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SEX PHEROMONE OF ORIENTAL BEETLE, *Exomala orientalis*: IDENTIFICATION AND FIELD EVALUATION

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Abstract—A gas chromatograph coupled with a behavioral bioassay was used to identify two sex pheromone components, 7-(*Z*)- and 7-(*E*)-tetradecen-2-one of the Oriental beetle (OB), *Exomala orientalis*. Field experiments showed that the blend of the two isomers (*Z*:*E*, 7:1) was not significantly more attractive than the *Z* component alone. The best performance of traps baited with the synthetic sex pheromone was achieved when they were set with the pheromone device at 30 cm above the ground. Catches in traps baited with 1 and 10 mg were not significantly different, but they were higher (2.9-fold) than captures in traps loaded with 0.1 mg of the pheromone. Further investigations by GC-EAD revealed the presence of a possible minor component, but the small amount of material prevented its identification. 2-(*E*)-Nonenol, with the same retention time as the natural product, did not affect the attractancy of the synthetic sex pheromone. GC-EAD screening of previously identified sex pheromones of scarab beetles showed that male antennae of the Oriental beetle responded to japonilure, but it showed neither synergism nor inhibition to the OB sex pheromone.

Key Words—*Exomala orientalis, Blitopertha orientalis, Phyllopertha orientalis*, Coleoptera, Scarabaeidae, Oriental beetle, 7-tetradecen-2-one, 6-tetra-

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decen-2-one, 5-tetradecen-2-one, 2-(*E*)-nonenol, japonilure, GC-EAD, GC-BB

INTRODUCTION

The Oriental beetle (OB), *Exomala orientalis* (Waterhouse) (Coleoptera: Scarabaeidae), is probably a native of the Philippine Islands that was carried to Japan and was introduced from Japan to the United States. Sometime before 1908, it was introduced to the Hawaiian Island of Oahu, where it became a serious pest of sugarcane. On the mainland of the United States, adults were first collected in 1920 in a New Haven, Connecticut, nursery, having presumably been imported directly from Japan in infested balled nursery stock (Tashiro, 1987). In Japan, no scientific evidence has been documented in the old literature regarding the origin of the OB (Japanese name: *semadarakogane*). However, the beetle has been recorded for a long time, although its scientific name has changed over the years: *Phyllopertha orientalis* Waterhouse (Murayama, 1950), *Anomala orientalis* (Waterhouse) (Anonymous, 1980), and currently *Blitopertha orientalis* (Waterhouse) (Anonymous, 1987).

A revision of the subfamily Anomalinae (Scarabaeidae) has been proposed for the genus occurring in the United States and Canada, with *Blitopertha* considered a synonym of *Anomala*, tribe Anomalini (Potts, 1974) and, therefore, the OB is named *Anomala orientalis* in the United States (Stoetzel, 1989).

Given the economic importance of the OB in Japan, and the need for alternative methods of control, we initiated a project aimed at identifying the sex pheromone of the beetle and evaluating its potential in practical applications. A similar project was also launched in the laboratory of Dr. Wendell L. Roelofs (Cornell University at Geneva, New York) with the American population of OB. Although these beetles were supposed to be a different species, the facts that ''*Anomala orientalis*'' males responded in a wind tunnel to a sex pheromone identified from ''*Blitopertha orientalis*'' (Leal, 1993) and that the active chemical isolated from the former had the same capillary GLC retention times and MS as the latter (Leal et al., 1993a; to be reported in detail by Roelofs' group), led us to compare the two species taxonomically.

Specimens were sent to the United States, and the taxonomists consulted referred to a recent revision of the genus *Blitopertha* (Baraud, 1991), in which *B. orientalis* is newly classified as *Exomala orientalis*. As Baraud's paper refers only to the species occurring in Japan, Korea, and Hawaii, it appears that the external characters of aedeagus that led to the new genus classification have not been examined in the American population of OB.

We describe here the identification and field evaluation of the sex pheromone of the Japanese population of the OB (*semadarakogane*, formerly *Blitopertha orientalis*), *Exomala orientalis*.

METHODS AND MATERIALS

Chromatographic, Mass, and Infrared Spectral (MS, IR), Analyses. GC analyses were performed on a Hewlett-Packard 5890 equipped either with a DB-wax column (30 m × 0.25 mm; 0.25 μm) or an HP-1 column (12 m × 0.2 mm; 0.33 μm), operated in a splitless mode at 50°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 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 the same type of capillary columns under the same conditions as described for the GC. GC-FTIR was done on a Hewlett-Packard 5965B equipped with a DB-wax capillary column operated at 70(1)–150(1)/5–240(10)/10. The light pipe was operated at 250°C and the transfer line at 270°C.

Gas Chromatography–Electroantennographic Detector (GC-EAD). The response of *Exomala orientalis* antennae were recorded with a GC-EAD system (Leal et al., 1994a). The previously described acrylic EAD station (Leal et al., 1992a) was modified in order to have an adjustable opening space between the two holes (for holding the pedicel and last flagellum) to be regulated according to the size of the antenna.

Gas Chromatography–Behavior Bioassay (GC-BB). Coupled chromatographic resolution and behavioral observations were done as previously described (Leal et al., 1992b). Males (10) were placed in a plastic box (17 × 12 × 6 cm) fixed to the outlet of the GC (Figure 1) and their response to GC eluents was observed.

Insects. Eggs laid by field-collected female beetles were transferred to wet sand (10% water) in ice cream cups (60 ml), which were kept at 25°C. After hatching, grubs were individually kept in ice cream cups loaded with a 1:1 mixture of sand and humus obtained from leaves of *Quercus acutissima* (a kind of oak; *kunugi* in Japanese). This was humidified (ca. 10% water) and the grubs supplied with slices of sweet potato. After the third stage, grubs reached the yellow stage (stop feeding) and were chilled to 10°C for over two months. The temperature was then raised again to 25°C. Adults were kept in culture dishes (90 mm OD × 60 mm high) at 25°C, 70% relative humidity, and 14L:10D photoperiod, and provided with saturated sucrose solution on cotton.

Aeration. The airborne volatiles of either male or female beetles were collected according to a previously reported method (Leal et al., 1992a).

Isolation of Pheromone. Crude extract of female volatiles was 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. Pheromonal activity was monitored by a simplified bioassay (Leal et al., 1992c). Males were placed inside culture dishes (90 mm OD × 60 mm high), the bottom of which was

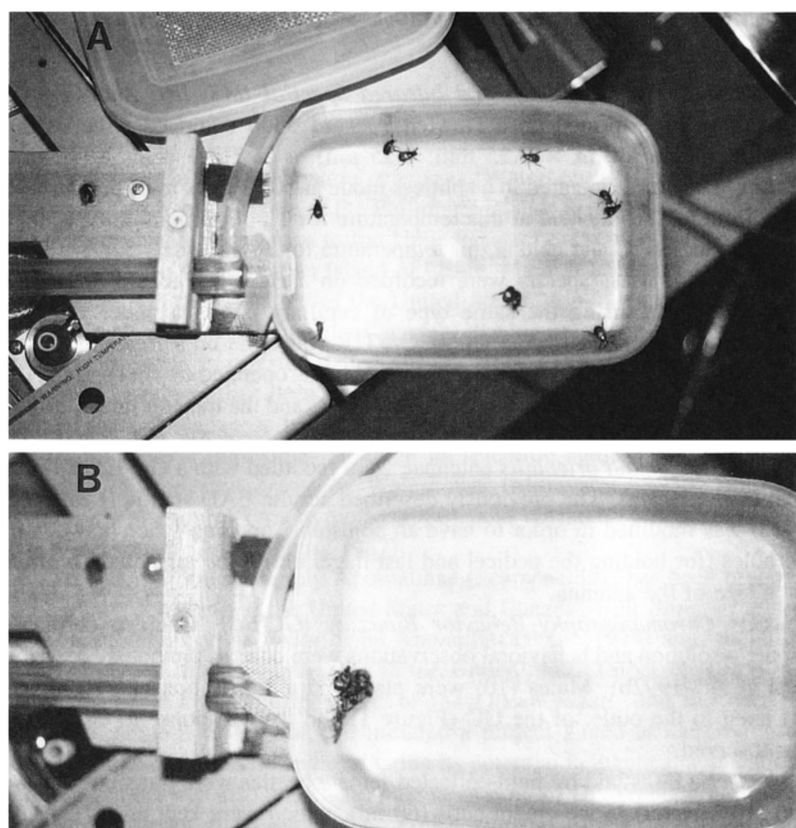

FIG. 1. Behavioral response of *E. orientalis* males in a GC-BB. (A) Males randomly walking inside the arena and (B) gathered on the outlet of the GC system in response to female-released semiochemicals separated on the GC capillary column.

covered with wet filter paper. Samples were transferred to a filter paper (1 × 1 cm), set inside the dish, and the insect response was recorded.

Synthesis. 7-Tetradecen-2-one was synthesized as reported (Leal, 1993). Pure *Z* isomer was obtained by separation on silver nitrate on a silica gel (10%, 200 mesh) column.

5- and 6-Tetradecen-2-one were prepared by Wittig reaction of dicarbo-ethoxy aldehydes with appropriate ylides. These dicarboethoxy products were

transformed into the corresponding acids by hydrolysis, and then they were treated with methyl lithium to yield the desired ketones.

5-Tetradecen-2-one was synthesized starting from the reaction of ethyl malonate with sodium hydride in DMSO, followed by coupling with 2-bromoacetaldehyde diethylacetal to produce 3,3-dicarboethoxypropionaldehyde diethylacetal. After deprotection of the aldehyde, it was reacted with the ylide formed by the action of butyl lithium in THF on nonyltriphenylphosphonium bromide. Alkaline hydrolysis of the product, 1,1-dicarboxy-3-dodecene, followed by heating (135°C), gave 4-tridecenoic acid. Reaction of the acid with methyl lithium in THF yielded 5-tetradecen-2-one (85% *Z*; 15% *E*).

Synthesis of 6-tetradecen-2-one (90% *Z*; 10% *E*) was achieved by the same route, starting from 3-bromopropionaldehyde ethylene acetal and using octyltriphenylphosphonium bromide in the Wittig reaction.

2-(*E*)-Nonenol was obtained by the reduction of 2-(*E*)-nonenal with $LiAlH_4$ in dry ether (Leal et al., 1992b). 2-Tetradecanone was commercially available (Tokyo Kasei Kogyo Co., Tokyo).

