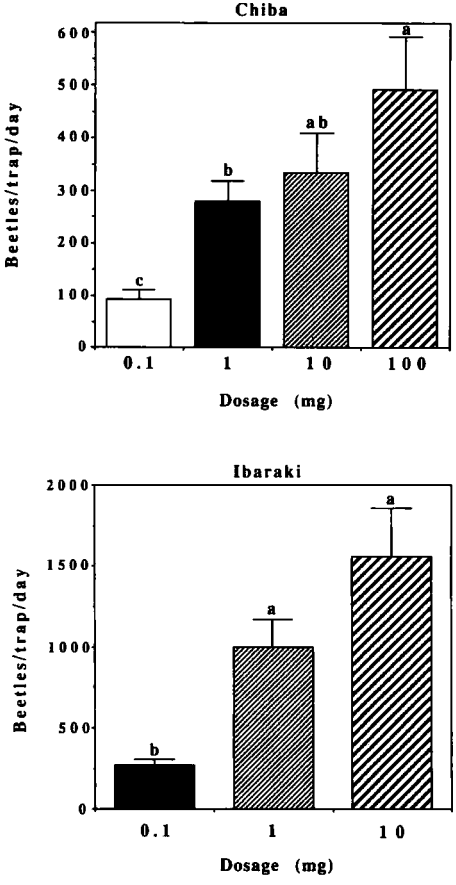

FIG. 3. Captures of *A. octiescostata* with different dosages of synthetic sex pheromone incorporated into plastic pellets.

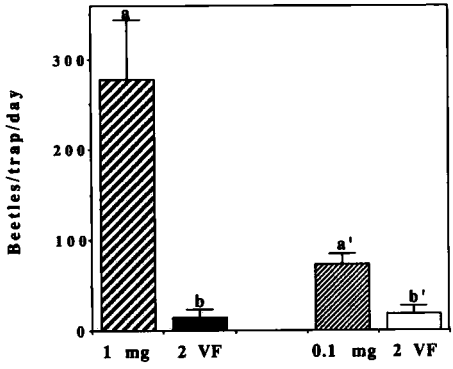

FIG. 4. Comparative catches of *A. octiescostata* in traps baited with two virgin female beetles (2 VF) and 0.1 and 1 mg of synthetic sex pheromone.

components in the pheromone system of scarab beetles could not be experimentally determined for *A. cuprea* (Leal et al., 1993a) and *Holotrichia parallela* (Leal et al., unpublished). In order to rule out the possibility of error incurred by experimental design, we conducted experiments on the effect of the ratio of the two components on the catches of *A. octiescostata* not only in randomized blocks (as in the two cases cited), but also in a Latin-square design, which has been demonstrated to be more efficient than other designs for quantitative comparison of attractants (Perry et al., 1980). The former was done in Chiba (April 28–May 4) and the latter in Ibaraki (April 23–28), where a larger area was available for the field tests. Neither in Latin-square nor in randomized-blocks experiments was a significant difference found in the response of male *A. octiescostata* to traps baited with the two components in the ratios of 9:1, 8:2, 7:3, and 6:4 (Figure 5).

It has been long known that the optimum blend ratio of pheromone components of Lepidoptera is that which most closely approximates the natural ratio emitted by females. Behavioral responses diminish markedly even with slight deviations from this proportion (Baker, 1989). Deviation from the natural ratio (8:2) of sex pheromone emission by female *A. octiescostata* did not significantly diminish the attraction of males to traps in the field. One possible explanation is that the dosage of the synthetic bait was higher (Ibaraki, 1 mg; Chiba, 10 mg) than the level at which male beetles can discriminate different ratios of the sex pheromone. This hypothesis must await future field testing at lower dosages.

The ratios of two components of other scarab sex pheromone systems have been demonstrated to change, according to the physiological condition of the female beetle (Leal et al., 1993a,b). That the ratio of buibuilactone/japonilure emitted by virgin *A. cuprea* changed considerably after mating and isolation

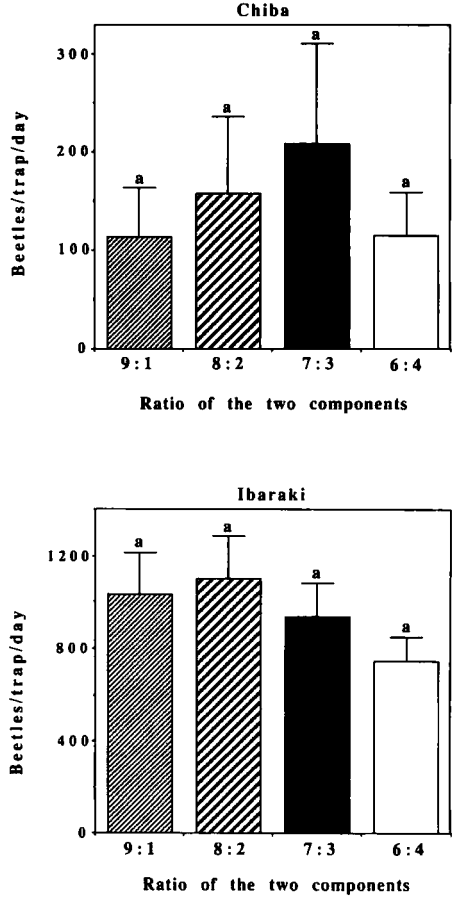

FIG. 5. Response of *A. octiescostata* to traps baited with different ratios of buibuilactone and japonilure in two experimental designs: randomized blocks (Chiba) and Latin square (Ibaraki).

was considered to be a selective advantage of virgin against mated females, giving the former a higher mating probability (Leal et al., 1993a). Therefore, it may be also possible that males of different physiological conditions respond to different ratios of the sex pheromone.

Seasonal and Daily Flight Activity. Seasonal captures of *A. octiescostata* was investigated in Ibaraki with buried traps in a fairway. Catches reached a peak at the end of April 1993 (Figure 6), when over 10,000 beetles per trap

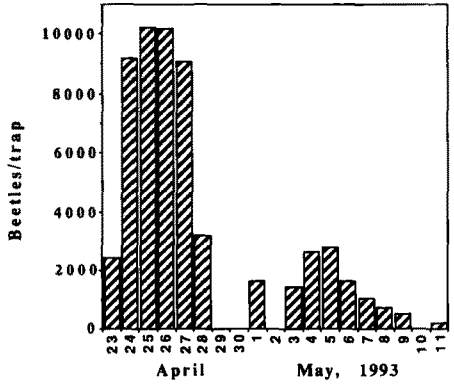

FIG. 6. Seasonal flight activity of male *A. octiescostata*, as revealed by trap catches in Ibaraki Prefecture. A very small peak was also detected in autumn.

and day were captured. These catches reflect not only the high response of male *A. octiescostata* to sex pheromone, but also the level of beetle infestation at that golf course. The highest flight activity of the beetle occurred during a holiday season in Japan, "golden week," when many golf competitions take place.

In mid-September, flight activity by the beetles was observed by personnel of Edosaki Country Club. We set traps for one week (September 22–28) in three different places. One trap in a fairway (same height condition as for the data in Figure 6) captured a total of 111 beetles, whereas catches in traps at a tee positioned 50 cm above the ground numbered 66. Another trap at 150 cm above the ground captured 51 beetles. In other words, two peaks were found in the seasonal flight activity of *A. octiescostata*, the one in autumn being very small.

Daily flight activity was investigated in Chiba (May 5, 1993) utilizing traps baited with 0.1, 1, 10, and 100 mg. The pattern of activity was almost the same (regardless of the dosage). Beetles were active from 7:00 AM to 3:00 PM, showing a peak of flight activity from 9:00 to 10:00 (Figure 7). The flight activity was affected by weather conditions. This experiment was conducted on a sunny day, but it become cloudy at about 11:00 AM and the temperature decreased, influencing the flight activity of *A. octiescostata* (decrease in catches; valley at ~11:00 AM). Wind was stronger at ~12:30 and ~1:00 PM, and this was reflected in the decrease in captures during this period (valley in between 12:00 and 1:00).

In conclusion, male *A. octiescostata* are highly attracted to the synthetic sex pheromone system, which may be a valuable tool for monitoring and integrated pest management.

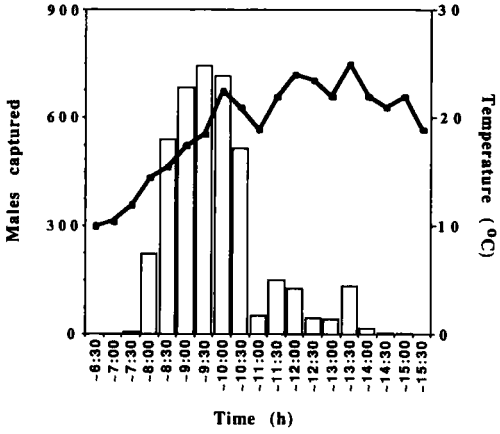

FIG. 7. Daily flight activity of male *A. octiescostata* in relation to the ambient temperature. Data were recorded at a golf club in Chiba on a sunny day.

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PLANT RESPONSE TO EGGS VS. HOST MARKING PHEROMONE AS FACTORS INHIBITING OVIPOSITION BY *Pieris brassicae*

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Abstract—*Pieris brassicae* L. butterflies secrete miriamides onto their eggs. These avenanthramide alkaloids are strong oviposition deterrents when sprayed onto a cabbage leaf. However, these compounds could not be detected in cabbage leaves from which egg batches had been removed two days after deposition and that still showed oviposition deterrency. It was concluded that the miriamides were not directly responsible for the avoidance by females of occupied leaves while searching for an oviposition site. Evidence was obtained that cabbage leaves themselves produce oviposition deterrents in response to egg batches. Fractions containing potent oviposition deterrents could be isolated from surface extracts of leaves from which previously laid egg batches had been removed. The term host marking pheromone that was used previously is not applicable in this case.

Key Words—Lepidoptera, Pieridae, *Pieris brassicae*, Host Marking Pheromone, Cruciferae, *Brassica oleracea*, induction, oviposition deterrency.

INTRODUCTION

Several phytophagous insect species belonging to different orders deposit chemical markers on or around their eggs. These are called host marking pheromones (HMPs) and constitute a chemical signal that deters conspecific females from

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laying eggs at that site (Prokopy et al., 1976; Roitberg and Prokopy, 1987). The chemical nature of the substances involved has been established in only a few cases and was found to be profoundly different for different insect species (Hurter et al., 1987; Imai et al., 1990).

More than a century ago Kirby and Spence (1863) observed that oviposition by *Pieris brassicae*, commonly occurring on cruciferous plants, was influenced by the presence of previously laid eggs. Rothschild and Schoonhoven (1977) confirmed this observation under more controlled conditions. In a choice situation between cabbage leaves with or without conspecific eggs, the butterfly prefers to oviposit on the latter. An oviposition-deterring mixture can be collected by washing the eggs with water or methanol. A methanolic egg wash of 100 eggs, when sprayed onto cabbage leaves, is much more deterrent to females than the presence of 100 intact eggs (Klijnstra, 1986). Recently Blaakmeer et al. (1994) isolated and identified three novel avenanthramide alkaloids, which together explained the oviposition-deterring effect of a crude egg wash. The compounds were found to be secreted onto the eggs by the accessory gland of the female during oviposition (Blaakmeer, unpublished results). The question arises how the deterrent signal is spread over the leaf, as from the behavior it is clear that a female perceives the presence of an egg batch without actually contacting it. Moreover, the miriamides are not volatile (Blaakmeer et al., 1994). This study was aimed at investigating the putative translocation of these molecules through cabbage leaves. At the outset it became clear that no such translocation occurred. This led us to look into the possible involvement of chemicals in the leaf surface, induced by previously laid egg batches, that deter *P. brassicae* females from oviposition.

METHODS AND MATERIALS

Plant Material. Brassica oleracea L. var. *gemmifera* cv. Titurel plants were reared in a greenhouse (20–30°C, 50–80% relative humidity, 16L:8D) in standard potting soil. Illumination consisted of natural daylight supplemented by high-pressure sodium vapor lamps hanging 0.75 m above pot level.

Insects. Pieris brassicae L. adults were obtained from a laboratory colony maintained on *Brassica oleracea* L. This culture was established in 1981 and since then 18 generations have been produced each year. Field-collected adults have been introduced several times during this period. Rearing conditions were similar to those described by David and Gardiner (1952).