Field Experiments. Evaluation of baits was conducted at the fields of NISES (Tsukuba) and Chiba Prefectural Agricultural Experiment Station (Chiba) in the summer of 1993. Funnel traps (Japan Tobacco Inc., Tokyo), 7 or 10 m apart, were baited with synthetic lures incorporated into plastic pellets (4–5 mm in diameter) made of a polyethylene–vinyl acetate. These pellets were placed inside pellet holders (Fuji Flavor Co., Tokyo) and set 2 cm above the trap lip. Unless otherwise mentioned, traps were positioned with the pheromone dispenser at 30 cm above the ground. The candidate lures were replicated in randomized blocks and capture data were transformed to log $(x + 1)$ before differences among means were tested for significance by ANOVA with 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. In the figures, means of captures are untransformed and error bars show one SE.

RESULTS AND DISCUSSION

Identification of Sex Pheromone. Pheromonal activity was observed in the crude extract of airborne volatiles collected from the headspace of 20 female beetles, and this activity was recovered in a hexane–ether (90:10) fraction after separation on a silica gel column. In order to identify the active peak(s) (out of at least 38 candidates), this fraction was analyzed by GC-BB. While inactive compounds were eluted, male beetles walked randomly inside the arena (Figure 1A), and they gathered in the outlet of the GC system (Figure 1B) in response to peak(s) appearing at ca. 31.5 and 29.1 min on an HP-1 and DB-wax columns,

respectively. The same activity was elicited only with the crude extract and 90:10 fraction.

GC-MS analyses of the 90:10 fraction demonstrated the occurrence of at least four possible peaks in the active region by GC-BB (Figure 2A). The peaks at R_t 32.51, 32.41, 29.25, 27.67, and 25.86 were ruled out because they were found also in the (inactive) crude extract collected from male beetles. The peaks at R_t 31.73 and 31.91 min gave similar mass spectra (Figure 2B and C). The occurrence of a base peak at *m/z* 43 was initially considered due to an acetate structure (with the *m/z* 61 peak missed), but the fact that the compounds did not undergo alkaline hydrolysis ruled out this possibility. Hydrogenation with Adam's catalyst gave rise to 2-tetradecanone, which was also found in the volatiles of male and female beetles (peak at R_t 32.41 min, Figure 2A). Therefore, the major peak was considered to be tetradec-?-en-2-one. The peak at *m/z* 58 was small because the position of the double bond may not favor McLafferty rearrangement. The occurrence of peaks at *m/z* 145 and 159 in the MS of the dimethyl disulfide derivative suggested that the major peak was either 6- or 7-tetradecen-2-one.

Although not available at the time of the structure elucidation, the vapor phase IR of the major peak (Figure 3A and B) gave a characteristic profile of a long-chain ketone: C—H stretching (2935–6 cm^{-1}) predominant over the carbonyl band (C=O st, 1731–2 cm^{-1}). A library search suggested a possible structure to be 2-undecanone (Figure 3C), which basically differed from the natural product in that the former did not show the band at 3013–4 cm^{-1} of a double bond in the *cis* configuration (Leal, 1991; Leal et al., 1992d).

Synthetic 7-(Z)-tetradecen-2-one was identical to the major peak in terms of R_t, MS, and pheromonal activity in the GC-BB, whereas 6-tetradecen-2-one differed not only in the MS, but also in retention times. Based on its R_t on the two capillary columns as well as on its MS, the minor peak at R_t 31.91 min was characterized as 7-(*E*)-tetradecen-2-one. Therefore, the sex pheromone of *Exomala orientalis* was identified as a mixture of 7-(Z)- and 7-(*E*)-tetradecen-2-one in a natural ratio of 7:1.

The possibility of chemically related compounds being used as minor components was also exploited. Although the saturated ketone, 2-tetradecanone, was found to be released by both male and female beetles, its attractancy was tested in the field in Tsukuba (June 17–20). There was no significant difference in captures of the OB in traps baited with the synthetic sex pheromone alone, 7-(Z)-tetradecen-2-one, or in combination with 2-tetradecanone (Figure 4A).

Because the occurrence of 6-tetradecen-2-one as a minor component could not be ruled out on the basis of the MS of the dimethyl disulfide derivative, this compound was tested in the field (Tsukuba, June 25–28). A combination of 7-(Z)-tetradecen-2-one and 6-tetradecen-2-one was not a significantly better lure than 7-(Z)-tetradecen-2-one alone (Figure 4B).

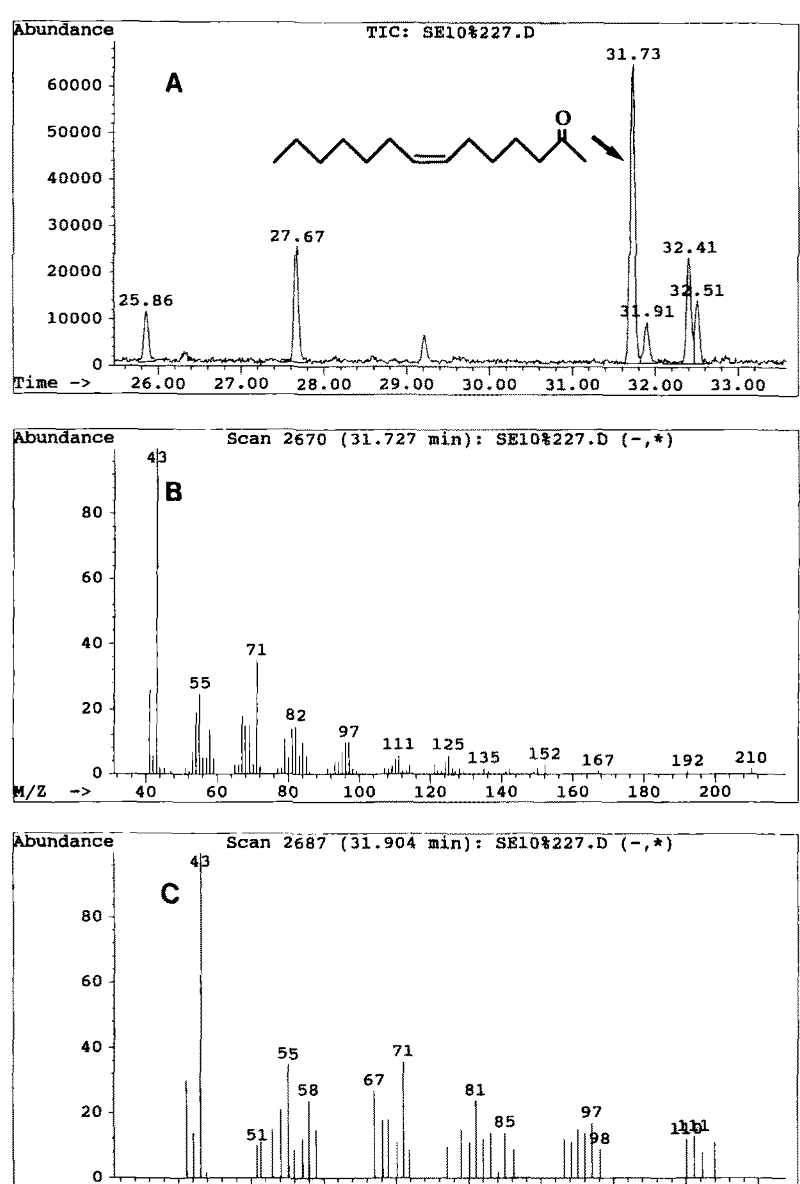

FIG. 2. Reconstructed total ion monitor profile of the active hexane–ether (90 : 10) fraction separated on a DB-wax capillary column (A). EI-MS of the two female-specific active peaks at R_t 31.73 min (B) and 31.91 min (C).

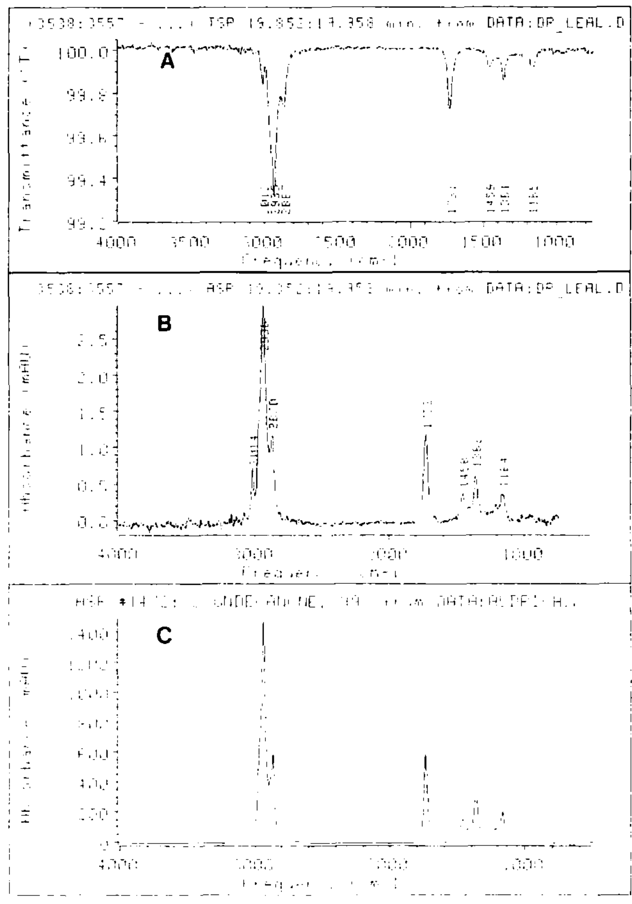

FIG. 3. Vapor-phase infrared spectrum of the major peak (A, B) compared to the best library fitting (C).