Bioassays. Oviposition preferences were tested in wooden cages with walls of muslin and doors of glass measuring 80 × 50 × 100 cm high. The cages were placed in a conditioned greenhouse, with temperatures fluctuating between 22 and 25°C. In addition to natural daylight, each cage was illuminated from

0700 to 1500 hr by a 400-W sodium vapor lamp hanging 30 cm above the glass roof of the cage. In each cage, eight females and four males were introduced just after eclosion. The butterflies were repeatedly used for bioassays over a 10-day period. In the bioassays, 1 ml of solvent or fraction was sprayed on the upper leaf surface only using a chromatographic solvent sprayer. One control and one treated leaf were placed in diagonally opposite corners, and positions were alternated between replicate cages to minimize positional effects. In the first isolation attempt of putative deterrents, control leaves were sprayed with leaf surface extracts of control plants and fractions obtained from them. In the other attempts, control leaves were sprayed with methanol only. Females could oviposit on the leaves for 5 hr (8 AM–1 PM). The preference of the butterflies was measured by comparing the number of egg batches deposited on the leaves sprayed with different fractions with those on control leaves. On any one day, eight replicates were run. The significance of preference was tested using the Wilcoxon's matched-pairs signed-rank test (Siegel, 1956).

Deterrency of Leaves that had Carried Egg Batches. To determine the degree of avoidance of leaves that had carried egg batches [previously documented by Rothschild and Schoonhoven (1977)], we used a bioassay in which the oviposition preference of about 40 individuals was measured. In this bioassay, females were given a choice between control leaves and leaves from which egg batches (3–15 batches per leaf, an average batch consists of 45 eggs) that had adhered to the leaf for 24, 48, or 72 hr since oviposition were removed just prior to the bioassay. These leaves had been on intact plants and were excised just prior to removal of the eggs. After a female butterfly had made a choice for one of the leaves and had started to lay eggs, the female and the one or two eggs she had already deposited were immediately removed. Significance of preference for control leaves was tested by a chi-square test for expected frequencies (Sokal and Rohlf, 1981).

HPLC. The pumps (models 302 and 303), the manometric module (model 802C), dynamic mixer (model 811), and UV-detector (model 116) were from Gilson. A software HPLC system manager (model 702) from Gilson was used on an Apple II computer. The column used was a Microsorb RP C18, 250 × 10 mm, 5 μm particle size and 100 Å pore size (Rainin Instrument Co.).

Extraction and Fractionation of Surface of Leaves that had Carried Egg Batches. Eggs were laid by *P. brassicae* on leaves of intact *B. oleracea* L. var *gemmifera* cv. Titurel plants (8–10 weeks old). After 48 hr, leaves carrying four to six egg batches were harvested. The egg batches were gently removed with a brush, and the leaves were dipped in 500 ml dichloromethane for 3 sec followed by a dip in 500 ml methanol, also for 3 sec. The crude methanol dips of 1000 leaves (ca. 6000 g) collected during the months of June, July, and August were combined.

The methanol extract was then evaporated to dryness and redissolved in 10

ml of methanol. This crude methanol extract was separated into six fractions using reverse-phase C-18 HPLC. The mobile phase contained 0.05% trifluoroacetic acid (TFA). The solvent composition changed in 20 min linearly from MeCN–H_2O 10:90% to MeCN–H_2O 70:30% and was kept at that composition for 5 min, at a flow rate of 3.0 ml/min. After 4 min, a fraction was collected every 3 min (six in total).

Fractions 4, 5, and 6 were further separated by means of HPLC using the same column but different gradients. The flow rate of the solvents (containing 0.05% TFA) was 3.0 ml/min. For fraction 4, the solvent composition changed in 30 min linearly from MeCN–H_2O 20:80% to MeCN–H_2O 35:65%. After 4 min, a fraction (four in total) was collected every 5 min. For fraction 5, the solvent composition changed in 30 min linearly from MeCN–H_2O 25:75% to MeCN–H_2O 55:45%. After 4 min, two fractions were collected, each for 8 min. For fraction 6, the solvent composition changed in 25 min linearly from MeCN–H_2O 35:65% to MeCN–H_2O 65:35%. After 4 min, three fractions were collected, each for 6 min.

RESULTS

Cabbage leaves from which egg batches had been removed 24, 48, or 72 hr after egg laying were avoided as an oviposition substrate in favor of clean cabbage leaves in two-choice situations (Table 1). In two-choice situations the crude methanol extract of the leaf surface still deterred oviposition (Figure 1). Surprisingly, HPLC analysis of the crude methanol extract of the leaf surface failed to demonstrate the presence of the three miriamides, which are the only oviposition-deterring compounds obtained from the egg washes of *P. brassicae* (Blaakmeer et al., 1994).

The separation procedure used to isolate oviposition-deterring fractions and/or compounds of the crude methanol extract is shown in Figure 2. The methanol extract was separated into six fractions using reverse-phase C-18 HPLC. Fractions 3, 4, 5, and 6 contained oviposition-deterring activity (Figure 1), while fractions 1 and 2 did not. Fraction 3 was not further analyzed because of its low oviposition-deterring activity compared to the three other fractions. Fraction 4 was further subdivided into four fractions, and only fraction 4.2 showed oviposition-deterring activity. Fraction 5 was further separated into two fractions, of which only 5.1 contained oviposition-deterring activity. Fraction 6 was separated into three fractions, of which only 6.1 contained oviposition-deterring activity. The oviposition-deterring activity was lost by further purification of the three active subfractions. Two other attempts to isolate and identify components responsible for the oviposition-deterring activity in the methanol extracts of leaves that had carried egg batches for two days (removed prior to extraction),

TABLE 1. OVIPOSITION DETERRENCE OF LEAVES THAT HAD CARRIED EGG BATCHES FOR TIME INDICATED (TREATED LEAVES)[a]

Number of egg batches	Residence time (hr) of egg batches on leaves	Ovipositions	
		On treated[b] leaves (*N*)	On control leaves (*N*)
7	24	15	33[d]
15	24	4	36[d]
5	24[c]	14	30[d]
8	48	11	29[d]
3	72	14	36[d]

[a] Groups of six to eight females were observed individually while having a choice between treated and control leaves. As soon as a female started to oviposit, she was removed from the cage. This was repeated for seven groups. Numbers of ovipositions are totals over seven groups.

[b] Treatment signifies the adherence of egg batches (numbers indicated in the first column) during the periods indicated in the second column.

[c] Egg batches were removed after 24 hr and leaf was tested 24 hours later. In all other cases, leaves were offered directly after removal of the egg batches.

[d] Number of ovipositions on treated leaves significantly lower than that on control leaves (chi-square test).

gave exactly the same result, i.e., activity was reproducibly found in the same subfractions but vanished when further purification was undertaken.

DISCUSSION

The strongly oviposition-deterring miriamides, which are constituents of eggs of *P. brassicae* (Blaakmeer et al., 1994), could not be detected in surface extracts from leaves from which egg batches had been removed (detection limit of the miriamides is 0.15 μg/leaf). Because at least 1.5 μg/leaf of one of the two most active miriamides is necessary to achieve an oviposition-deterring activity comparable to that reported here (Blaakmeer et al., 1994), we conclude that the miriamides are not responsible for the oviposition-deterring activity of a leaf after oviposition by *P. brassicae*. It is doubtful whether the three miriamides are involved in the avoidance of leaves carrying an egg batch under natural conditions. It may be possible that the miriamides associated with an egg batch remain tightly bound to the leaf surface after removal of egg batches. However, in separate experiments, application of a droplet containing the three pure miriamides in a dose equivalent to 100 eggs at the lower side of a leaf at five different spots did not render this leaf less acceptable to the females when tested two days later. This is additional evidence for the absence of a role for the miriamides in inducing the apparent changes in leaf surface chemistry. There-

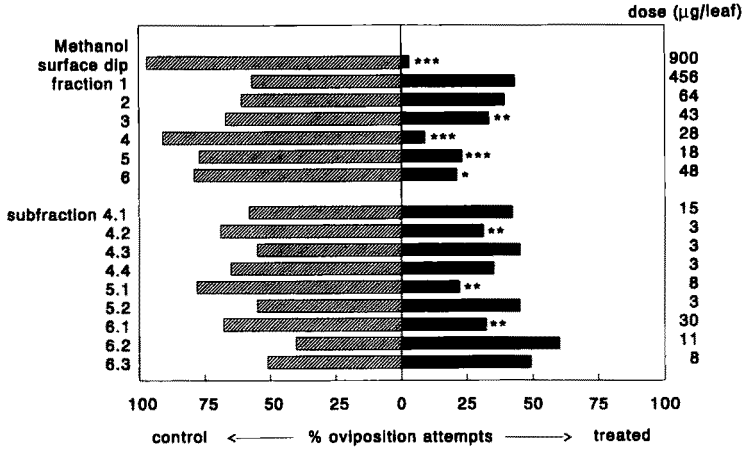

FIG. 1. Oviposition preferences displayed by *Pieris brassicae* female butterflies in a two-choice situation. Results obtained during the first isolation attempt. Asterisks (*) indicate that treated leaves were significantly less preferred according to Wilcoxon's matched-pair signed-rank test (two-tailed) (Siegel, 1956), under the null hypothesis that egg batches were distributed evenly over control and treated leaves. $*P < 0.05$; $**P < 0.02$; $***P < 0.01$. The amount of dry material applied to test the activity of a certain fraction originated from fractionation of dry material present in the original methanolic leaf dip of two cabbage leaves.

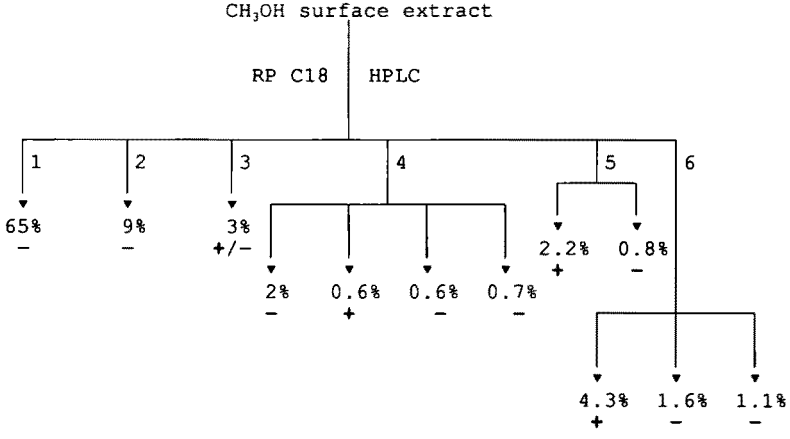

FIG. 2. Purification scheme of the methanol surface extract and the dry material distribution (%) in the six fractions obtained. Fractions significantly deterring oviposition are marked with a "+" sign. The 9% loss of dry matter during fractionation was caused by sampling errors in weighings.

fore, the term HMP does not correctly describe the phenomenon of *Pieris* butterflies avoiding host plants already carrying conspecific eggs (Schoonhoven, 1990).

The loss of oviposition-deterring activity after purification of subfractions 4.2, 5.1, and 6.1 could be due to instability of the active compounds. Lack of synergism can be excluded as a cause for the loss of activity because recombination of fractions of subfractions 4.2, 5.1, and 6.1 did not show any oviposition-deterring activity.

We interpret these behavioral effects of leaf surface fractions as deterrency caused by plant compounds and not by compounds of insect origin. We can exclude the possibility that the adherence of eggs to the leaf surface reduces the concentrations of glucosinolates, known to be the major oviposition stimulants to *P. brassicae* (Van Loon et al., 1992). We first determined that spray application of exogenous glucosinolates, doubling the amount present on the surface of a normal cabbage leaf, did not induce a preference for leaves thus treated. This justified, in the second and third isolation attempts, our spraying the control leaves in the two-choice assay with methanol only. Nevertheless, females significantly preferred the latter. This proves that the glucosinolates applied only on the treated leaves, albeit in reduced amounts, cannot account for the preference for the control leaves.

The HPLC procedure used was identical to that described by Blaakmeer et al. (1994) for the separation of the crude egg wash. The HPLC fractions of the crude surface leaf dip that contained the activity were compared with the corresponding fractions of an egg wash. The latter was found to contain only the three miriamides as active compounds.

In the bioassay used, the reaction to the egg batches was studied only in those leaves that actually had carried eggs, not in other leaves of the same plant. However, when a more sensitive bioassay was used, in which individual females were followed, other leaves of the same plant were found to become less acceptable than control leaves from a plant that never received any eggs (Van Loon, unpublished observations).

A hypersensitivity reaction to eggs is found for other *Brassica* species (Shapiro and DeVay, 1987). Some individual plants of *B. nigra* produce a necrotic zone at the base of freshly laid eggs of *P. rapae* and *P. napi*, thereby desiccating them.