Although we do not have any direct evidence of the biosynthetic pathways, some sex pheromones of scarab beetles seem to be derived from fatty acids (Leal et al., 1994a). Based on this biochemistry reasoning, 5-tetradecen-2-one was considered to be a possible minor component. Since it possessed a retention time that is very close to 7-tetradecen-2-one, the candidate chemical was tested in the field in Tsukuba (June 25–29). Interestingly, trap catch of OB to its sex pheromone was reduced by the presence of 5-tetradecen-2-one. It has been found (Ono, unpublished data) that captures of the soybean beetle, *Anomala rufocuprea*, were greatly decreased by the use of its sex pheromone, methyl 5-(*Z*)-

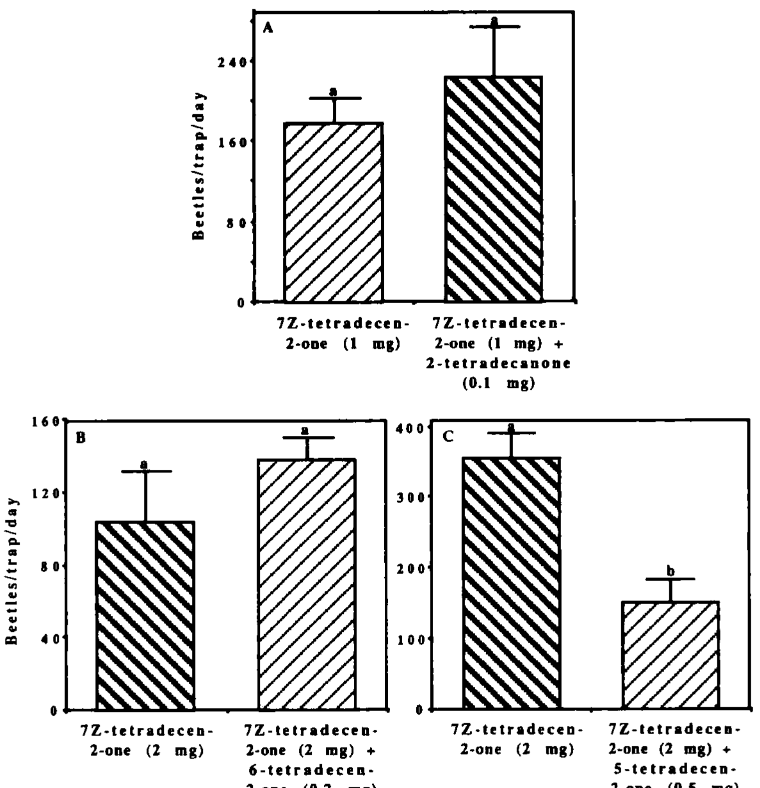

FIG. 4. Effect of possible minor components (A) 2-tetradecan-one, (B) 6-tetradecen-2-one, and (C) 5-tetradecen-2-one on the catches of the OB.

tetradecenoate, in combination with japonilure, the sex pheromone of the Japanese beetle. Therefore, we considered that 5-tetradecen-2-one may be involved in the pheromonal communication of related species.

Evaluation of Synthetic Sex Pheromone. Preliminary field tests of the synthetic sex pheromone (5 mg) loaded in a rubber septum (July 30–August 12) revealed that a $Z{:}E$ (7:1) mixture was attractive to males of the OB (Leal, 1993). It remained unclear, however, whether both geometric isomers were essential for attraction. Experiments conducted in Chiba (July 15–30, 1993) demonstrated that captures in traps baited with the $Z{:}E$ mixture were not significantly different from the catches with the Z isomer only (Figure 5).

The effect of the dosage on the catches of the OB was tested in field experiments in Chiba (June 24–27) by comparing the captures with 0.1, 1, and

10 mg of a Z:E (7:1) mixture of the sex pheromone. Traps baited with 1 mg of the pheromone captured significantly more beetles (2.9-fold) than those with 0.1 mg of the lure. However, there was no significant difference in catches in traps with 1 and 10 mg of the synthetic sex pheromone (Figure 6). We have found that catches of *Anomala octiescostata* with the synthetic sex pheromone were not significantly different in traps baited with 1 and 10 mg, but 100 mg captured significantly more beetles than 1 mg (Leal et al., 1994b). So far, we

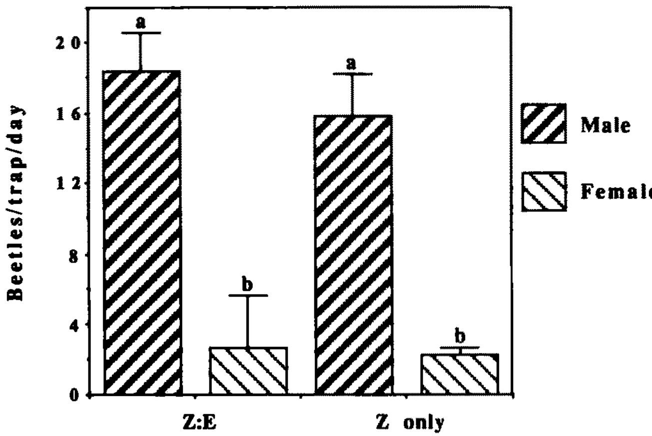

FIG. 5. Captures of *E. orientalis* male and female beetles with the two naturally occurring geometric isomers and the Z component only.

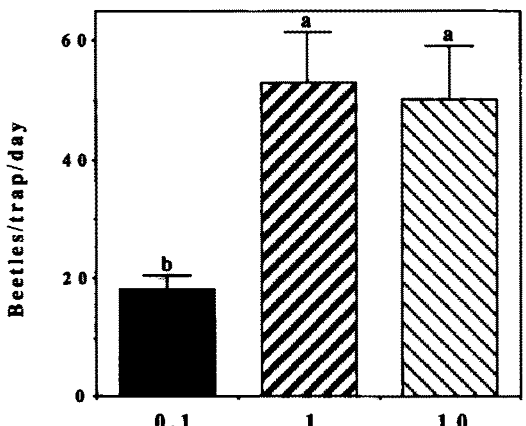

FIG. 6. Effect of the dosage on the catches of the *E. orientalis*.

have not found any case of a decrease in catches of scarab beetles due to the dosage of the pheromone in the range from 0.1 to 100 mg.

Catches of *Exomala orientalis* were compared in experiments conducted in Chiba (July 2–9), setting traps with the pheromone dispenser at 30, 100, and 160 cm above the ground. The best performance was achieved with traps at 30 cm (Figure 7), in which 86% of the male beetles were captured. This has also been shown to be the best height for captures of *Anomala schonfeldti* with synthetic sex pheromone (Hasegawa et al., 1993). Interestingly, 11% of the OB captured in these experiments were female beetles. Furthermore, nearly the same ratio was found in another experiments (Figure 5). In our experiments, these female catches are significantly higher in traps baited with the synthetic sex pheromone than in control traps. Therefore, it seems that male-released semiochemicals may also be involved in the communication of scarab beetles (see Leal et al., 1994a for further discussion).

Investigation of Occurrence of Minor Component(s). Sex pheromone systems of nine scarab species have been identified so far (Leal et al., 1994a), of which only four have been identified as binary mixtures (the others were single components). In the cupreous chafer, the occurrence of two components has been demonstrated not only by chemical identification (Leal et al., 1993b), but also by the existence of two receptor cells in the pheromone-sensitive sensilla as well as the occurrence of two spike amplitudes in the impulses from the pheromone receptor (Leal and Mochizuki, 1993). We believe that, as a rule, minor component(s) may be involved in the pheromonal communication of scarab

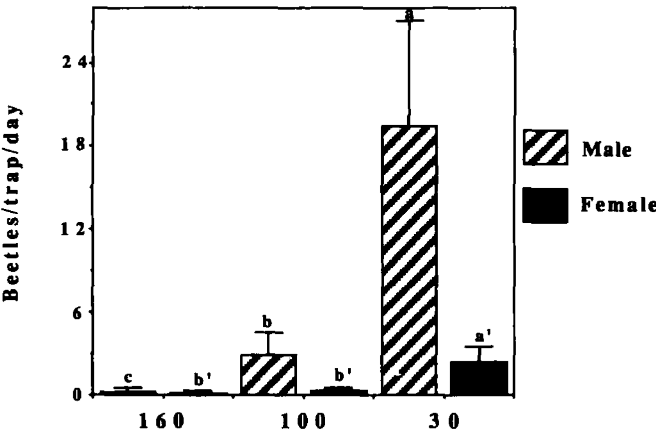

FIG. 7. Performance of the sex pheromone-baited traps on the captures of the OB at different heights.

beetles, although the existence of minor constituent(s) may be masked by a highly attractive major pheromone (Leal et al., 1993c).

As minor components are unlikely to be detected by GC-BB, we investigated the occurrence of minor component(s) in the pheromone system of *Exomala orientalis* by means of GC-EAD. Male antennae of the OB (settled in an improved EAD station) responded to two peaks in the crude extract of the airborne volatiles from female beetles (Figure 8). The major peak at R_t 28.12 min was confirmed to be 7-(Z)-tetradecen-2-one, whereas the minor peak had the same retention time as 2-(*E*)-nonenol (22.05 min) on the polar capillary column. Due to the small amount of this EAD-active peak, it was not possible to obtain an MS out of pooled extract from the headspace of female beetles. Nevertheless, the fact that some scarab species utilize sex pheromone of other species as a minor component of their own blends (Leal et al., 1993d; 1994a) and that 8% of the total capture in traps baited with 2-(*E*)-nonenol, the sex pheromone of *A. schonfeldti*, were males of the OB (Hasegawa, unpublished) led us to test its attractiveness.

Field experiments carried out in Tsukuba (June 17–20) showed that there was no significant difference in catches of the OB with 7-(*Z*)-tetradecen-2-one alone or in combination with 2-(*E*)-nonenol (Figure 9A). Further experiments will be carried out in the next seasons in order to characterize the EAD-active peak at R_t 20.02 min and to clarify its role.

The use of traps baited simultaneously with the sex pheromones of two different species, although desirable for economic reasons, has not been possible in some cases due to the antagonism caused by the sex pheromone of one species on the captures of the other. Simultaneous monitoring of *E. orientalis* and *A. schonfeldti* may be possible, but the effect of the combined lure on the catches of the latter are yet to be tested.

An EAD screening of the previously identified sex pheromones of scarab

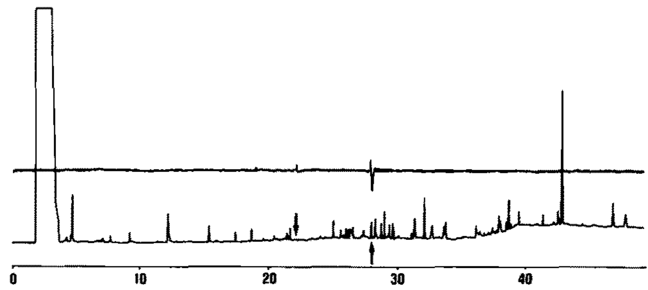

FIG. 8. Coupled GC-EAD response of male antenna to the airborne volatiles of virgin female beetles showing two EAD-active peaks. Upward and downward arrows indicate the peak of 7-(*Z*)-tetradecen-2-one and an unidentified chemical, respectively.