In contrast to what we suggested previously (Blaakmeer et al., 1994), the ecological function of the miriamides on the egg surface of *P. brassicae* eggs remains unclear. The avenanthramides, compounds related to miriamides, isolated from oat groats and hulls (Collins, 1989) and from infected carnation stems (Niemann et al., 1992; Niemann, 1993), have strong antifungal activity. Miriamides could possibly protect the eggs of *P. brassicae* against various fungal diseases or against certain predators. The egg-induced changes in leaf surface

chemistry documented here are, to our knowledge, the first example of a plant response to an insect product and a subsequent effect on insect behavior without prior injury being inflicted to the plant.

In conclusion, the three miriamides, isolated from the eggs of *P. brassicae* (Blaakmeer et al., 1994) are not responsible for the oviposition-deterring effect of leaves that carry egg batches. Instead, evidence was obtained that the leaves react to contact with eggs or to compounds emanating from the eggs, which then act as elicitors. The elicitors of insect origin and the mechanism via which they operate to cause chemical changes in the plant surface will be subject to future studies.

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THE SCARAB BEETLE *Anomala albopilosa sakishimana* UTILIZES THE SAME SEX PHEROMONE BLEND AS A CLOSELY RELATED AND GEOGRAPHICALLY ISOLATED SPECIES, *Anomala cuprea*

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Abstract—Two components were identified in the sex pheromone system of the green chafer, *Anomala albopilosa sakishimana* Nomura: (*R*,*Z*)-5-(−)-(oct-1-enyl)oxacyclopentan-2-one (buibuilactone) and (*R*,*Z*)-5-(−)-(dec-1-enyl)oxacyclopentan-2-one (japonilure), which have been previously identified as sex pheromone constituents of *A. cuprea* and *A. octiescostata*. A female-specific minor component, (*R*,*E*)-5-(−)-(oct-1-enyl)oxacyclopentan-2-one, did not seem to be involved in pheromonal communication because it was not EAD active, but its role remained unclear. A synthetic blend of the two components captured significantly more beetles than any other treatments. Nevertheless, the fact that both the synthetic sex pheromone and field-captured female beetles were weak lures convinced us that the sex pheromone system may be only part of a complex communication system, probably involving plant volatiles. Although the sex pheromone was released during both the scotophase and photophase, there was an increase of 60% in the photophase.

Key Words—Coleoptera, Scarabaeidae, (*R*,*Z*)-5-(−)-oct-1-enyl)oxacyclopentan-2-one, (*R*,*Z*)-5-(−)-(dec-1-enyl)oxacyclopentan-2-one, (*R*,*E*)-5-(−)-(oct-1-enyl)oxacyclopentan-2-one, *Anomala albopilosa sakishimana*, *Anomala octiescostata*, *Anomala cuprea*, pheromone release, GC-EAD.

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INTRODUCTION

In spite of the fact that many scarab beetle species (Coleoptera: Scarabaeidae) are economically important pests throughout the world—causing damage to crops, pastures, turfgrass, trees, and fruits—and that alternative methods of control are highly sought after, studies designed to identify sex pheromones and evaluate their potential in scarab pest management are still rather rare. One of us (W.S.L.) has initiated a comprehensive cooperative project to investigate the possibility of applying semiochemicals in the integrated pest management (IPM) of various scarab beetles. As a result, the sex pheromones of some economically important pests in Japan have recently been identified (Leal et al., 1993a, and references therein).

Although these studies revealed a remarkable chemical diversity in the semiochemicals, they also demonstrated that some species utilize pheromones produced by other species as constituents of their own blend (Leal et al., 1993a,b). Recently, *Anomala octiescostata* Burmeister was found to utilize the same sex pheromone constituents as *A. cuprea* Hope, but the two species are seasonally isolated (Leal et al., 1993c). We therefore became interested in getting a better insight into the communication channel of closely related, but geographically isolated species. In this regard, the green chafer, *Anomala albopilosa sakishimana* Nomura, was an interesting case study because it is an agricultural pest—attacking mainly sugarcane—native to Miyakojima Island and is taxonomically very close to the most important scarab species in Japan, *A. cuprea*, whose sex pheromone has been identified already (Leal et al., 1993a). The presence of the latter species has not been recorded on that island but has been reported in many parts of the main island (Honshu) of the Japanese archipelago. We report here that *A. albopilosa sakishimana* utilizes the same sex pheromone system as *A. cuprea* and that this attractant seems to be only part of a more complex aggregation system.

METHODS AND MATERIALS

Gas Chromatographic (GC) and Mass Spectral (MS) Analyses. GC analyses were performed on a Hewlett-Packard 5890 equipped with a DB-wax column (30 m × 0.25 mm; 0.25 μm), operated in splitless mode at 50°C for 1 min, programmed at 4°C/min, held at this temperature for 1 min, programmed again at 10°C/min to 230°C and held at this temperature for 20 min [i.e., 50(1)–180(1)/4–230(20)/10]. Mass spectra were recorded on a Hewlett-Packard 5891 mass selective detector using either an HP-1 column (12 m × 0.2 mm; 0.33 μm) operated at 100(1)–240(10)/5 or a DB-wax column with the same conditions as for GC. Enantiomeric resolution of the lactones was achieved on a Chiraldex GTA column (20 m × 0.25 mm; 0.125 mm, Astec reactivated by Tokyo Kasei),

operated in split mode at 130°C, using helium as carrier gas (2 kg/cm^2 head pressure, flow rate of 4.4 ml/min). Both internal and external standards were used for quantitative analyses.

Gas Chromatography–Electroantennographic Detector (GC-EAD). The responses of *A. albopilosa sakishimana* antennae were recorded with a GC-EAD system as previously described (Leal et al., 1992a). The effluent from the capillary column was diluted with helium makeup gas (30 ml/min) into megabore tubing (0.15 m × 0.53 mm) and then split by a glass connector to a flame-ionization detector (FID) and the biodetector, EAD.

Insects. Beetles were collected in a field on Miyakojima Island at the beginning of the flight season (May 18–19, 1993). Since the collected females did not lay fertilized eggs, they were assumed to be unmated. Insects were segregated by sex, before shipment to the Tsukuba laboratory for chemical analysis. There, they were kept on grape leaves or artificial diet for the wild silk moth (Nihon Chlorella Co.) at 25°C, 70% relative humidity, and 14L:10D photoperiod.

Aeration. The airborne volatile of either eight male or 13 female beetles fed on grape leaves, *Vitis vinifera* L., were collected according to a previously reported method (Leal et al., 1992a). The amounts of pheromone released during scotophase and photophase were determined by collecting the volatiles from the same group of 12 female beetles fed on grape leaves for 10 hr in each phase, starting from the scotophase.

Isolation of Pheromone. Headspace volatiles collected from female beetles fed on grape leaves for one day were separated on a silica gel column (Wako C-200) by successive elution with hexane–ether mixtures, 100:0, 95:5, 90:10, 80:20, 50:50, and 0:100 (2 ml for each fraction).

Synthesis. (*R,Z*)-5-(−)-(Oct-1-enyl)oxacyclopentan-2-one was synthesized as previously reported (Leal, 1991). (*R,Z*)-5-(−)-(Dec-1-enyl)oxacyclopentan-2-one was prepared starting from protected D-ribose (Koseki et al., 1993). (*S,Z*)-5-(+)-(Oct-1-enyl)oxacyclopentan-2-one and (*S,Z*)-5-(+)-(dec-1-enyl)oxacyclopentan-2-one were synthesized by the method of Doolittle et al. (1980), starting from (*S*)-(+)-5-oxo-2-tetrahydrofurancarboxylic acid (Aldrich Chemical Company, Inc.). The optical purity of all synthetic pheromones was determined to be >97% ee by chiral chromatography (Leal, 1991).

Field Evaluation. Synthetic sex pheromone was evaluated in a sugarcane field at the Okinawa Prefectural Experiment Station, Miyako Branch, during the summer of 1993. Funnel traps (Japan Tobacco Inc.) were baited with synthetic sex pheromone incorporated either into pellets (4–5 mm in diameter) made of a polyethylene–vinyl acetate copolymer or in a device made of ES fiber (Chisso Co. Ltd.) and covered with a polyethylene film. The experiments were done in randomized blocks with three spatial and 12 temporal replications. Traps with an intertrap distance of 10 m were suspended with the pheromone dispenser

at either 1.5 or 0.9 m above the ground. Capture data were transformed to $\log(x + 1)$ and the differences between means were tested for significance by ANOVA using JMP software (Version 2) (SAS Institute, 1992). In this paper, treatments followed by the same letters are not significantly different at a 5% level in the Tukey-Kramer honestly significant difference test.

RESULTS AND DISCUSSION

Identification of Pheromone. GC-EAD analyses of female volatiles showed the occurrence of some EAD-active peaks (Figure 1), which were either volatiles derived from the foodstuff (R_t 7.21 and 9.72 min) or female-released semiochemicals (R_t 38.49 and 41.67 min). Volatiles collected from the headspace of male beetles under the same conditions gave the two EAD-active peaks of shorter retention times; however, the peaks at R_t 38.49 and 41.67 min were not detected. By column chromatography, the two female-specific, EAD-active chemicals were recovered in the ether fraction and they appeared on an HP-1 column at R_t 11.03 and 15.19 min. Their mass spectra were identical to those of (*R*,*Z*)-5-(−)-(oct-1-enyl)oxacyclopentan-2-one [(*R*,*Z*)-5-dodecen-4-olide, common name: buibuilactone] and (*R*,*Z*)-5-(−)-(dec-1-enyl)oxacyclopentan-2-one [(*R*,*Z*)-5-tetradecen-4-olide, common name: japonilure], previously identified as sex pheromone constituents of other scarab beetle (Leal et al., 1993a). Authentic buibuilactone and japonilure coeluted with the natural products and they were also EAD active.

The stereochemistry of the two components was investigated by a chiral column (Leal, 1991). (*R*)-Buibuilactone appeared at R_t 19.25 min (k' = 17.24)

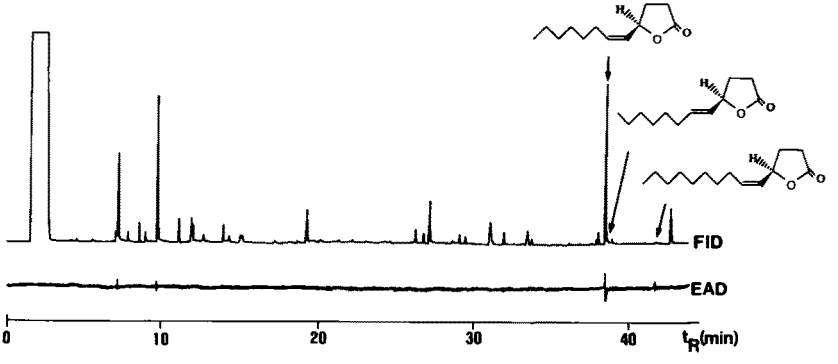


FIG. 1. Coupled FID and male EAD chromatograms of the airborne volatiles (0.01 female-equivalent per day) collected from female *A. albopilosa sakishimana* fed on grape leaves, using a DB-wax capillary column.

and the *S* enantiomer at 21.25 min ($k' = 19.24$), $\alpha = 1.12$). At 120°C, (*R*)- and (*S*)-buibuilactone showed the following retention times: 33.96 and 40.09 min, respectively. Since the major component of the sex pheromone gave the same R_t as synthetic (*R*)-buibuilactone, no trace of its enantiomer was detected, and the natural product coeluted with the *R* enantiomer, it was fully identified as (*R*,*Z*)-5-(−)-(oct-1-enyl)oxacyclopentan-2-one.

Enantiomeric resolution of japonilure was also achieved on the chiral column under the same conditions: (*R*,*Z*)-5-(−)-(dec-1-enyl)oxacyclopentan-2-one, R_t 55.67 ($k' = 51.76$) and (*S*,*Z*)-5-(+)-(dec-1-enyl)oxacyclopentan-2-one, R_t 59.88 min ($k' = 55.76$, $\alpha = 1.08$). Nevertheless, it was not possible to determine the absolute configuration of the natural product isolated from a pooled sample, due to the small amount of the minor component.

A minor component appeared at R_t 38.61 min on the polar column (R_t 11.19 min on HP-1) and gave a MS similar to the one of the major component. It was identified as the geometric isomer of the major component, i.e., (*R*,*E*)-5-(−)-(oct-1-enyl)oxacyclopentan-2-one, which is also obtained as a by-product (ca. 5%) in the synthesis of (*R*,*Z*)-5-(−)-(oct-1-enyl)oxacyclopentan-2-one. Although the chirality of the natural product could not be determined, it was assumed to be the same as in the major component. Whether this isomer plays any pheromonal role is yet to be determined; however, neither the natural nor the synthetic chemical gave positive EAD responses, even when their chromatographic resolution was improved by operating the column at 50(1)–180(1)/4–210(20)/10. (*R*,*E*)-5-(−)-(Oct-1-enyl)oxacyclopentan-2-one was also found in the volatiles collected from *A. cuprea* virgin females (Leal et al., 1993a, Figure 1).

Sex Pheromone Release. The amount of the two EAD-active, female-specific chemicals released by female beetles fed on grape leaves in a preliminary experiment was ca. 60% higher during the photophase (buibuilactone, 1300 ng/beetle; japonilure, 43 ng/beetle) than during the scotophase (buibuilactone, 790 ng/beetle; japonilure, 28 ng/beetle). The major/minor ratio differed slightly between the two phases (scotophase 28:1; photophase 30:1), probably due to experimental error (especially in the measurement of the minor component).

This pattern of release is close to the one observed for a related species, *A. cuprea*, which releases nearly the same amount of pheromone in the two phases, and is remarkably different from the one of the large black chafer, *Holotrichia parallela* (Motschulsky), whose amount of the major sex pheromone (L-isoleucine methyl ester) increased more than three times from the photo- to the scotophase (Leal et al., 1993d). Little is known about the ecology of the green chafer, but the fact that the beetle is highly attracted to light has led to the belief that it is active only at night. Studies on the behavior of the beetle in the field are needed, but on the basis of pheromone release, it appears likely that it is active during both phases.

Evaluation of Synthetic Sex Pheromone. Field experiments were carried out for 10 days (June 4–13, 1993) to determine the response of *A. albopilosa sakishimana* to traps baited with 3 mg of the following chemicals: major, (*R*,*Z*)-5-(−)-(oct-1-enyl)oxacyclopentan-2-one only; minor, (*R*,*Z*)-5-(−)-(dec-1-enyl)oxacyclopentan-2-one only; major + minor (9:1); and minor + major (9:1).

Traps baited with buibuilactone and japonilure (9:1) captured significantly more male beetles than the other baits (Figure 2). No beetles were captured in the control or the trap baited with the minor component only. Beetles were captured with the major component only and the major + minor (1:9) baits, but these catches were not significantly different from the control. Therefore, buibuilactone and japonilure are constituents of the sex pheromone system of the green chafer. On the other hand, catches with light traps in the same area were 5–10 times greater than those in traps baited with the binary pheromone system.

That the proportion of the minor component was higher in the synthetic than in the natural blend may have accounted only for a slight decrease in attractiveness. As previously reported (Leal et al., 1993a,c) the ratio of two components in scarab beetle sex pheromones is not as critical as it is in moth pheromones. This hypothesis must await future field experiments.

As the chirality of the minor component was not analytically determined, the effect of the stereochemistry of the minor component was investigated by evaluating catches by traps baited with (*R*)-buibuilactone + (*S*)-japonilure (9:1, 3 mg), in which however, no beetles were captured. The weak response of

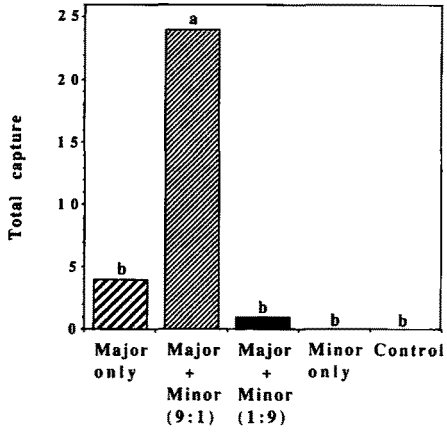


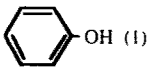
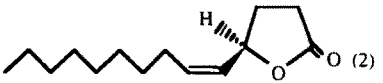
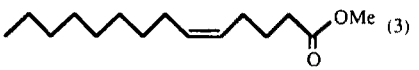
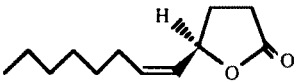
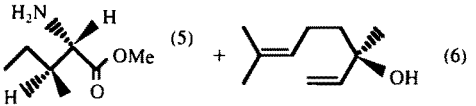
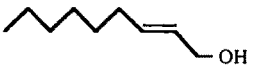
FIG. 2. Response of males *A. albopilosa sakishimana* to traps baited with one or two components of the sex pheromone system. Major: (*R*,*Z*)-5-(−)-(oct-1-enyl)oxacyclopentan-2-one; minor: (*R*,*Z*)-5-(−)-(dec-1-enyl)oxacyclopentan-2-one.

A. albopilosa sakishimana to the synthetic sex pheromone may be due to missing minor component(s), but the fact that in an experiment carried out from July 12 to August 3, 1993, no beetles were captured in traps baited with field-captured female beetles without foodstuff convinced us that the sex pheromone plays only a part in the aggregation behavior of this species. Recently, it has been demonstrated that the response of *Maladera matrida* Argaman, an exotic species in Israel, to the sex pheromone can be dramatically increased by the presence of plant volatiles. Live female beetles or volatiles collected from them in combination with food (cut peanuts leaves) attracted more males than females (in a 3:1.5–2 ratio), whereas attraction to female-released chemicals alone was weak (Yarden and Shani, 1993). It has been demonstrated that aggregation of the green June beetle is mediated by a sex pheromone (Domek and Johnson, 1987) as well as by semiochemicals released by feeding of both sexes (Domek and Johnson, 1988). Interestingly, the green chafer responded to two green volatiles emitted by grape leaves: 2-(*E*)-hexenal, R_t 7.26 min, and 3-(*Z*)-hexenyl acetate, R_t 9.74 min (Figure 1). Clear EAD responses were also obtained for these and other synthetic green volatiles: 2-(*E*)-, 2-(*Z*)-, 3-(*E*)-, and 3-(*Z*)-hexenol. Whether green volatiles could enhance the response of *A. albopilosa sakishimana* to its synthetic sex pheromone will be investigated during the next season(s).

It has been suggested that chemical cues are probably involved in the aggregation of *H. serrata* (Fabricius), but these are probably produced by both sexes, as female-released sex pheromone alone would not explain the formation of pure male clumps (as opposed to male–female clumps), accounting for 10% of two beetles clumps recorded in a field observation in Bangalore, India (Ganeshiah and Kumar, 1993). It has also been reported that both sexes of the Japanese beetle, *Popillia japonica* Newman (tethered on a leaf), attracted males in the field, resulting in their accumulation. The same attraction was observed with ether extracts of the body surface of both sexes, suggesting that an airborne substance from the former occupants is responsible for the participation of males in the formation of an aggregation (Iwabuchi and Takahashi, 1983). In addition, it has been observed in field experiments with the sex pheromone of the Oriental beetle, *Exomala orientalis* (Waterhouse), that ca. 20% of the beetles captured were female beetles (Leal, 1993; Leal et al., 1993e). This has been attributed to female attraction to male-released semiochemical(s) (Sawada et al., unpublished). Therefore, it seems that female-released sex pheromones are only part of a more complex communication system in scarab beetles. Nevertheless, the role of other semiochemicals may be masked in species that utilize sex pheromones highly attractive to conspecific males.

Species Isolation and Pheromone Chemistry. Surprisingly, *A. albopilosa sakishimana* utilizes the same pheromone system as *A. cuprea*, a closely related species. So far, three species of the genus *Anomala* have been found to have

TABLE 1. SEX PHEROMES OF SCARAB BEETLES

Beetle	Structure	Reference
Costelytra zealandica	 (1)	Henzell and Lowe (1970)
Popillia japonica	 (2)	Tumlinson et al. (1977)
Anomala rufocuprea	 (3)	Tamaki et al. (1985)
Anomala cuprea	 (4) + 2	Leal (1991); Leal et al. (1993a)
Holotrichia parallela	 (5) + (6)	Leal et al. (1992c; 1993d)
Anomala schonfeldti	 (7)	Leal et al. (1992b)
Anomala daimiana	7 + 4	Leal et al. (1993b)
Exomala orientalis	 (8)	Leal (1993)
Anomala octiescostata	4 + 2	Leal et al. (1993c)

buibuilactone and japonilure as sex pheromone constituents (Table 1). *A. cuprea* and *A. octiescostata* are seasonally isolated; the former is active in summer, whereas the flight activity of the latter occurs mainly in early spring (Leal et al., 1993c). On the other hand, *A. albopilosa sakishimana* is geographically isolated from the other two species. Therefore, there was probably no selective pressure for the evolution of sex pheromone systems with different constituents. Field experiments carried out in various regions in Hungary revealed that two European species, *A. vitis* Fabricius and *A. dubia* Scopoli utilize 2-(*E*)-nonenol, the sex pheromone of a Japanese species, *A. schonfeldti* Ohaus (Table 1), at least as one component of their sex pheromone systems (Miklós and Leal, unpublished). It has been also observed that the Japanese beetle is attracted to and attempts to mate with a large brown scarab, *Pelidnota* sp., native to the United States, and this pheromonal mix-up has been attributed to the fact that *P. japonica* is an exotic species in the United States and there was never selection for divergent pheromones (D. Whitman, personal communication).

Although only a few sex pheromone constituents of scarab beetles have been identified thus far, the present scenario is that species of the same genus utilize biosynthetically related, if not the same, sex pheromones. This is the case of *Anomala* spp., whose sex pheromone constituents are likely derived from fatty acids. The same is true for the Japanese beetle of the same subfamily Rutelinae, but not for the Oriental beetle, *Exomala orientalis* (Table 1). Biosynthesis of (Z)-tetradec-7-en-2-one is unlikely related to the fatty acid-derived pheromones. Melolonthinae species seem to have an even more diverse chemistry of pheromone, as highlighted by the two species identified having phenolic [*C. zealandica* (White)], amino acid derivative, and terpenoid chemicals (*H. parallela*).

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CUTICULAR HYDROCARBONS OF THE PAPER WASP, *Polistes fuscatus*: A SEARCH FOR RECOGNITION PHEROMONES

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Abstract—The cuticular chemicals of 124 individual wasps (foundresses and workers) from 23 colonies of *Polistes fuscatus* were analyzed. The compounds identified, all of which were hydrocarbons, were similar to those of other vespid wasps in that the bulk of the hydrocarbons were 23–33 carbons in chain length. However, the hydrocarbon profile of *P. fuscatus* differed from those of its congeners in its proportions of straight-chain alkanes, methylalkanes, and alkenes. Three of the 20 identified hydrocarbons, 13- and 15-MeC_{31}, 11,15- and 13,17-diMeC_{31}, and 13-, 15-, and 17-MeC_{33}, had properties postulated for recognition pheromones: colony specificity, efficacy in assigning wasps to the appropriate colony, heritability, lack of differences between foundresses and workers, and distinctive stereochemistry.

Key Words—*Polistes fuscatus*, Hymenoptera, Vespidae, cuticular hydrocarbons, discriminant function analysis, nestmate recognition, social wasps.

INTRODUCTION

The ability to recognize relatives has been documented in a variety of animals, including social insects (Fletcher and Michener, 1987; Hepper, 1991). In insects, chemoreception is the only sensory channel known to mediate the recognition

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of kin (Breed and Bennett, 1987; Smith and Michener, 1987). It is thought that insects detect the cuticular chemicals of kin by contact or close approach (Hölldobler and Michener, 1980).

Behavioral studies of the paper wasp, *Polistes fuscatus*, have established that nestmate (kin) recognition is based on chemoreception. Specifically, laboratory studies have confirmed that nestmate recognition pheromones are learned (and can be acquired) from the comb within several hours after wasps emerge from their cells (Pfennig et al., 1983; Gamboa et al., 1986). Espelie and Hermann (1990) and Espelie et al. (1990) reported that for *P. annularis* and *P. metricus*, the mixture of hydrocarbons on the comb is the same as that found on individual wasps from the colony. A recent chemical and behavioral study of the paper wasp, *P. metricus*, demonstrated that nestmate recognition pheromones are, indeed, cuticular hydrocarbons (Singer and Espelie, 1992). Nevertheless, the specific identities of those hydrocarbons that serve as recognition pheromones are unknown for any insect (Gamboa, 1994).

We examined the cuticular hydrocarbons of *P. fuscatus* because its kin recognition system is the most studied of any wasp, and one of the best understood of any animal (Gamboa, 1994). We wanted to know which cuticular hydrocarbons were most likely to be recognition pheromones. We reasoned that recognition pheromones should have the following properties: (1) they should be the most colony-specific hydrocarbons as well as the most efficacious hydrocarbons for assigning wasps to their appropriate colony; (2) they should be heritable, since behavioral studies have documented that recognition pheromones of *P. fuscatus* have a genetic component (Gamboa et al., 1987; Gamboa, 1988; Bura and Gamboa, 1994); (3) they should not differ markedly between the foundresses and workers of a colony because recognition pheromones proclaim colony identity; and (4) they would be compounds that have a distinctive stereochemistry, which may make them easier to distinguish than other cuticular hydrocarbons (Butts et al., 1993).