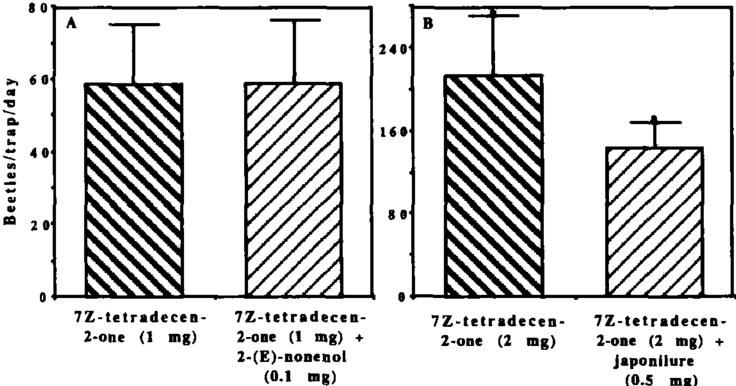

FIG. 9. Effect of the pheromones of two other scarab species on the catches of *E. orientalis*: (A) 2-(*E*)-nonenol, the sex pheromone of *A. schonfeldti*, and (B) japonilure, the sex pheromone of *Popillia japonica*.

beetles (Leal et al., 1994a) was carried out to explore possible attractants or antagonists. Only japonilure gave a significant and reproducible EAD response. Both the signal-to-noise ratio and reproducibility of the response generated by 2-(*E*)-nonenol were very low. Field tests in Tsukuba (June 28–30) demonstrated, however, that japonilure neither increases nor decreases trap catch of the sex pheromone of *E. orientalis*.

In conclusion, 7-(Z)- and 7-(*E*)-tetradecen-2-one were identified as sex pheromone constituents of the Japanese population of the OB, *E. orientalis*. This pheromone system is a potent lure for studies on the chemical ecology as well as management of the OB.

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TRAIL PHEROMONE OF LEAF-CUTTING ANT *Acromyrmex subterraneus subterraneus* (FOREL)

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Abstract—The trail pheromone of *Acromyrmex subterraneus subterraneus* consists simply of the heterocyclic ester methyl 4-methylpyrrole-2-carboxylate in the venom reservoir of workers at about 1.2 ng/ant. No pyrazines were detected, and no enhancement of trail-following was observed when pyrazines were added to the pyrrole compound.

Key Words—Trail pheromone, Hymenoptera, Formicidae, methyl 4-methylpyrrole-2-carboxylate, poison gland.

INTRODUCTION

The leaf-cutting ants of the New World (tribe Attini of the subfamily Myrmicinae) are noted for their destructive effects on cultivated plants and for their ability to lay down and follow lengthy trails between their nests and the plant material, which provides the substrate for their fungus gardens. The chemical source of the pheromone used for these trails has been identified for only five species. Tumlinson et al. (1971, 1972) first identified the trail pheromone of *Atta texana* as methyl 4-methylpyrrole-2-carboxylate (M4MPC). Later, the same substance was identified as the trail pheromone of *Atta cephalotes* (Riley et al., 1974a,b), and of *Acromyrmex octospinosus* (Cross et al., 1982). A different

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substance, 3-ethyl-2,5-dimethylpyrazine (EDMP) was identified as the pheromone in another *Atta* species, *A. sexdens rubropilosa* (Cross et al., 1979). All of these investigations were carried out on large (kilogram) samples of insects by lengthy isolation and purification procedures. Using our own techniques for analyzing individual insect glands (Morgan and Wadhams, 1972; Morgan, 1990), we identified EDMP as the trail pheromone in the venom glands of *A. sexdens sexdens* with small numbers of insects (Evershed and Morgan, 1983). There are reports, less readily accessible, on trail pheromones of *Atta laevigata* (M4MPC) and *A. robusta* (M4MPC) (Oliveira 1975; Oliveira et al., 1990) and *A. bisphaerica* (probably M4MPC and EDMP) (Oliveira et al., 1990). The trail pheromone of only one of the 24 recorded species of *Acromyrmex* (Hölldobler and Wilson, 1990) has been identified, although from behavioral tests a mixture of M4MPC and EDMP was considered to be the trail pheromone of *Acromyrmex subterraneus molestans* (Martinez, 1988).

By a combination of trail-following experiments, chemical analysis of individual glands or small groups of glands, and experiments with synthetic compounds, we have identified the trail pheromone of a second species of *Acromyrmex*, *A. subterraneus subterraneus* (Forel 1893) and quantified the amount of it in the venom gland.

METHODS AND MATERIALS

A. subterraneus subterraneus were collected in the vicinity of the University of Viçosa. In some cases the insects were immediately dissected and the venom glands sealed in glass capillaries (Morgan, 1990) and transported to Keele. In others, live colonies were taken to Keele University and maintained in the laboratory there in artificial nests.

In trail-following tests carried out in Brazil, we confirmed that the venom gland was the source of the trail pheromone (Moreira and Della Lucia, 1993a), as described by Blum et al. (1964) generally for attine ants, and that the contents of a venom gland laid as an artificial trail on paper retained potency for 96 hr (Moreira and Della Lucia, 1993b).

Trail-following tests with workers of *A. s. subterraneus* were carried out at Keele on circular trails (radius 5 cm) marked on paper using the method of Pasteels and Verhaeghe (1974). Hexane extracts (100 μl) of dissected parts of worker abdomens or hexane solutions of pure chemicals were laid on the circumference of the circle. After 2 min to allow the solvent to evaporate, the paper was presented to the ants and observed until at least 20 ants had contacted the trail. The number of arcs of 1 cm that each ant walked on the trail before it lost contact with it were recorded and the median value calculated.

Gas chromatography was carried out on a fused silica capillary column

(12 m × 0.32 mm) coated with dimethylsilicone grease (film thickness 0.25 μm) in a Carlo Erba Fractovap Series 4160 chromatograph. The oven was programmed from 50°C at 15°C/min to 250°C, using a helium (1 ml/min) as carrier gas.

Detection was with a Carlo Erba nitrogen–phosphorus flame ionization detector operating in the nitrogen mode with maximum suppression of carbon response. Injection was made by the solvent-less technique of Morgan and Wadhams (1972) using dissected venom glands sealed in glass capillaries in groups of one to three glands.

RESULTS

We have found that our linked gas chromatograph–mass spectrometer (Hewlett Packard MSD) has rather poor sensitivity of detection for methyl 4-methylpyrrole-2-carboxylate (M4MPC) (limit of about 5 ng injected), so it was not a suitable technique for examining poison reservoirs for this compound. However, the nitrogen–phosphorus GC detector has a detection limit of less than 100 pg for M4MPC. We therefore used the N-P detector for an examination of worker venom glands. No lower pyrazines (e.g., dimethyl or trimethylpyrazine or EDMP) were detected (limit of detection of EDMP was < 100 pg). The only peak visible was that due to the pyrrole M4MPC, which gave a sharp peak at 5.6 min (Figure 1), identical in retention to the pure substance (Sigma, Gillingham, Dorset). By comparison of peak areas from gland samples with a solution of synthetic M4MPC (1 ng/μl), the mean amount of M4MPC per gland was found to be 1.2 ± 0.3 ng ($N = 10$). When glands were injected onto a

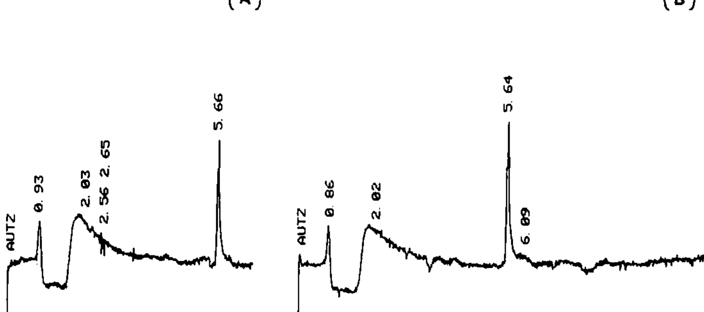

FIG. 1. Gas chromatograms using the nitrogen–phosphorus detector in the nitrogen-specific mode: (A) pure M4MPC (1 ng) injected. (B) poison gland reservoir of one worker of *A. subterraneus subterraneus* using the solid sampling method. Other conditions are as described in the text.

linked gas chromatograph–mass spectrometer (Hewlett Packard MSD) under similar conditions, no substances were detected at all, but it was then known that the quantities of M4MPC present were below the level of detection.

In trail-following assays, only the venom gland showed activity and, of the pure synthetic compounds tested, only M4MPC was active (Table 1). There was no significant difference between the activity of one venom reservoir and 1 ng of M4MPC.

When we used mixtures of EDMP and M4MPC in trail-following tests with *Atta sexdens sexdens*, we found that a mixture of these compounds was more active than either alone (Billen et al., 1992). We therefore carried out tests with simple alkylpyrazines, 2,5-dimethylpyrazine (DMP), trimethylpyrazine

TABLE 1. TRAIL-FOLLOWING TESTS ON GLANDULAR EXTRACTS AND SYNTHETIC COMPOUNDS[a]

Tested extract or compound	Median ± SD (cm)[b]
One Dufour gland	0
One Venom gland	47 ± 18.2[c]
Venom gland, 0.1	3.5 ± 3.4
Venom gland, 0.01	0
M4MPC, 10 ng	36 ± 12.9[c]
M4MPC, 1 ng	31 ± 11.9[c]
M4MPC, 0.1 ng	1.5 ± 1.3
M4MPC, 0.01 ng	0
EDMP, 10 ng	0
EDMP, 1 ng	0
EDMP, 0.1 ng	0
DMP, 1 ng	0
DMP, 0.1 ng	0
TMP, 1 ng	0
TMP, 0.1 ng	0
M4MPC (10) + EDMP (10)[d]	11.4 ± 2.9
M4MPC (1) + EDMP (1)[d]	4.0 ± 2.3
M4MPC (0.1) + EDMP (0.1)[d]	1.0 ± 0.8
M4MPC (10) + DMP (10) + TMP (10) + EDMP (10)[d]	10.5 ± 3.5
M4MPC (1) + DMP (1) + TMP (1) + EDMP (1)[d]	3.0 ± 0
M4MPC (1) + DMP (0.1) + TMP (0.1) + EDMP (0.1)[d]	12 ± 2.0
M4MPC (1) + DMP (0.01) + TMP (0.01) + EDMP (0.01)[d]	13 ± 2.0

[a] In each case the extract, compound, or mixture of compounds was dissolved in 100 μl hexane and applied to a circular trail of radius 5 cm. Twenty-nine observations were made to obtain each median value.

[b] Average of five determinations for each experiment.

[c] No statistical different between these values.

[d] Quantities are all in nanograms.

(TMP), and EDMP, and mixtures of these with M4MPC (Table 1) to see if there was any enhancement of activity through the presence of small quantities of pyrazines below the level of chemical detection. The pyrazines showed no activity alone and inhibited that of M4MPC when they were mixed together.