In terms of numbers of wasps and colonies, our study is the most extensive analysis of cuticular hydrocarbons for any social wasp.

METHODS AND MATERIALS

In September 1991, we marked 712 gynes (potential queens) of *P. fuscatus* (F.) from 20 colonies nesting in plywood nestboxes at two sites. Twelve colonies were on the natural campus of Oakland University in Rochester, Michigan, and eight colonies were at a site 30 miles north of Rochester near Metamora, Michigan. Gynes were marked with colony-specific, thoracic paint marks. After these females had overwintered and initiated colonies the following May, we surveyed nestboxes at our field sites and recorded 60 colonies, 20 of which had marked

sister colonies. Sister colonies were those colonies whose queens had the same thoracic mark, i.e., the queens were former nestmates. We began to mark workers with wing marks unique to their colony on June 26.

Between August 8 and 10, 1992, we collected 124 wasps from 23 colonies, of which 15, representing five different sister groups, had known sister colonies. The collection protocol consisted of placing a nestbox into an ice chest for approximately 40 min or until wasps became inactive. We then used tweezers to place each wasp into an individual 4-ml glass vial, labeled the vial, and placed it in another ice chest. In order to prevent the transfer of cuticular hydrocarbons among wasps, we rinsed tweezers with clean, 99.9% assay grade hexane after each use. Vials containing wasps were transferred directly from ice chests into a −20°C freezer on the day of collection. On August 20, the vials containing wasps were shipped air express on Dry Ice to the University of Georgia for hydrocarbon analysis. Wasps were kept frozen in vials at the University of Georgia until their cuticular hydrocarbons were extracted.

Hydrocarbon Analyses. Wasps were extracted individually in redistilled hexane for 60 sec at room temperature. Extracts were taken to dryness under a stream of nitrogen. Samples were dissolved in hexane and aliquots (5%) were analyzed on a Hewlett Packard 5890A gas chromatograph with a 25-m cross-linked methyl silicone Hewlett Packard HP-1 capillary column with helium as the carrier gas. The column was held at 55°C for 3 min after injection (splitless), and then the temperature was increased at a rate of 15°C/min until it reached 305°C where it was held for 30 min. The column was connected to a Hewlett Packard 5970 mass spectrometer, and mass spectra were taken every 0.8 sec at 70 eV. Components were characterized by determination of their equivalent chain lengths and analysis of their mass spectra (Nelson, 1978; Blomquist et al., 1987). Quantitation was based upon integration of total ion chromatograms (Espelie and Bernays, 1989).

Statistical Analyses. Univariate (ANOVA) analyses using individual wasps' percent signal for each of the 20 identified hydrocarbons were conducted using the SPSS^x statistical system (SPSS Inc., 1986). The significance of alpha for multiple ANOVA tests was adjusted using the sequential Bonferroni correction (Rice, 1989).

Stepwise discriminant function analysis (DFA) was performed on percent signal of hydrocarbons from individual wasps using the BMDP statistical package (Dixon, 1981). DFA determined which hydrocarbons were most important in distinguishing groups of wasps and the degree to which a multivariate model would correctly classify wasps into groups. DFA was conducted using two *a priori* classifications. The first analysis used colony as the grouping variable, and the second analysis pooled colonies headed by sister queens and used sister groups as the grouping variable. F values used to enter and remove variables as determined by the Bonferroni correction were 2.3 and 2.2, respectively.

Comparisons of cuticular hydrocarbons between foundresses and workers were made using two-tailed Wilcoxon matched-pairs signed-ranks tests. For each colony, we calculated a mean percent signal for foundresses and a corresponding mean value for workers for each identified cuticular hydrocarbon.

RESULTS

We identified 20 hydrocarbons in the hexane extracts of *Polistes fuscatus* (Table 1, Figure 1). These compounds, which ranged in mean relative abundance from 0.01 to 32.26%, comprised 94.38% of the extracted hydrocarbons. The compounds identified ranged in length from 21 carbons (n-C_{21}) to 33 carbons (11,15-diMeC_{33} and 13-, 15-, and 17-MeC_{33}). Eleven of the 20 compounds

TABLE 1. CUTICULAR HYDROCARBON IDENTIFICATION (WORKERS AND FOUNDRESSES COMBINED) OF THE PAPER WASP, *Polistes fuscatus* (N = 124 FEMALES FROM 23 COLONIES).[a]

TIC peak No.	ECL	Component
1	21.0	n-Heneicosane (n-C_{21})
2	22.0	n-Docosane (n-C_{22})
3	23.0	n-Tricosane (n-C_{23})
4	24.0	n-Tetracosane (n-C_{24})
5	25.0	n-Pentacosane (n-C_{25})
6	26.0	n-Hexacosane (n-C_{26})
7	27.0	n-Heptacosane (n-C_{27})
8	28.0	n-Octacosane (n-C_{28})
9	28.9	Nonacosene ($C_{29:1}$)
10	29.0	n-Nonacosane (n-C_{29})
11	29.2	13-Methylnonacosene (13-Me$C_{29:1}$)
12	29.3	11- and 13-Methylnonacosane (11- and 13-MeC_{29})
13	29.6	11,15-Dimethylnonacosane (11,15-diMeC_{29})
14	30.0	n-Triacontane (n-C_{30})
15	31.0	n-Hentriacontane (n-C_{31})
16	31.2	13-Methylhentriacontene (13-Me$C_{31:1}$)
17	31.3	13-* and 15-Methylhentriacontane (13-* and 15-MeC_{31})
18	31.6	11,15-* and 13,17-Dimethylhentriacontane (11,15-* and 13,17-diMeC_{31})
19	33.3	13-, 15-,* and 17-Methyltritriacontane (13-, 15-,* and 17-MeC_{33})
20	33.6	11,15-Dimethyltritriacontane (11,15-diMeC_{33})

[a]Total ion chromatogram (TIC) peak numbers correspond to those in Figure 1. ECL (equivalent chain length) is listed for each component. Listed components were identified by mass spectral analysis. An asterisk denotes the most prevalent positional isomer.

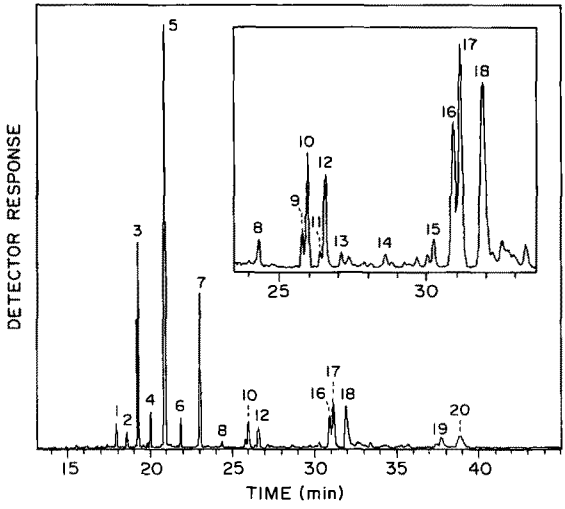

FIG. 1. Total ion chromatogram of the hexane extract of an individual female *Polistes fuscatus*. Numbered components were identified by their mass spectra and are listed in Table 1. Inset shows components with retention times between 24 and 33 min.

were straight-chain alkanes, six were methyl-branched alkanes, and three were alkenes. In terms of abundance, 67.09%, 23.32%, and 3.97% of the identified hydrocarbons were straight chain alkanes, methyl-branched alkanes, and alkenes, respectively. The three most abundant hydrocarbons, n-C_{25} (32.26%), n-C_{27} (13.46%) and n-C_{23} (12.14%), were straight-chain alkanes. Of the 20 hydrocarbons, nine were detected in all 124 wasps. The remaining 11 hydrocarbons were detected in 1.6–98.4% (i.e., 2–122) of the 124 wasps (Table 2).

Statistical Analyses. Based on univariate analyses (ANOVA), 13 cuticular hydrocarbons had statistically significant differences in percent signal among the 23 colonies (Table 3). The ranks of the univariate F statistics were not significantly correlated with the abundance of the compound ($r = -0.085$, $P >> 0.05$). The six compounds having the highest ranks for colony specificity based on univariate statistics were 13-, 15-, and 17-MeC_{33} (rank 1); 13- and 15-MeC_{31} (rank 2), $C_{29:1}$ (rank 3), n-C_{23} (rank 4), 13-$MeC_{31:1}$ (rank 5), and 11,15- and 13,17-diMeC_{31} (rank 6).

Both the colony- and sister group-based discriminant function analyses (DFA) gave canonical classification functions that included the same nine hydrocarbons: 13-, 15-, and 17-MeC_{33}; 13-$MeC_{31:1}$; 11,15- and 13,17-diMeC_{31}; n-C_{23}; n-C_{22}; n-C_{30}; $C_{29:1}$; n-C_{26}; and 11,15-diMeC_{33}. The functions had Wilk's lambda values of 0.00110 and 0.00465, which were both significant at $P = 0.0000$. The DFA produced combinations of variables similar to but slightly

TABLE 2. MEAN (±SE) AND RANGE OF PERCENT SIGNAL (PERCENTAGE OF TOTAL CUTICULAR HYDROCARBONS) FOR EACH COMPOUND IDENTIFIED IN CUTICULAR EXTRACTS OF THE PAPER WASP, *Polistes fuscatus* (N = 124 FEMALES FROM 23 COLONIES).

Compound	Percent signal (mean ± SE)	Range of % signal	Wasps with compound (%)
n-C_{21}	1.32 ± 0.09	0–6.86	96.8
n-C_{22}	0.52 ± 0.03	0–2.50	98.4
n-C_{23}	12.14 ± 0.46	2.62–26.78	100.0
n-C_{24}	1.59 ± 0.05	0.48–3.40	100.0
n-C_{25}	32.26 ± 0.60	17.64–53.63	100.0
n-C_{26}	1.84 ± 0.04	1.15–4.46	100.0
n-C_{27}	13.46 ± 0.27	12.93–13.99	100.0
n-C_{28}	0.41 ± 0.03	0–3.81	89.5
$C_{29:1}$	0.17 ± 0.03	0–1.86	30.6
n-C_{29}	2.76 ± 0.12	0.97–7.47	100.0
13-Me$C_{29:1}$	2.94 ± 0.10	1.07–6.22	100.0
11- and 13-MeC_{29}	1.07 ± 0.08	0–4.15	88.7
11,15-diMeC_{29}	0.01 ± 0.01	0–0.76	1.6
n-C_{30}	0.05 ± 0.01	0–1.35	18.5
n-C_{31}	0.74 ± 0.06	0–4.19	87.1
13-Me$C_{31:1}$	0.86 ± 0.11	0–6.17	57.3
13- and 15-MeC_{31}	7.34 ± 0.25	2.59–22.66	100.0
11,15- and 13,17-diMeC_{31}	8.87 ± 0.32	0.97–16.51	100.0
13-, 15-, and 17-MeC_{33}	1.52 ± 0.15	0–11.62	87.1
11,15-diMeC_{33}	4.51 ± 0.24	0–13.59	97.6
Total	94.38		

different from those shown to be significant with univariate F tests (Table 2). The differences were due to the structure of multiple correlations between variables. For example, both 13- and 15-MeC_{31} and 13-, 15-, and 17-MeC_{33} were highly significantly different among colonies in univariate F tests, but these two variables showed a 90% correlation. Thus, information for one compound accounted for 90% of the information for the other compound.

DFA using colony as the *a priori* grouping variable gave a 61.3% correct classification of wasps to colony. DFA using sister groups as the *a priori* grouping variable gave a 60.5% correct classification to sister group. A jackknifed classification (the group to be classified was not used to derive the classification function) yielded a 31.5% correct classification to colony and a 38.7% correct classification to sister group. Similar correct classification results were obtained when the DFA used both increased and decreased F values for stepwise selection of the canonical function.

TABLE 3. RANKINGS OF 20 HYDROCARBONS FOR COLONY SPECIFICITY BASED ON UNIVARIATE STATISTICS AND MULTIVARIATE STATISTICS (COLONY AND SISTER GROUP AS GROUPING VARIABLES)[a]

Compound	Univariate rank	Multivariate rank (colony)	Multivariate rank (sister-group)	Sum of ranks	Overall rank
n-C_{21}^{*}	9	20	6	35	12
n-C_{22}^{*}	8	9	8	25	7
n-C_{23}^{*}	4	7	3	14	4
n-C_{24}	15	16	17	48	19
n-C_{25}	14	14	14.5	42.5	13.5
n-C_{26}^{*}	13	19	13	45	17
n-C_{27}^{*}	12	8	9	29	8
n-C_{28}	17	11	14.5	42.5	13.5
$C_{29:1}^{*}$	3	15	4	22	5
n-C_{29}	16	5	12	33	10.5
13-$MeC_{29:1}^{*}$	11	18	16	45	17
11- and 13-MeC_{29}	7	10	7	24	6
11,15-diMeC_{29}	19	3	21	43	15
n-C_{30}	20	6	19	45	17
n-C_{31}	18	13	18	49	20
13-$MeC_{31:1}^{*}$	5	17	11	33	10.5
13- and 15-MeC_{31}^{*}	2	1	2	5	1
11,15- and 13,17-diMeC_{31}^{*}	6	2	5	13	3
13-, 15-, and 17-MeC_{33}^{*}	1	4	1	6	2
11,15-diMeC_{33}^{*}	10	12	10	32	9

[a]The overall rank is a composite rank that is based on the cumulative ranks of all three statistical analyses. An asterisk indicates that there were significant differences among colonies for this hydrocarbon based on analysis of variance (ANOVA).

The six compounds having the highest ranks based on DFA using colony as the *a priori* grouping variable were: 13- and 15-MeC_{31} (rank 1); 11,15- and 13,17-diMeC_{31} (rank 2); 11,15-diMeC_{29} (rank 3); 13-, 15-, and 17-MeC_{33} (rank 4); n-C_{29} (rank 5); and n-C_{30} (rank 6) (Table 2). The six compounds having the highest ranks based on DFA using sister group as the *a priori* grouping variable were: 13-, 15-, and 17-MeC_{33} (rank 1); 13- and 15-MeC_{31} (rank 2); n-C_{23} (rank 3), $C_{29:1}$ (rank 4); 11,15- and 13,17-diMeC_{31} (rank 5); and n-C_{21} (rank 6, Table 3).

DISCUSSION

The hydrocarbon profile of *P. fuscatus* is similar to profiles of other vespid wasps in that the bulk of the hydrocarbons are 23–33 carbons in chain length (Espelie and Hermann, 1990; Espelie et al., 1990; Bonavita-Cougourdan et al., 1991; Butts et al., 1991; Lorenzi, 1992; Singer et al., 1992). However, the hydrocarbon profile of *P. fuscatus* differs markedly in some respects from that of its cogeners, *P. metricus*, *P. annularis*, *P. exclamans*, and *P. dominulus*. In particular, the cuticular hydrocarbons of *P. fuscatus*, unlike those of the other wasps, are dominated by straight-chain alkanes. In fact, 67.09% of the cuticular hydrocarbons of *P. fuscatus* are straight chain alkanes as compared to 33.5–50.8%, 14.1%, 17.8–20.0%, and 22.2% for *P. metricus*, *P. annularis*, *P. exclamans*, and *P. dominulus*, respectively. *P. fuscatus* differs from *P. metricus*, *P. annularis*, *P. exclamans*, and *P. dominulus* in having a lower proportion of methylalkanes. For example, in *P. fuscatus*, 13.71% of its cuticular hydrocarbons are methylalkanes, while in *P. metricus*, *P. annularis*, *P. exclamans*, and *P. dominulus*, the percentages are 43.7–45.9%, 41.5%, 61.2–69.8%, and 77.0%, respectively (Espelie and Hermann, 1990; Espelie et al., 1990; Bonavita-Cougourdan et al., 1991; Singer et al., 1992). Finally, *P. fuscatus* has a higher percentage of alkenes (4%) than do *P. metricus* (0%), *P. annularis* (0%), *P. exclamans* (0.7%), and *P. dominulus* (0.3%).

Candidates for Recognition Pheromones. Based on the four criteria we advanced in our introduction, three cuticular hydrocarbons have the properties we postulated for recognition pheromones. These compounds (13- and 15-MeC_{31}, 11,15- and 13,17-diMeC_{31}, and 13-, 15-, and 17-MeC_{33}) are fairly colony specific and are efficacious for assigning wasps to their appropriate colony when compared to other identified hydrocarbons (Table 3). The three compounds also rank highly among identified hydrocarbons for assigning individual wasps to appropriate sister groups (Table 3). This latter finding suggests that these compounds are heritable, a property that has been demonstrated for recognition pheromones of *P. fuscatus* (Gamboa et al., 1987; Gamboa, 1988; Bura and Gamboa, 1994). In addition, these three hydrocarbons do not differ significantly between foundresses and workers, which is consistent with a recognition pheromone that proclaims colony identity (Table 4). Finally, the three compounds display a stereochemistry that may make them especially suitable as recognition pheromones. For example, it may be easier for a wasp to distinguish between hydrocarbons that have different branching methyl groups than between different *n*-alkanes that differ simply in chain length. Interestingly, methyl-branched hydrocarbons were among the most heavily weighted compounds in a discriminant analysis of the cuticular lipids of the baldfaced hornet, *Dolichovespula maculata* (Butts et al., 1993).

One might ask why the discriminant function analysis using colony as the

TABLE 4. MATCHED INTRACOLONY COMPARISONS OF CUTICULAR HYDROCARBONS (MEAN PERCENT ± SE) BETWEEN FOUNDRESSES AND WORKERS OF *Polistes fuscatus* (N = 19 COLONIES)[a]

Compound	Foundresses	Workers	P
n-C_{21}	0.98 ± 0.09	1.59 ± 0.18	0.003
n-C_{22}	0.38 ± 0.03	0.58 ± 0.07	0.003
n-C_{23}	13.94 ± 1.16	12.39 ± 1.11	NS
n-C_{24}	1.67 ± 0.16	1.59 ± 0.09	NS
n-C_{25}	29.65 ± 1.14	33.76 ± 1.03	NS
n-C_{26}	1.66 ± 0.07	1.95 ± 0.08	NS
n-C_{27}	13.93 ± 0.72	13.40 ± 0.49	NS
n-C_{28}	0.42 ± 0.04	0.42 ± 0.06	NS
$C_{29:1}$	0 ± 0	0.16 ± 0.04	0.003
n-C_{29}	3.45 ± 0.37	2.52 ± 0.19	NS
13-$MeC_{29:1}$	3.07 ± 0.25	2.76 ± 0.18	NS
11- and 13-MeC_{29}	1.17 ± 0.13	0.96 ± 0.11	NS
11,15-diMeC_{29}	0 ± 0	0.02 ± 0.01	NS
n-C_{30}	0.06 ± 0.02	0.06 ± 0.02	NS
n-C_{31}	1.01 ± 0.17	0.71 ± 0.12	NS
13-$MeC_{31:1}$	0.05 ± 0.02	1.00 ± 0.21	0.003
13- and 15-MeC_{31}	6.88 ± 0.37	6.88 ± 0.40	NS
11,15- and 13,17-diMeC_{31}	9.61 ± 0.77	8.31 ± 0.50	NS
13-, 15-, and 17-MeC_{33}	1.11 ± 0.14	1.35 ± 0.18	NS
11,15-diMeC_{33}	5.80 ± 0.66	3.89 ± 0.47	NS

[a] Significance levels have been adjusted for multiple comparisons using the sequential Bonferroni correction (Rice, 1989).

grouping variable gave only a 61.3% correct classification to colony. We believe that this classification efficiency is consistent with the fact that wasps from 15 of the 23 colonies had known sister colonies. Thus, many wasps in our sample were nonnestmate sisters, aunts, nieces, and first cousins. Gamboa (1988) and Bura and Gamboa (1994) reported that a significant number of nonnestmate sisters, aunts, nieces, and first cousins are treated like nestmates and admitted to a colony. Presumably this occurs because nestmates and nonnestmate kin share genetically specified recognition odors. It is also likely that some colonies not identified as sister colonies were actually related. For example, some colonies could have been headed by queens that were first cousins. This, in turn, might result in a decreased efficiency of assigning wasps to the appropriate sister group.

If the three compounds we have identified are, indeed, recognition pheromones, then it cannot be the presence or absence of a compound that provides a colony signature to individual wasps. Because all or nearly all wasps possessed

these three compounds, it is the relative amounts of the compounds that likely determine the colony specificity of recognition odors. Two of the three putative recognition pheromones were highly correlated with each other, and thus we may have tentatively identified only two independent recognition pheromones. Obviously, there may be other cuticular hydrocarbons, some of which we failed to identify, that may function as recognition pheromones.

We emphasize that our attempt to identify the recognition pheromones of *P. fuscatus* is preliminary. Further studies, including combination behavioral and chemical studies, are required to confirm the identity of recognition pheromones in *P. fuscatus*.

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MIMOSINE-INHIBITED SEED GERMINATION, SEEDLING GROWTH, AND ENZYMES OF *Oryza sativa* L.

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Abstract—The effect of mimosine (50 ppm and 100 ppm concentrations) on *Oryza sativa* (rice) seed germination; root and shoot growth, i.e., length and fresh weight of seedlings; activities of nitrate reductase, peroxidase, catalase, and IAA oxidase were investigated. Significant inhibition in seed germination and shoot length was noted. Root length was inhibited by 100 ppm mimosine; however, the 50 ppm was not significant. Root and shoot fresh weight was not significantly inhibited by the tested concentrations of mimosine. Significant inhibition in activities of nitrate reductase, peroxidase and its isoenzymes, catalase, and IAA oxidase was observed. Ecophysiological implications of mimosine phytotoxicity are discussed.

Key Words—Mimosine, *Oryza sativa*, nitrate reductase, peroxidase, isoenzymes, catalase, IAA oxidase, allelopathy.

INTRODUCTION

Mimosine is a nonprotein amino acid that occurs in leaves and seeds of *Leucaena* species and *Mimosa pudica* (Chou and Kuo, 1986; Smith and Fowden, 1966). Auxiliary roles of *Leucaena* species, particularly cultivars of *L. leucocephala* in agroforestry attracted global attention for their multiple uses (Budelman and Vandapol, 1992). Variations in *Leucaena* mimosine concentrations up to about 5% have been recorded in the literature. Phytotoxicity of mimosine has been reported (Chou and Kuo, 1986; Tawata and Hongo, 1987). Mimosine toxicity

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is attributed to presence of —OH in the three position and —O in the four position of the pyridine ring (Ward and Harris, 1976). It is known to inhibit iron-containing enzymes and competes with tyrosine (Vickery and Vickery, 1981).

The objectives of this study were: to investigate the effect of mimosine on *O. sativa* seed germination, root and shoot growth (length and fresh weight), the activity of iron-containing enzymes (nitrate reductase, peroxidase and its isoenzymes, catalase, and growth promoting IAA oxidase) to explain the physiological basis of phytotoxicity.

METHODS AND MATERIALS

O. sativa seeds (var. Hamsa) (25) were surface-sterilized with sodium hypochlorite (2.5%) for 15 min, soaked in distilled water for 12 hr, and sowed in polypropylene boxes. From days 1 to 6, the surface was wetted with 3–5 ml of 50 ppm and 100 ppm mimosine (Sigma Chemical Co.) in Hoagland's solution. Hoagland's solution was used as a control. On the seventh day, the root and shoot length, fresh weight, and percentage of germination were measured. Nitrate reductase, peroxidase, catalase, and IAA oxidase activities were assayed. Triplicates were maintained for each treatment. The data were subjected to Student's *t* test with the help of the Epistat ver. 3.0 statistical package.

Enzyme Preparation

Leaf/root tissue (1 g) was macerated in 5 ml of suitable medium (for nitrate reductase 1 mM EDTA, 25 mM cystein, and 25 mM potassium phosphate, adjusted to a final pH 8.8 with KOH; phosphate buffer of 100 mM, pH 7 for peroxidae, 50 mM, pH 7 for catalase, and 200 mM, pH 6 for IIA oxidase). Sand was used as an abrasive and homogenate was centrifuged at 20,000 rpm for 20 min in a Beckman J2-21 M/E centrifuge at 4°C. The supernatant was used as the enzyme source. Protein content was estimated by Lowry's method. Enzymes were assayed using a Shimadzu UV-VIS spectrophotometer (model UV-160A).

Enzyme Assays

Nitrate reductase. Its activity was assayed following Hageman and Reed (1980). The assay mixture contained potassium phosphate (50 μmol), potassium nitrate (20 μmol), NADH (0.25 μmol), and enzyme extract in a final volume of 2 ml. The reaction was initiated by addition of NADH. Omission of enzyme in the reaction mixture served as a control. The reaction mixture was incubated at 30°C for 30 min and was terminated by rapid addition of 1 ml of 1% sul-

fanilamide followed by 1 ml of 0.02% NED (*N*-1-naphthyl-ethylene diamine dihydrochloride) reagent. The color was allowed to develop for 30 min prior to reading at 540 nm.