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PLANT-NATURAL ENEMY ASSOCIATION IN THE TRITROPHIC SYSTEM, *Cotesia rubecula–Pieris rapae–* BRASSICEAE (CRUCIFERAE): I. SOURCES OF INFOCHEMICALS

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Abstract—The role of airborne infochemicals in host selection by the parasitoid *Cotesia rubecula* (Marshal) (Hymenoptera: Braconidae) was examined in a wind tunnel. To elucidate the role of volatile chemicals in attracting *C. rubecula* to cabbage infested by the host [*Pieris rapae* L. (Lepidoptera: Pieridae)], the potential sources of volatiles related to *P. rapae* infestation on cabbage were tested individually. The responses of females to nonhost plant species, bean and geranium, as well as to frass of a nonhost lepidopteran were also examined. *C. rubecula* was attracted to cabbage previously infested by *P. rapae* and to frass and regurgitate of *P. rapae*. No attraction was observed to larvae of *P. rapae* alone. Females were also attracted to mechanically damaged cabbage, cabbage previously infested by *Plutella xylostella* L. (Lepidoptera: Plutellidae) (a nonhost lepidopteran herbivore), and cabbage previously infested by snails (a nonhost, noninsect herbivore). Intact cabbage, bean, and geranium plants elicited no attraction. A low frequency of attraction was observed to mechanically damaged bean and geranium. Attraction was also observed to frass of *P. xylostella*. Volatiles from cabbage related to damage, and volatiles from frass and regurgitate of the host seem to play an important role in guiding *C. rubecula* to plants infested by its host.

Key Words—*Cotesia rubecula*, Hymenoptera, Braconidae, Lepidoptera, Pieridae, Plutellidae, *Pieris rapae*, *Brassica oleracea*, *Plutella xylostella*, *Phaselus vulgaris*, *Geranium molle*, tritrophic interactions, infochemicals, volatiles.

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INTRODUCTION

The chemical defense of plants against herbivorous insects can be divided into two categories, a direct defense related to a systemic production of toxins and an indirect defense related to attraction of natural enemies of the herbivores (Dicke and Takabayashi, 1991). Attraction of natural enemies (insect predators and parasitoids) to volatile chemicals emitted by host plants indicates that natural enemies use these chemicals during one stage of their search for prey or hosts (Vinson, 1984; Roland, 1990; Dicke et al., 1990b; Vet and Dicke, 1992). Recent research has shown that plants release a different blend of volatile chemicals according to their damage state. Intact plants emit different volatile compounds from mechanically damaged ones, and mechanically damaged ones from plants damaged by herbivores (Dicke et al., 1990a; Turlings et al., 1990, 1991; Whitman and Eller, 1990). It is not known what part of the volatile chemical blend the natural enemies utilize and what information each chemical, or group of chemicals, conveys.

The aim of the present work was to investigate the role of the host plant in host location by the parasitoid *Cotesia rubecula* (Marshal) (Hymenoptera: Braconidae).

C. rubecula is native to the Palaearctic region. Despite a distinct preference for *Pieris rapae* L. (Lepidoptera: Pieridae) as a host, the range of other hosts that geographically separated populations of the species utilize is not well known. The species was originally described by Marshall in 1885 as a solitary parasitoid of *P. rapae* under the name *Apanteles rubecula* (Shenefelt, 1972). According to the reports from other parts of Europe, Wilkinson (1945) redescribed the species, adding two more host species, *Pieris napi* L. (Lepidoptera: Pieridae) and *Plutella xylostella* L. (= *maculipennis*) (Lepidoptera: Plutellidae). Shenefelt (1972) reported eight hosts of *C. rubecula* based on a review of literature.

C. rubecula was released in North America, New Zealand, and Australia to control the cabbage white butterfly where it is now established (Wilkinson, 1966; Wilson, 1960; Biever, 1992).

Our work refers to the Australian population of *C. rubecula*, found around Adelaide in South Australia and which originated from Switzerland (Wilson, 1960). This population has only been collected from *P. rapae*, despite the great abundance of *P. xylostella* in the field. Laboratory observations verify the field findings that *C. rubecula* from Adelaide does not attack *P. xylostella* (personal observations).

Females of *C. rubecula* are known to be attracted to cabbage plants infested by *P. rapae* and intact cabbage leaves inside a vial (Nealis, 1986; Keller, 1990; Kaiser and Cardé, 1992). The aim of the present work was to investigate how different sources of volatile chemicals influence the attraction of *C. rubecula* to cabbage plants infested by *P. rapae*. The potential sources of volatile chemicals

were divided into two categories. The first category includes volatile compounds originating from the host and the second category includes volatile compounds originating from the plant. Host volatile chemicals were subdivided into those emitted from the body of the host, from frass, and from regurgitate. Regurgitate was used as a potential source of attractive volatiles as the larvae of *P. rapae* often regurgitate when they are disturbed. The second category is a complex one, as the volatile emissions of a plant change according to its age (Wallbank and Wheatley, 1976; Cole, 1980) and state (Dicke et al., 1990a; Turlings et al., 1990). This category includes volatile compounds emitted by cabbages that were intact or damaged by mechanical means, the host, a nonhost lepidopteran, and a nonhost mollusk. The attraction of female *C. rubecula* to potential sources of attractive volatile chemicals were observed in a wind tunnel. The attraction of females was also observed for different states of nonhost plant species and the frass of a nonhost lepidopteran herbivore.

METHODS AND MATERIALS

Plants. Cabbage (*Brassica oleracea capitata* cv. Green Coronet) was grown under greenhouse conditions. Because it is not known how nutrient availability affects the chemistry of plants, a hydroponic system was used to grow the cabbages so that nutrient depletion associated with soil leaching and consumption of nutrients by the developing plant would not be a factor in the experiment. The plants were grown in unfertilized soil and were watered with Hoagland nutrient solution (Silsbury, 1984) at regular intervals depending on the developmental stage of the plant. The main aim of the plant culture was to obtain intact cabbage plants. During development, plants were regularly checked for root exposure or other accidental damage. Damaged plants were rejected as it is not known how damage affects the physiology and subsequent emission of volatiles by the plant. Beans (*Phaseolus vulgaris* L.) and geranium (*Geranium molle* L.) were also grown under these conditions.

Insects. P. rapae (Lepidoptera: Pieridae) and *P. xylostella* were collected in Adelaide, South Australia, and reared on cabbage at 25°C and a photoperiod of 14L:10D. *C. rubecula* (Hymenoptera: Braconidae) was collected in Adelaide and reared on *P. rapae*. Wasp cocoons were isolated in vials together with a drop of honey. On emergence, the insects were sexed and the experimental females mated.

Parasitoid Experience. After mating, naive females were held in a screened cage (17 × 17 × 21 cm) for 48 hr with water and honey. To obtain experienced females, newly emerged, mated females were exposed to odors emanating from cabbage infested by *P. rapae* in a wind tunnel (Keller, 1990). A net separated them from the infested plant. The females were released downwind and every

individual was observed to take a complete flight inside the odor plume (wind speed 54.3 cm/sec, temperature 25°C) (see below for the definition of a complete flight). The experienced females were collected and transferred to a cage (17 × 17 × 21 cm) where they remained for 48 hr with water and honey.

Experimental Procedure. Wasps were released in the wind tunnel from a glass tube (diameter 2.5 cm, length 7 cm). One end was covered with mesh, allowing air to pass through and preventing escape of insects. The other end was closed with a plug of cotton wool, which could easily be removed to release the insects. The tube was held horizontally so that when the cotton was removed the insects could perceive any volatile infochemicals carried by air while still inside the vial. Before the initiation of an observation, the insects were held inside the tube until becoming calm. The observation started when the cotton was removed. To avoid attraction originating from the color of the plant, a white mesh net (0.5 × 0.5-mm mesh opening) was installed perpendicular to the flow of air, 3 cm away from the stimulus and 77 cm away from the releasing tube. Smoke (a mixture of acetic acid and diaminoethane) was used to visualize the odor plume and to make sure that the releasing tube was inside the odor plume. Smoke was also used to mark the area of the net where the odor plume was passing through. The wind speed used was 54.3 cm/sec. All experiments took place from 9:00 AM to 12:00 noon.

Bioassays. Females were tested individually in the presence of each stimulus. Two flight categories, "complete flight" and "incomplete flight," were recorded. Complete flight occurred when wasps flew inside the odor plume and either successfully landed on the marked area of the net when the net was used or reached the stimulus in cases where the net was not used. Females that flew inside the odor plume, reached the marked area of the net, and continued flying inside the limits of the marked area without landing were also considered to have achieved complete flight. Incomplete flight (no attraction to the stimulus) occurred when the females flew upwards, outside the odor plume and landed on the walls of the wind tunnel. A second chance was given to females that had exhibited an incomplete flight the first time. The maximum time of observation was 5 min.

Test 1. The response of experienced females to different ages of intact and mechanically damaged host plants, as well as to intact and mechanically damaged nonhost plant species was examined in the first test. Cabbage (host plant) was tested at three ages: I (5–6 leaves), II (8–9 leaves), and III (12–13 leaves). Beans and geranium (nonhost plants) were tested when they had four leaves. Mechanical damage was inflicted by removing 2 cm^2 from each of the leaves located on the same level on opposite sides of the plant. Care was taken not to damage the main veins of the leaves. To test the attraction of females to intact cabbage leaves inside a vial, three leaves were cut and their petioles were

wrapped in cotton wool and placed in a vial of water. Plants were located behind the net during this experiment.

Test 2. The response of experienced females to plants that were damaged by different herbivores was examined in the second test. Before the experiment, the herbivores and their by-products were removed and the plant was washed with water. Cabbages were damaged by *P. rapae*, the nonhost lepidopteran *P. xylostella*, and snails (common garden snail, *Helix aspera* Müller). The lepidopteran larvae were isolated on two leaves on opposite sites of each plant and left to consume approximately 4 cm^2 of foliage. With *P. rapae*, 10–12 second instars per leaf were left to feed for 24 hr. With *P. xylostella*, 10–12 fourth instars per leaf were left to feed for 24 hr. Snails were observed while feeding on the leaf and were removed when they had consumed the required amount of leaf area. During the experiment, the test plant was located behind the net.

Test 3. The responses of experienced females to host larvae alone and to host and nonhost by-products were documented in the third test. Odor sources were: (I) 20 second-instar *P. rapae*, starved for 48 hr and washed with water before the experiment, (II) regurgitate of *P. rapae*, (III) 350 mg frass of *P. rapae*, and (IV) 350 mg of frass of *P. xylostella*. Frass was collected by placing a sheet of paper under an infested cabbage for 24 hr. Regurgitate of *P. rapae* was collected by forcing 10 fourth and fifth instars to vomit on a glass slide. Caution was taken to avoid contact of the caterpillar's mouthparts with the glass slide. All stimuli were presented inside the wind tunnel on a glass slide. The net was not used for this test.