Sodium nitrite concentrations ranging from 10–100 nmol were used as standards. To 2 ml of sample containing nitrite, 1 ml of 1% sulfanilamide and 1 ml of 0.02% NED were added, mixed well using a vortex mixer, and absorbance was recorded at 540 nm after incubation at room temperature.

Catalase. Its activity was monitored according to the method of Aebi (1975). The assay mixture contained enzyme extract, phosphate buffer (100 mM, pH 7), and H_2O_2 (10 mM) in a final volume of 1 ml. The assay mixture devoid of H_2O_2 served as a blank. The change in absorbance was recorded for 1 min after addition of H_2O_2. A change of 1 OD unit represents 25 μmol of H_2O_2 degraded per milliliter.

IAA Oxidase. Its activity was measured by the method of Talwar et al. (1985). The assay mixture contained 0.02 ml IAA (20 μg), enzyme extract (50 μg), 0.02 ml 2,4-dichlorophenol (0.1 mM), 0.02 ml $MnCl_2$ (0.1 mM), and 0.2 M potassium phosphate buffer (pH 6) in a final volume of 1 ml. Omission of IAA and enzyme extract from the assay mixture served as a blank. The reaction mixture was incubated in the dark at 30°C for 15 min and stopped by adding Salkowski reagent (2 ml) to each tube and again incubating in the dark at 30°C for 15 min. The color was read at 530 nm. Salkowski reagent was prepared by adding 1 ml freshly prepared $FeCl_3$ (8% w/v) to 50 ml 35% $HClO_4$. One unit of activity represents 1 μg of IAA oxidized/ml in 15 min.

Peroxidase. Its activity was assayed according to the method of Shinshi and Naguchi (1975). The reaction mixture consisted of 3 ml in 0.1 M phosphate buffer, pH 7 containing 0.2 mM guaiacol, 2 mM H_2O_2 and 5–25 μl enzyme in buffer. Addition of H_2O_2 starts the reaction, which is run at 25°C. Omission of H_2O_2 in the assay mixture served as a blank. One unit of peroxidase activity represents the change in absorbance of 1 per minute.

Detection of Isozymes of Peroxidase

Nondenaturing polyacrylamide gel electrophoresis was carried out in an alkaline buffer system (pH 8.3) in 7.5% polyacrylamide gel to visualize the isoenzyme pattern of peroxidase (Wetter and Dyck, 1983). Enzyme extract containing 100 units of peroxidase in 10% sucrose was loaded in each lane. Bromophenol blue was used as a tracking dye. Electrophoresis was done at 4°C at 15 mA for 30 min. and later 150 V for 8 hr. Peroxidase isoenzymes were developed following Reddy and Prasad (1992). Gels were rinsed with phosphate buffer and incubated for 10 min in freshly prepared solutions of 1% benzedine in 25% acetic acid and developed with 3% H_2O_2.

RESULTS

Seedling bioassay revealed that mimosine inhibited *Oryza sativa* seed germination significantly. Mimosine at 50 ppm concentration was noninhibitory to root length; however, shoot length inhibition was significant with the tested concentrations of mimosine (Table 1). Mimosine inhibition of fresh weight of root and shoot was not significant (Table 1). Mimosine inhibited significantly the activity of nitrate reductase in *O. sativa* seedling leaves (Table 2). Inhibition to the extent of 50–60% was observed over the control with 50 ppm and 100 ppm of mimosine. The peroxidase activity decreased with mimosine in both roots and leaves (Table 2). In leaves, 23–30% and in roots, 54–57% inhibition was observed with 50 and 100 ppm, respectively. Nondenaturing polyacrylamide gel electrophoresis of peroxidase showed five isoenzymes (A–E) in control leaves (Figure 1, lane 1). Mimosine, at 50 ppm concentration inhibited the synthesis of isoenzyme D (Figure 1, lane 2), and partially suppressed isoenzyme E (Figure 1, lane 2). Mimosine, at 100 ppm concentration totally inhibited the isoenzyme E (Figure 1, lane 3). In control roots, three isoenzymes (A, C, and E) appeared (Figure 1, lane 4). Mimosine treatment with 50 and 100 ppm concentrations completely inhibited isoenzyme E (Figure 1, lanes 5 and 6).

Significant inhibition of catalase activity in roots (36–67%) and leaves (43–61%) with 50 and 100 ppm of mimosine was observed (Table 2). IAA oxidase activity significantly decreased in leaves by 38–85%; however, there was a slight increase in roots (1.9% and 2.4%) (Table 2).

DISCUSSION

Nitrate reductase is a multicomponent enzyme containing flavin (FAD), heme (cyt b), and molybdenum. Mimosine, being a tyrosine analogue, might have been incorporated into polypeptide chains during protein synthesis. Hence, the altered physicochemical properties of the new protein might have inhibited the enzymatic activity. Another possibility is that mimosine is a metal-chelating agent and thus forms a complex with Mo^{2+} or Fe^{3+} in the cytochrome system, thereby inhibiting the activity of nitrate reductase.

Peroxidase and catalase are peroxide-scavenging enzymes widely distributed in living organisms and occur in the form of a number of isoenzymes (Machakova et al., 1975); all contain protohemin IX as a prosthetic group. The active site of the enzyme includes the apoprotein as well as the heme group (Weinryb, 1966). Reduction in the activity of peroxidase and catalase might be due to the chelation of the heme group present in the enzymes as mimosine is known to chelate metals, especially iron (Vickery and Vickery, 1981).

Reduction in the activities of peroxidase and catalase by mimosine treatment accumulates peroxides in the system. Peroxide accumulation changes plant

TABLE 1. MIMOSINE-INHIBITED SEED GERMINATION AND SEEDLING GROWTH IN *D. sativa*[a]

Mimosine treatment	Germination (%)	Length (cm)		Fresh weight (mg)	
		Root	Shoot	Root	Shoot
Control	96	6.2 ± 1	9.5 ± 0.8	366 ± 43	790 ± 15
50 ppm	92[ab]	5.9 ± 1c (−4)	8.5 ± 0.7e (−11)	355 ± 54c (−3)	618 ± 5c (−12)
100 ppm	88b	5.5 ± 1d (−11)	7.3 ± 0.4f (−23)	294 ± 33c (−20)	559 ± 76c (−20)

[a] Figures in parentheses indicate percentage of decrease (−) over control. Average of 25 seedlings
[b] *P* values significantly different from respective controls: a = 0.03, b = 0.0006, c = not significant, d = 0.04, e = 0.02, f = <10 (−6).

metabolism in several ways: i.e., oxidation of sulfhydryl groups (Stonier and Yang, 1973) and, in combination with superoxides, formation of hydroxyl radicals that are involved in senescence (Beauchany and Fridovich, 1979).

The inhibition of IAA oxidase might lead to the accumulation of IAA, an important growth regulator. It is clear that IAA is normally degraded by enzymes of the peroxidase type. Gelinas (1973) reported the peroxidase-catalyzed oxidation of IAA. Peroxidase activity was also reduced by mimosine; thus, there is no possibility of oxidation of IAA in the system. The possible mechanism of action of mimosine in inhibiting these enzymes might be due to the chelation of metals. The reduction in activities of these enzymes either alone or synergistically would have retarded the growth of *O. sativa* seedlings.

Smith and Fowden (1966) explained mimosine toxicity partly as complexation between mimosine and pyridoxal phosphate. Thus, complex formation would limit the amount of pyridoxal phosphate available for use as enzyme prosthetic groups; alternatively, complex formation may involve pyridoxal phosphate moieties already associated with holoenzymes. Both processes would lead to the decreased activities of pyridoxal phosphate transaminases. Mimosine-induced growth inhibition of mung bean seedlings was reversed by a simultaneous supply of pyridoxal phosphate and Fe^{3+} and suggested that supplementation of iron limits the degree of chelation in vital enzymes such as those in the cytochromes. Mimosine chelates several divalent metal ions, and supplementation of such ions exogenously resulted in restoration of mimosine toxicity (Lin and Ling, 1961). Chelation of iron by mimosine could be the possible mechanism for inhibiting the activities of nitrate reductase, peroxidase, and catalase activities since these contain iron.

Mimosine is one of the 10 allelochemicals identified in *Leucaena leuco-*

TABLE 2. MIMOSINE EFFECT ON NITRATE REDUCTASE, PEROXIDASE, CATALASE, AND IIA OXIDASE IN *D. Sativa* SEEDLINGS[a]

Mimosine treatment	Nitrate (nmol nitrite formed/g FW/min) reductase leaves	Peroxidase (units/mg protein/hr)		Catalase (μmol H_2O_2 degraded/mg protein/hr)		IIA Oxidase (units/mg protein/hr)	
		Roots	Leaves	Roots	Leaves	Roots	Leaves
Control	178 ± 5	1220 ± 35	180 ± 35	1253 ± 265	2700 ± 85	1396 ± 1	1450 ± 7
50 ppm	91 ± 14[b]	558 ± 89a	138 ± 14b	786 ± 11d	1530 ± 42e	1423 ± 20g	895 ± 33a
	(−49)	(−54)	(−23)	(−36)	(−43)	(+1.9)	(−38)
100 ppm	65 ± 17a	520 ± 70a	126 ± 6c	413 ± 11a	1050 ± 212f	1430 ± 45h	218 ± 56a
	(−63)	(−57)	(−30)	(−67)	(−61)	(+2.4)	(−85)

[a] Figures in parentheses indicate percentage of decrease (−)/ increase (+) over control. FW = fresh weight

[b] *P* values significantly different from respective controls: a = <10 (−6), b = 0.0001, c = 0.000002, d = 0.004, e = 0.0003, f = 0.00003, g = 0.0005, h = 0.008.

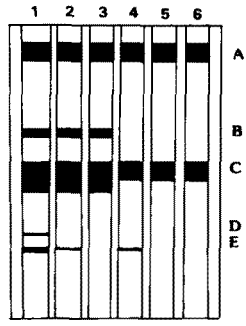

FIG. 1. Zymogram of nondenaturing polyacrylamide gel electrophoresis showing the mimosine-inhibited isozymes of peroxidase. Lanes 1 and 4 = control, 2 and 5 = treated with 50 ppm mimosine; 3 and 6 = treated with 100 ppm mimosine, Lanes 1–3 = leaves. Lane 1, control leaves show five isoenzymes (A–E). Lane 2, isoenzyme D was suppressed by mimosine (50 ppm) and isozyme E was partially inhibited. Lane 3, isozymes D and E were suppressed completely with mimosine 50 ppm mimosine and 100 ppm concentrations. Lanes 4–6 = roots. Lane 4 control roots show only three isoenzymes (A, C, and E). Note the disappearance of isoenzyme E with mimosine treatment (50 ppm and 100 ppm concentration) in lane 5 and lane 6, respectively.

cephala plant parts (Chou and Kuo, 1986). It is the most preferred perennial in tropical agroforestry to supplement essential nutrients such as nitrogen by regular pruning of foliage and leaf mulch application (Mittal et al., 1992). the leaf mulch of *L. leucocephala* has a weed control property, due to the presence of potential allelochemicals (Budelman, 1988). Therefore, plant, soil, and crop interactions need to be investigated critically for optimal utilization of the multiple-use *L. leucocephala* in agroecosystems in view of its toxic constituent viz. mimosine.

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KAIROMONE FROM DANDELION, *Taraxacum officinale*, ATTRACTANT FOR SCARAB BEETLE *Anomala octiescostata*

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Abstract—The attraction of the scarab beetle *Anomala octiescostata* to dandelion, *Taraxacum officinale*, was demonstrated to be chemically mediated by a mixture of *cis*-3-hexenyl acetate, benzaldehyde, phenylacetaldehyde, benzyl alcohol, phenethyl alcohol, phenylacetonitrile, and benzyl benzoate, in the ratio 4:8:14:3:5:19:11. Combination of the synthetic kairomone and sex pheromone (buibuilactone + japonilure, 8:2), significantly increased the total catches of *A. octiescostata*. Catches of male (but not female) beetles were significantly higher with the kairomone–pheromone blend than with kairomone alone. The synergistic effect of the kairomone from dandelion on the attractiveness did not significantly differ from that of a food-type lure, anethol, geraniol, and phenethyl propionate (9:0.5:0.5). The latter combined with the synthetic sex pheromone resulted in better attraction of female (but not male) *A. octiescostata* than the sex pheromone alone.

Key Words—*cis*-3-Hexenyl acetate, benzaldehyde, phenylacetaldehyde, benzyl alcohol, phenethyl alcohol, phenylacetonitrile, benzyl benzoate, anethol, geraniol, phenethyl propionate, kairomone, sex pheromone, dandelion, *Taraxacum officionale*, Coleoptera, Scarabidae, *Anomala ortiescostata*, scarab beetle.

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INTRODUCTION

Outbreaks of the scarab beetle *Anomala octiescostata* Burmeister (Coleoptera: Scarabaeidae) have been recently recorded in the Kanto area in Japan, with the population rapidly increasing in numbers and destructiveness. Although we do not have direct evidence to prove this hypothesis, it is likely that the beetle has invaded the new habitat because, before it was found in Chiba and Ibaraki prefectures, it was known to occur only in the south of Japan. Furthermore, the pattern of annual population increase is very similar to those of exotic scarab species, devoid of natural enemies, exploiting new habitats.

Since the beetle can not be controlled with insecticides currently registered for scarab beetles in Japan and because there is a trend to use alternative methods of control, especially for turf pests, a great deal of effort has been utilized looking for new strategies. One of the promising techniques is the use of the nematode, *Steinernema glaseri*, for grub control (S. Yamanaka, personal communication).

The high attraction of males to the synthetic sex pheromone of *A. octiescostata* (Leal et al., 1994a) motivated us to investigate the possibility of using sex pheromones in mass trapping as part of integrated pest management programs. In that respect, chemicals that could be used in combination with sex pheromones and that would result in improved response of female beetles are of utmost importance.

We observed that adult *A. octiescostata* are attracted to and voraciously feed on dandelion, *Taraxacum officinale* Weber (Japanese name: *seiyotanpopo*). As described here, this attraction is semiochemically mediated, and these semiochemicals in combination with the sex pheromone significantly increase the catches of female *A. octiescostata* in the field.

METHODS AND MATERIALS

Kairomone Extraction. Flowers of dandelion (10 g) were extracted with hexane (100 ml) for 5 min. This was filtered and concentrated to 1 flower-equivalent for bioassay; an aliquot was diluted to 0.1 flower-equivalent for GC-MS.

Field Evaluation. Attractiveness of candidate lures to adult *A. octiescostata* was evaluated in tests conducted on golf courses in Chiba (Chiba Springs Country Club) and Ibaraki (Edosaki Country Club) in the spring of 1993.

Dandelion extract was transferred to pieces of cotton, which were placed on sticky plates (30 × 30 cm). Baited and unbaited (control) traps were tested in randomized blocks on the ground. After 20 min, they were reloaded and rerandomized.

Synthetic sex pheromone was incorporated into pellets (4–5 mm in diam-

eter) made of a polyethylene–vinyl acetate copolymer. One pellet, having 1 mg of the sex pheromone, was placed inside a pellet holder (Fuji Flavor Co.) and placed 2 cm above the trap lip. Different devices were used for floral compounds; synthetic dandelion kairomone (500 mg) was loaded in hand-made glass vials (3 cm long; 0.5 ID) and allowed to evaporate from the open end of the vial, whereas the food-type lure anethol + geraniol + phenethyl propionate (500 mg) was incorporated into pellets. These devices were placed inside plastic bottles (Fuji Flavor Co.), which were then attached to funnel traps (Japan Tobacco Inc.) 7 cm above the trap lip. Each test was replicated in randomized blocks with traps spaced 10 m apart and 30 cm above the ground. Capture data were transformed to $\log(x + 1)$ and differences between means were tested for significance by ANOVA using JMP software (Version 2) (SAS Institute, 1989). In this paper, treatments followed by the same letters are not significantly different at a 5% level in the Tukey-Kramer honestly significant difference test (SAS Institute, 1989).

Gas Chromatography–Mass Spectrometry (GC-MS) Analyses. Mass spectra were recorded on a Hewlett-Packard 5891 mass selective detector equipped with a DB-wax column (30 m × 0.25 mm; 0.25 μm), operated in splitless mode at 50°C for 1 min, programmed at 4°C/min, held at this temperature for 1 min, programmed again at 10°C/min to 230°C and held at this temperature for 20 min.

Chemicals. (*R*,*Z*)-5-(−)-(Oct-1-enyl)oxacyclopentan-2-one was synthesized as previously reported (Leal, 1991) and (*R*,*Z*)-5-(−)-(dec-1-enyl)oxacyclopentan-2-one was prepared starting from protected D-ribose (Kosecki et al., 1993). The other chemicals were obtained from commercial sources; their purities were >95%, as revealed by GC-MS.

RESULTS AND DISCUSSION

During the flight activity of *A. octiescostata* (Leal et al., 1994a), we have observed that adults feed voraciously on flowers of dandelion, *T. officinale* (Figure 1). Examination on a sunny day of the sex of the feeding beetles revealed that the number of males was nearly equal to that of females. This observation was repeated three times: at 10:00 AM, 49 males and 48 females were counted on flowers, whereas these figures were 45 and 35 at noon, and 9 and 12 at 2:00 PM JST.

These yellow flowers are conspicuous against the green background of a golf course, and visual stimuli may play a part in the attraction. However, our data support that the attraction was mainly semiochemically mediated. Traps baited with flower extracts of dandelion were significantly more attractive than unbaited traps. Soon after the traps were placed on the ground, beetles started

FIG. 1. Clumps of the scarab beetle *A. octiescostata* feeding on flowers of the common weed dandelion.

to fly into the baited ones. In 2 min, 10 beetles/trap were captured in kairomone-baited traps, and catches increased to 36 beetles/trap in 10 min. Control traps captured 4 beetles/trap in the same period.

Flower extracts of dandelion were analyzed by GC-MS. Based mainly on their mass spectra, the major constituents were identified as the following chemicals (proportions in parentheses): *cis*-3-hexenyl acetate (4), benzaldehyde (8), phenylacetaldehyde (14), benzyl alcohol (3), phenethyl alcohol (5), phenylacetonitrile (19), and benzyl benzoate (11). Their identities were confirmed by comparing their retention times and mass spectra to those of authentic compounds. These chemicals comprise 78.6% of the extract; the other components are mainly saturated hydrocarbons and other minor components.

Attractiveness of a synthetic mixture of these chemicals in the natural proportion was tested in the field. This was compared with the major sex pheromone

of the scarab beetle, *Holotrichia parallela*, L-isoleucine methyl ester (LIME) (Leal et al., 1992), which has been demonstrated to be attractive for other flower-feeding scarab species, *Protaetia orientalis* (Leal et al., 1993). In addition, we compared the attraction to a mixture of anethol, geraniol, and phenethyl proporionate (A + G + PEP, 9:0.5:0.5), which has been found to be attractive to other scarab species (Ono, unpublished data). Traps baited with the candidate lures were buried in a fairway in Edosaki Country Club and captures were recorded on May 3–9, 1993.

Traps baited with the synthetic dandelion kairomone and the food-type lure, A + G + PEP, captured significantly more beetles than traps baited with LIME, but no significant difference was found in the catches between the two types of floral attractants (Figure 2).

The synthetic sex pheromone of *A. octiescostata*, buibuilactone and japonilure (8:2), has been demonstrated to be highly attractive in the field (Leal et al., 1994a). We investigated the effect of the combination of this synthetic sex pheromone with the kairomone from dandelion in field tests conducted in Chiba (May 9–13) and Ibaraki (May 1–6).

In Ibaraki, catches in traps baited with synthetic sex pheromone and kairomone (277 ± 71 beetles/trap) were significantly higher (×2.5) than those in traps baited with sex pheromone only (107 ± 22 beetles/trap). In Chiba, the captures were analyzed by sex and demonstrated that catches with sex pheromone + kairomone lure were significantly higher than captures with the kairomone alone only in relation to male beetles. There was no significant increase in captures of female beetles due to the addition of the pheromone (Figure 3).

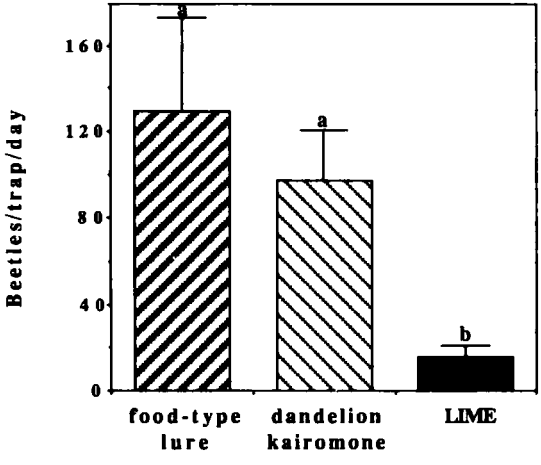

FIG. 2. Attractiveness of selected baits to the scarab beetle *A. octiescostata*.

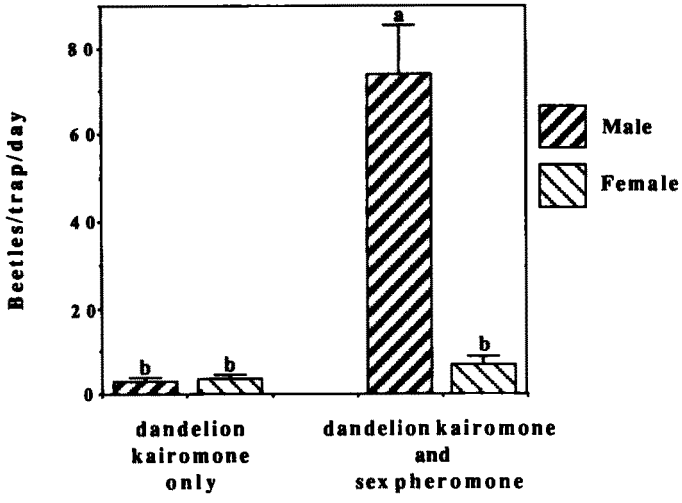

FIG. 3. Captures of *A. octiescostata* in traps baited with the synthetic kairomone from dandelion and the kairomone combined with the synthetic sex pheromone (buibuilactone and japonilure, 8:2).

The synergism of the food-type lure, A + G + PEP, was also investigated in Chiba (May 9–13) by comparing captures in traps baited with the sex pheromone only or sex pheromone in combination with floral lure. The addition of floral lure did not significantly increase the captures of male beetles, but significantly more female beetles (×4.3) were captured with sex pheromone in combination with floral lure (Figure 4). The fact that traps baited with sex pheromone only captured significantly more female beetles than control traps has already been discussed, and this has been attributed to the occurrence of male-released pheromone (Leal et al., 1994b).

Investigations of the response of the Japanese beetle, *Popillia japonica*, to sex pheromone and a food-type lure, phenethyl propionate + eugenol, revealed that throughout the season the combination of food-type lure and sex pheromone was a better lure than the two lures used independently. However, a decrease in attractiveness of the synthetic sex pheromone was detected after the period of heavy emergence (Klein et al., 1981). Our experiments were carried out at the end of the flight season of *A. octiescostata*, and therefore the effect of the kairomone to increase the attraction of the synthetic sex pheromone may be underestimated in our field data. Nevertheless, our findings demonstrated that floral lures in combination with synthetic sex pheromone provide a more effective lure for trapping *A. octiescostata*. This combined lure system may be val-

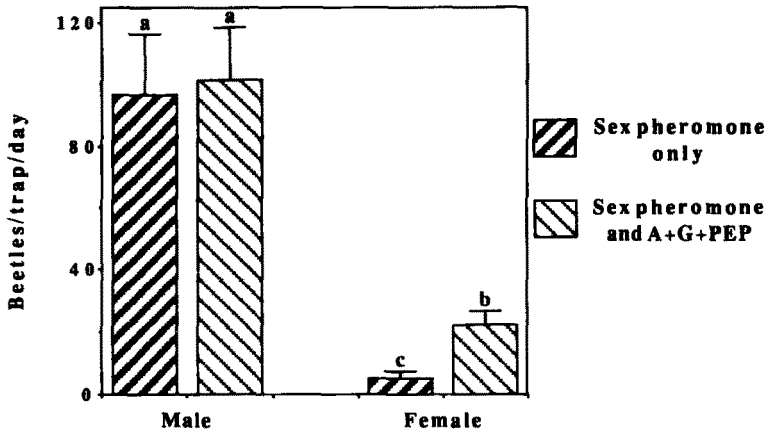

FIG. 4. Effect of the food-type lure anethol–geraniol–phenethyl propionate on the catches of male and female *A. octiescostata*.

uable in integrated pest management, and it will also pave the way for studies designed to get better insight in the chemical ecology of this scarab pest.

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