Test 4. To test preference in landing between the damaged and the undamaged part of the plant, the net was removed and the landing site of the female was recorded on a mechanically damaged cabbage (age II).

Test 5. Naive females were tested in the presence of different odors to determine if they behaved differently from experienced females. Odors were produced by cabbage that was damaged mechanically, by *P. rapae*, by *P. xylostella* and by snails, and by frass and regurgitate of *P. rapae*.

RESULTS

Females responded differently to intact and mechanically damaged cabbage plants (Table 1). Complete flight was achieved in all cases when mechanically damaged cabbages were present, but few females made complete flights to mechanically damaged bean and geranium. None of the females tested displayed complete flight when intact cabbages, beans, and geranium were used as a stimulus. No differences were observed in the response among the different plant ages tested. No differences in the response of females was detected when dif-

ferent herbivores damaged the cabbage plants. Females showed complete flight to cabbages damaged by both hosts and nonhosts (Table 2). Host by-products related to feeding on cabbage plants (frass, regurgitate) resulted in complete flight to the odor source. Frass from the nonhost Lepidoptera species, *P. xylostella*, elicited the same response as the host frass. No attraction was observed to larvae of *P. rapae* alone (Table 3). Females discriminated between damaged and undamaged leaves while they were flying. Thirty females landed on the damaged leaves and none on the undamaged leaves. Naive females showed the same sort of attraction as experienced females (Table 4).

TABLE 1. RESPONSES OF EXPERIENCED FEMALES OF *C. rubecula* TO INTACT OR MECHANICALLY DAMAGED CABBAGE PLANTS AND NONHOST PLANT SPECIES (N = 30/TREATMENT)

Odor source	Complete flight	Incomplete flight
Intact cabbage		
Age I	0	30
Age II	0	30
Age III	0	30
Mechanically damaged cabbage		
Age I	30	0
Age II	30	0
Age III	30	0
Intact bean	0	30
Intact geranium	0	30
Mechanically damaged bean	20	10
Mechanically damaged geranium	9	21
Cabbage leaves in a vial	18	12
Air only	0	30

TABLE 2. RESPONSES OF EXPERIENCED FEMALES OF *C. rubecula* TO CABBAGE PLANTS DAMAGED BY DIFFERENT HERBIVORES (N = 30/TREATMENT)

Odor source	Complete flight	Incomplete flight
Cabbage damaged by *P. rapae*	30	0
Cabbage damaged by *P. xylostella*	30	0
Cabbage damaged by snails	30	0
Air only	0	30

TABLE 3. RESPONSES OF EXPERIENCED FEMALES OF *C. rubecula* TO HOST AND NONHOST BY-PRODUCTS (N = 30/TREATMENT)

Odor source	Complete flight	Incomplete flight
Frass of *P. rapae*	30	0
Frass of *P. xylostella*	30	0
Regurgitate of *P. rapae*	30	0
Larvae of *P. rapae*	0	30
Air only	0	30

TABLE 4. RESPONSES OF NAIVE FEMALES OF *C. rubecula* TO HOST PLANT, HOST LARVAE, AND HOST BY-PRODUCTS (N = 30/TREATMENT)

Odor source	Complete flight	Incomplete flight
Intact cabbage, age I	0	30
Mechanically damaged cabbage, age II	30	0
Frass of *P. rapae*	30	0
Regurgitate of *P. rapae*	30	0
Cabbage damaged by *P. rapae*	30	0
Cabbage damaged by *P. xylostella*	30	0
Cabbage damaged by snails	30	0
Air only	0	30

DISCUSSION

Strong indications of plant involvement in host location are apparent in previous work on behavior of *C. rubecula*. Kaiser and Cardé (1992) suggested that it is likely plant volatiles are involved in host selection, when they showed that females were attracted to uninfested leaves held in a vial. A limited attraction to uninfested leaves was also observed by Keller (1990). Although Nealis (1986, 1990) found that *C. rubecula* was strongly attracted to host feeding damage irrespective of the presence of hosts, he attributed the attraction to host kairomones (possibly saliva) remaining on the feeding sites.

Our study showed that the observed attraction of *C. rubecula* to cabbage infested by the host *P. rapae* can be attributed to volatile chemicals originating from three different components of the infested plant, namely, the cabbage plant itself, the frass, and the regurgitate of the host. The plant volatile chemicals that attract *C. rubecula* are related to damage (mechanical or by herbivores) and they are responsible for directing the flying females to the host plant with great precision. Attraction of *C. rubecula* to mechanically damaged host plants is not

surprising as attraction of parasitoids to mechanically damaged plants is a well-documented phenomenon (Loke et al., 1983; Eller et al., 1988; Turlings et al., 1990, 1991). The ability of *C. rubecula* to differentiate between cabbages according to type of damage inflicted has already been tested during a series of choice tests (Agelopoulos and Keller, 1994).

C. rubecula, like the parasitoid species *Microplitis croceipes* (Whitman and Eller, 1990), can discriminate between intact and mechanically damaged host plants and shows attraction for the latter. Identification of the volatile chemical blends emitted by intact and mechanically damaged cabbage has already been completed (Agelopoulos and Keller, in preparation). The results showed that there are qualitative and quantitative differences between the two chemical blends of volatiles.

Host by-products relating to feeding on cabbage (frass and regurgitate) are also involved in directing *C. rubecula* to cabbage infested by *P. rapae*. The nature of the attractive chemicals is unknown. At this stage the attraction to host products cannot be attributed to plant chemicals or host kairomones. If the attractive volatile chemicals are indeed plant chemicals, then they may be those that pass through the alimentary canal of *P. rapae* unchanged, or modified plant chemicals characteristic of digestion by *P. rapae*, or a unique combination of both. *C. rubecula* is also attracted to frass from the nonhost species *P. xylostella*. Although we do not know what volatiles the frass of *P. rapae* and *P. xylostella* have in common, the involvement of plant chemicals is possible as the two Lepidoptera have the same feeding habits. Attraction to frass and regurgitate have also been observed for other parasitoid species (Lewis and Jones, 1971; Elzen et al., 1987; Eller et al., 1988; Navasero and Elzen, 1989; Turlings et al., 1991).

C. rubecula from the Australian stock showed no attraction to larvae of *P. rapae*, although *C. rubecula* from British Columbia, Canada, did (Kaiser and Cardé, 1992). Some reports indicate that parasitoids are somewhat attracted to host larvae (Elzen et al., 1987; Navasero and Elzen, 1989) while others do not (Elzen et al., 1987; Eller et al., 1988). From our experience with *C. rubecula*, the degree of starvation of the larvae is fundamental for attraction or indifference. Larvae of *P. rapae* tend to vomit or excrete when they have not been sufficiently starved. *C. rubecula* is not attracted to larvae of *P. rapae* that do not vomit or excrete.

C. rubecula was not only attracted to nonhost plant species but was able to differentiate between intact and mechanically damaged nonhost plants and showed little attraction to the latter. This suggests that some components of the volatile chemical blend emitted by the damaged host plant may be the same as that of damaged bean and geranium. Attraction to parts of nonhost plant species is not uncommon among parasitoids (Elzen et al., 1983; Navasero and Elzen,

1989). The role of nonhost plant species in host habitat location needs to be elucidated.

No difference in behavior was exhibited between naive females and experienced females that had been exposed to the odor of an infested plant. It seems that the amount of plant material used in our work was much above the olfactory threshold that Kaiser and Cardé (1992) had found.

Finally, caution should be taken in handling plant material. *C. rubecula* was not attracted to intact cabbage plants but did fly to intact cabbage leaves held in a vial of water. This attraction could be a response to plant volatiles released by the cut petioles or to volatiles released by the undamaged part of the leaves as a result of physiological changes that the plants undergo after the petioles were cut. The blend of volatiles emitted by the different states of cabbage plant, as well as the part of the chemical blend *C. rubecula* utilizes in each case, needs further investigation.

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PLANT-NATURAL ENEMY ASSOCIATION IN THE TRITROPHIC SYSTEM *Cotesia rubecula-Pieris rapae*-BRASSICACEAE (CRUCIFERAE): II. PREFERENCE OF *C. rubecula* FOR LANDING AND SEARCHING

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Abstract—The responses of the parasitoid *Cotesia rubecula* to differently damaged cabbages were recorded during a series of choice tests. To determine if flying *C. rubecula* can discriminate differences in the blend of volatiles emitted by cabbages damaged by different causes and how plant volatiles released from a distant source affect the searching behavior of *C. rubecula* once searching on a plant, wasps were presented with a choice of plants located one behind the other and separated by a distance of 15 cm. The sources of damage were: cabbage damaged by the host (*Pieris rapae*), by a nonhost lepidopteran herbivore (*Plutella xylostella*), by a nonhost, noninsect herbivore (snail), and by mechanical means. The results showed that the site of first landing and the time spent searching on the leaves was influenced by the type of damage inflicted on plants. Wasps preferred to land on cabbages damaged by host and nonhost species of Lepidoptera over those damaged by snails and mechanical means. No preference was observed for first landing between cabbages damaged by the two species of Lepidoptera or between cabbages damaged by snails and mechanical means. Cabbage damaged by *P. rapae* was searched most intensively, followed by cabbage damaged by *P. xylostella*, cabbage damaged by snails, and cabbage damaged by mechanical means. *C. rubecula* differentiates between the volatile blends emitted by differently damaged cabbages, and it is attracted to volatiles related to recent lepidopteran damage. Wasps searched longer on freshly damaged than on leaves with older damage.

Key Words—*Cotesia rubecula*, Hymenoptera, Braconidae, Lepidoptera, Pieridae, Plutellidae, *Pieris rapae*, *Plutella xylostella*, *Helix aspera*, *Brassica*

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oleracea, *Phaseolus vulgaris*, tritrophic interactions, synomones, infochemicals.

INTRODUCTION

The host plant has become one of the main components examined in studies of interactions between insect herbivores and their natural enemies. Increasing evidence suggests that the host plant is the main provider of airborne chemicals utilized by natural enemies to locate their hosts or prey (Vinson, 1975, 1984; Auger et al., 1989; Ramachandran et al., 1991; Dicke et al., 1990a,b; Turlings et al., 1990, 1991; Whitman and Eller, 1990; Dicke and Takabayashi, 1991). Although the idea of chemical communication between the natural enemies of herbivores and plants was discussed by Price et al. (1980), only recently has chemical evidence of that communication been provided. Identification of the volatile compounds emitted by infested plants showed that these compounds are plant chemicals and that feeding by herbivorous insects can induce plants to release a blend of volatile chemicals different from that released during the intact state or when mechanically damaged (Dicke et al., 1990a,b; Turlings et al., 1990, 1991; Whitman and Eller, 1990; Dicke and Takabayashi, 1991). Natural enemies can perceive these differences and prefer to search plants that are damaged by their herbivorous hosts or prey (Dicke et al., 1990a; Turlings et al., 1990; Steinberg et al., 1993).

The aim of the present study was to investigate further the involvement of plant volatile chemicals in the searching behavior of the parasitoid *Cotesia rubecula* (Marshall) (Hymenoptera: Braconidae). *C. rubecula* is a solitary endoparasitoid of a number of lepidopteran species that feed on cabbage, with a preference for *Pieris rapae* L. as the host species (Lepidoptera: Pieridae) (Shenefelt, 1972). Females of *C. rubecula* are attracted from a distance to volatiles emitted by cabbage infested by *P. rapae* (Nealis, 1986; Keller, 1990; Kaiser and Cardé, 1992). Volatile compounds related to damaged cabbage attract *C. rubecula* (Agelopoulos and Keller, 1994). Females are attracted to cabbage damaged by mechanical means, *P. rapae*, *Plutella xylostella* L. (Lepidoptera: Plutellidae, a nonhost herbivore), and snails (*Helix aspera* Müler, a nonhost, noninsect herbivore). *C. rubecula* is also attracted to mechanically damaged, nonhost plant species (bean and geranium), frass and regurgitate of *P. rapae*, frass of *P. xylostella*, but not to isolated larvae of *P. rapae* or intact cabbage, bean, and geranium.

Although we showed that *C. rubecula* is attracted to volatile chemicals from damaged cabbage, it was not determined in our previous study if the parasitoid responds differently to the volatile blends from different kinds of damaged plants. To investigate this matter, a novel choice test was developed

to investigate differences in the responses of *C. rubecula* to different types of plant damage.

METHODS AND MATERIALS

Culturing of plants and insects and their handling during experiments were the same as described in Agelopoulos and Keller (1994).

Experimental Procedure. Two plants were arranged inside a wind tunnel (Keller, 1990) in a series (one behind the other), separated by a distance of 15 cm (Figure 1). The releasing tube (Agelopoulos and Keller, 1994) was placed 80 cm away from the downwind plant. The advantage of a serial test over a conventional parallel choice test is that plants arranged in a series produce a differential distribution of infochemicals inside the wind tunnel, and the space of the wind tunnel can be separated into three parts. Part A is the space between the releasing tube and the downwind plant where the air conveys information from both plants. Part B is the space between the downwind plant and the upwind plant where the air conveys information only from the upwind plant. Part C is the remainder of the wind tunnel where the air conveys no information. The preference of the flying female during the first landing is related to similarities or differences in the blend of volatile compounds emitted by the two plants. The time spent on the damaged leaf of each plant is related to stimuli encountered on the damaged leaves and to volatile chemicals that the air conveys. When searching on the downwind plant, the decision to fly further is related to stimuli encountered on the damaged leaf and volatile chemicals released from the upwind plant. When on the upwind plant, the decision to fly further is related to stimuli present on the damaged leaf only.

The movements and the behavior of a female during a test were recorded on video tape and subsequently analyzed using an event recorder (The Observer 2.0; Noldus, 1991). The maximum time of an observation was set at 10 min after the release of the insect. Thirty experienced females (see Agelopoulos and Keller, 1994) were tested for every pair of plants. To understand how the

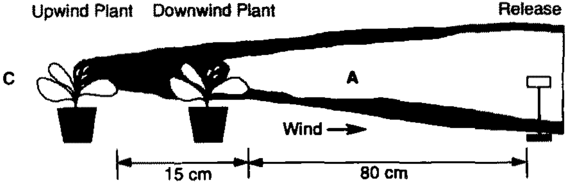

FIG. 1. Design of the serial choice test showing the distribution of plumes of volatile infochemicals from damaged leaves. A wasp in region A receives volatiles from both plants, in region B from the upwind plant only, and in region C from neither plant.

arrangement of the two plants (upwind vs. downwind) influences the movements of female wasps, 15 females were tested in one arrangement and 15 in the alternative arrangement. During an experimental day both arrangements were tested. The plant arrangement was changed every second observation. The cabbage plants used for an experiment were all of the same stage (seven leaves), while beans (*Phaseolus vulgaris* L.) had four leaves. The damage was restricted to only one leaf of the plant. Care was taken to ensure that the same amount of damage was present on each plant used for a test. During an experimental day, treated plants were replaced every hour. The wind speed was 54.3 cm/sec and the experiments took place between 8:00 AM and 12:00 noon. The components of behavior analyzed were the plant chosen for the first landing, the time spent on each damaged leaf during the first evaluation, and the overall time spent on the damaged leaf of each plant after multiple visits. For some females the first evaluation of a plant took place in one visit while for others the first evaluation took place in more than one visit. The first evaluation was defined as the time of all the visits (one or more) that females exhibited during their first encounter with a plant.

Treatments. Choice tests were separated into four groups (Table 1). In group A, the objective was to observe preferences of *C. rubecula* between mechanically damaged cabbages and those damaged by herbivores. In group B, the aim was to observe preferences of *C. rubecula* for cabbages damaged by different herbivores. In group C, the aim was to examine the effects of the age of damage on the preference of females. In group D, the objective was to record the responses of *C. rubecula* to mechanically damaged cabbage or bean (nonhost plant). The herbivores that damaged the cabbage plants were: *P. rapae* (host), *P. xylostella* (nonhost lepidopteran), and snails (*H. aspera*, a nonhost, noninsect herbivore). To obtain cabbage damage by lepidopteran larvae, 10 second instars of *P. rapae* or 10 fourth instars of *P. xylostella* were isolated on one leaf and left to feed for 24 hr. During this time the larvae consumed approximately 2 cm^2 of leaf tissue. Prior to the experiment the larvae and their by-products were removed and the plant was washed with water. To control the amount of damage done by snails, they were observed while feeding and removed when they had devoured approximately the same amount of leaf area as the other herbivores. After the removal of snails, the plant was washed. Mechanical damage was caused by removal of 2 cm^2 from the leaf using a razor blade.

Based on the time that the damage was inflicted on the plant, three different categories of damage were defined: fresh, recent, and old. Fresh damage was defined as the damage caused before the commencement of the tests such as mechanical damage on cabbage and bean and damage caused by snails. Recent damage was defined as the damage caused by *P. rapae* or *P. xylostella* after 24 hr of infestation. Old damage was obtained by holding a freshly damaged or

TABLE 1. RESPONSES OF *C. rubecula* TO CABBAGE DAMAGED BY DIFFERENT MEANS IN SERIAL CHOICE TEST[a]

Comparison		Downwind plant	Upwind plant
A. Mechanically-damaged cabbage vs. cabbage damaged by herbivores			
Fresh mechanical A vs. fresh mechanical B	I. Plant arrangement	Fresh mechanical A	Fresh mechanical B
	First landing (no.)	15	0
	Duration of first evaluation (min)[b]	0.79 ± 0.09[c]	0.04 ± 0.03
	Overall time on damaged leaf (min)	0.79 ± 0.09[c]	0.04 ± 0.03
	II. Plant arrangement	Fresh mechanical B	Fresh mechanical A
	First landing (no.)	13	0
	Duration of first evaluation (min)	1.38 ± 0.30[c]	0.42 ± 0.22
	Overall time on damaged leaf (min)	1.61 ± 0.33[c]	0.42 ± 0.22
	Preferred site of first landing	Downwind plant	
	Comparison of "first evaluation" between arrangements	Fresh mechanical A ns Fresh mechanical B[c]	
Fresh mechanical vs. recent *P. rapae*	I. Plant arrangement	Fresh mechanical	Recent *P. rapae*
	First landing (no.)	5	10
	Duration of first evaluation (min)	0.72 ± 0.29[c]	4.45 ± 0.94
	Overall time on damaged leaf (min)	1.05 ± 0.33[c]	7.07 ± 0.46
	II. Plant arrangement	Recent *P. rapae*	Fresh mechanical
	First landing (no.)	15	0
	Duration of first evaluation (min)	6.50 ± 0.88[c]	0.41 ± 0.22
	Overall time on damaged leaf (min)	7.82 ± 0.43[c]	0.41 ± 0.22
	Preferred site of first landing	Recent *P. rapae*	
	Comparison of "first evaluation" between arrangements	Fresh mechanical ns recent *P. rapae*[c]	
Fresh mechanical vs. recent *P. xylostella*	I. Plant arrangement	Fresh mechanical	Recent *P. xylostella*
	First landing (no.)	4	11
	Duration of first evaluation (min)	0.31 ± 0.07[c]	2.27 ± 0.58
	Overall time on damaged leaf (min)	0.40 ± 0.11[c]	4.94 ± 0.68
	II. Plant arrangement	Recent *P. xylostella*	Fresh mechanical
	First landing (no.)	15	0
	Duration of first evaluation (min)	3.65 ± 0.39[c]	0.96 ± 0.19
	Overall time on damaged leaf (min)	5.57 ± 0.43[c]	1.06 ± 0.24
	Preferred site of first landing	Recent *P. xylostella*	
	Comparison of "first evaluation" between arrangements	Fresh mechanical ns Recent *P. xylostella* ns	

Table 1. Continued

Comparison		Downwind plant	Upwind plant
A. Mechanically-damaged cabbage vs. cabbage damaged by herbivores			
Fresh mechanical vs. fresh snail	I. Plant arrangement	Fresh mechanical	Fresh snail
	First landing (no.)	15	0
	Duration of first evaluation (min)	2.70 ± 0.54 ns	2.14 ± 0.50
	Overall time on damaged leaf (min)	3.30 ± 0.55 ns	2.14 ± 0.50
	II. Plant arrangement	Fresh snail	Fresh mechanical
	First landing (no.)	14	1
	Duration of first evaluation (min)	2.12 ± 0.38 ns	1.87 ± 0.63
	Overall time on damaged leaf (min)	2.14 ± 0.64 ns	2.75 ± 0.40
	Preferred site of first landing	Downwind	
	Comparison of "first evaluation" between arrangements	Fresh mechanical ns Fresh snail ns	
B. Damage by different herbivores			
Recent *P. rapae* A vs. recent *P. rapae* B	I. Plant arrangement	Recent *P. rapae* A	Recent *P. rapae* B
	First landing (no.)	13	2
	Duration of first evaluation (min)	6.62 ± 0.78[c]	1.44 ± 0.57
	Overall time on damaged leaf (min)	6.84 ± 0.62[c]	1.44 ± 0.57
	II. Plant arrangement	Recent *P. rapae* B	Recent *P. rapae* A
	First landing (no.)	11	4
	Duration of first evaluation (min)	6.08 ± 0.62[c]	1.25 ± 0.64
	Overall time on damaged leaf (min)	6.17 ± 0.95[c]	2.68 ± 0.77
	Preferred site of first landing	Downwind	
	Comparison of "first evaluation" between arrangements	Recent *P. rapae* A[c] Recent *P. rapae* B[c]	
Recent *P. rapae* vs. recent *P. xylostella*	I. Plant arrangement	Recent *P. rapae*	Recent *P. xylostella*
	First landing (no.)	13	2
	Duration of first evaluation (min)	5.79 ± 0.87[c]	0.65 ± 0.24
	Overall time on damaged leaf (min)	7.69 ± 0.44[c]	0.71 ± 0.25
	II. Plant arrangement	Recent *P. xylostella*	Recent *P. rapae*
	First landing (no.)	14	1
	Duration of first evaluation (min)	0.99 ± 0.21[c]	5.80 ± 0.60
	Overall time on damaged leaf (min)	1.18 ± 0.25[c]	6.49 ± 0.49
	Preferred site of first landing	Downwind	
	Comparison of "first evaluation" between arrangements	Recent *P. rapae* ns recent *P. xylostella* ns	

TABLE 1. CONTINUED

Comparison		Downwind plant	Upwind plant
B. Damage by different herbivores			
Recent *P. rapae* vs. fresh snail	I. Plant arrangement	Recent *P. rapae*	Fresh snail
	First landing (no.)	14	1
	Duration of first evaluation (min)	7.56 ± 0.65[c]	0.55 ± 0.37
	Overall time on damaged leaf (min)	7.80 ± 0.50[c]	0.56 ± 0.37
	II. Plant arrangement	Fresh snail	Recent *P. rapae*
	First landing (no.)	5	10
	Duration of first evaluation (min)	0.57 ± 0.27[c]	7.34 ± 0.43
	Overall time on damaged leaf (min)	0.60 ± 0.29[c]	7.34 ± 0.43
	Preferred site of first landing	Recent *P. rapae*	
	Comparison of "first evaluation" between arrangements	Recent *P. rapae* ns Fresh snail ns	
Recent *P. xylostella* vs. fresh snail	I. Plant arrangement	Recent *P. xylostella*	Fresh snail
	First landing (no.)	15	0
	Duration of first evaluation (min)	4.18 ± 0.66[c]	1.37 ± 0.37
	Overall time on damaged leaf (min)	4.71 ± 0.66[c]	1.72 ± 0.39
	II. Plant arrangement	Fresh snail	Recent *P. xylostella*
	First landing (no.)	9	6
	Duration of first evaluation (min)	0.88 ± 0.26[c]	3.28 ± 0.56
	Overall time on damaged leaf (min)	0.95 ± 0.26[c]	4.45 ± 0.52
	Preferred site of first landing	Recent *P. xylostella*	
	Comparison of "first evaluation" between arrangements	Recent *P. xylostella* ns Fresh snail ns	
C. Old vs. fresh or recent damage			
Fresh mechanical vs. old mechanical	I. Plant arrangement	Fresh mechanical	Old mechanical
	First landing (no.)	12	3
	Duration of first evaluation (min)	1.07 ± 0.20 ns	2.27 ± 0.61
	Overall time on damaged leaf (min)	3.50 ± 0.61 ns	2.27 ± 0.61
	II. Plant arrangement	Old mechanical	Fresh mechanical
	First landing (no.)	11	4
	Duration of first evaluation (min)	2.85 ± 0.53[c]	0.65 ± 0.22
	Overall time on damaged leaf (min)	3.05 ± 0.65 ns	2.68 ± 0.53
	Preferred site of first landing	Downwind	
	Comparison of "first evaluation" between arrangements	Fresh mechanical ns Old mechanical ns	

TABLE 1. CONTINUED

Comparison		Downwind plant	Upwind plant
C. Old vs. fresh or recent damage			
Recent *P. rapae* vs. old *P. rapae*	I. Plant arrangement	Recent *P. rapae*	Old *P. rapae*
	First landing (no.)	15	0
	Duration of first evaluation (min)	6.77 ± 0.27[c]	0.06 ± 0.04
	Overall time on damaged leaf (min)	7.02 ± 0.23[c]	0.11 ± 0.08
	II. Plant arrangement	Old *P. rapae*	Recent *P. rapae*
	First landing (no.)	9	6
	Duration of first evaluation (min)	1.93 ± 0.43[c]	3.92 ± 0.59
	Overall time on damaged leaf (min)	2.66 ± 0.62[c]	4.47 ± 0.65
	Preferred site of first landing	Recent *P. rapae*	
	Comparison of "first evaluation" between arrangements	Recent *P. rapae*[c] Old *P. rapae*[c]	
Fresh mechanical vs. old *P. rapae*	I. Plant arrangement	Fresh mechanical	Old *P. rapae*
	First landing (no.)	15	0
	Duration of first evaluation (min)	2.12 ± 0.52 ns	1.99 ± 0.58
	Overall time on damaged leaf (min)	2.44 ± 0.39[c]	4.73 ± 0.28
	II. Plant arrangement	Old *P. rapae*	Fresh mechanical
	First landing (no.)	15	0
	Duration of first evaluation (min)	2.20 ± 0.53 ns	2.62 ± 0.51
	Overall time on damaged leaf (min)	5.43 ± 0.36[c]	1.07 ± 0.39
	Preferred site of first landing	Downwind	
	Comparison of "first evaluation" between arrangements	Fresh mechanical ns Old *P. rapae* ns	
D. Mechanically damaged cabbage vs. mechanically damaged bean			
Fresh mechanical vs. fresh bean	I. Plant arrangement	Fresh mechanical	Fresh bean
	First landing (no.)	13	0
	Duration of first evaluation (min)	1.37 ± 0.62 ns	0.19 ± 0.14
	Overall time on damaged leaf (min)	1.58 ± 0.66 ns	0.38 ± 0.33
	II. Plant arrangement	Fresh bean	Fresh mechanical
	First landing (no.)	12	0
	Duration of first evaluation (min)	0.58 ± 0.29 ns	0.59 ± 0.28
	Overall time on damaged leaf (min)	0.58 ± 0.29 ns	0.59 ± 0.28
	Preferred site of first landing	Downwind	
	Comparison of "first evaluation" between arrangements	Fresh mechanical ns Fresh bean ns	

[a] Observation period 10 min. $N = 15$/arrangement.
[b] Mean ± standard error.
[c] Significant difference ($P < 0.05$).

a recently damaged cabbage (without lepidopterans) for 15 hr at 25°C before it was used in the test.

Statistical Analysis. Within and between the arrangements, the total time spent searching on each damaged leaf was compared using paired and unpaired *t* tests, respectively. To decide which plant was the preferred site of first landing, the observed choices for first landing in the two arrangements of every choice test were compiled in a 2 × 2 contingency table and compared using χ^2 test (expected values were 7.5).

RESULTS

Group A: Mechanically Damaged Cabbage vs. Cabbage Damaged by Herbivores. Wasps preferred to land on plants damaged by the lepidopterans over those damaged by mechanical means (Table 1, A). No preference was observed when mechanically damaged cabbages were in a choice test with cabbages damaged by snails or by mechanical means. When cabbage damaged by the lepidopterans was paired with mechanically damaged cabbage, wasps searched longer on the damage caused by lepidopterans both during the first evaluation and overall. When mechanical damage was paired with mechanical damage or damage caused by snails, the time spent searching was equally distributed between the two damaged leaves.

Group B: Damage by Different Herbivores. Damage produced by the lepidopterans was preferred over that produced by snails (Table 1, B). No preference was observed between cabbage damaged by *P. rapae* and *P. xylostella.* Wasps spent longer times on leaves damaged by the lepidopterans than by snails, and when only damage by the lepidopterans was present, they spent more time on leaves damaged by *P. rapae.*

Group C: Old vs. Recent or Fresh Damage. Recent damage by *P. rapae* was preferred over old, but this was not the case for fresh mechanical damage vs. old mechanical damage or old damage caused by *P. rapae* (Table 1, C). Females stayed longer on recent damage of *P. rapae* than on old, and longer on old *P. rapae* than mechanical damage, but did not treat old and fresh mechanical damage differently.

Group D: Mechanically Damaged Cabbage vs. Mechanically Damaged Bean. No preference was observed for first landing, in both arrangements, and the females spent equal amounts of time on both damaged leaves (Table 1, D).

To understand better the results of these experiments, the treated plants were divided into three categories according to the preference for landing and the time spent searching on the damaged leaves. The first category includes those plants that, although located in the upwind position, were emitting a distinctive blend of volatile chemicals that stimulated the female to fly past the

downwind plant and land on the upwind one. The females spent more time on these plants. Such plants were, in group A: recently damaged by *P. rapae* (arrangement Fresh Mechanical—Recent *P. rapae*; Table 1) and recently damaged by *P. xylostella* (arrangement Fresh Mechanical—Recent *P. xylostella*); in group B: recently damaged by *P. rapae* (arrangement Fresh Snail—Recent *P. rapae*) and recently damaged by *P. xylostella* (arrangement Fresh Snail—Recent *P. xylostella*); and in group C: recently damaged by *P. rapae* (arrangement Old *P. rapae*—Recent *P. rapae*). The second category includes plants which, when in the upwind position in the arrangement, did not emit a distinctive blend of volatiles that directed the flying female over the downwind plant; the female landed on the first plant to be encountered, and the time spent on the damaged leaves of the two plants was equally distributed. Such plants are, in group A: freshly damaged by snails (arrangement Fresh Mechanical—Fresh Snail); in group D: fresh mechanically damaged bean (arrangement Fresh Mechanical—Fresh Bean); and in group C: fresh mechanically damaged cabbage (arrangement Old Mechanical—Fresh Mechanical), and old mechanically damaged cabbage (arrangement Fresh Mechanical—Old Mechanical). The third category is similar to the second category, the only difference being that the females spent longer times on one of the plants, although no preference in the first landing was observed. Such plants were, in group B: recently damaged by *P. rapae* (arrangement Recent *P. xylostella*—Recent *P. rapae*); and in group C: old damaged by *P. rapae* (arrangement Fresh Mechanical—Old *P. rapae*). Using the above divisions, the following hierarchy from most preferred to least preferred choice for first landing on damaged cabbage plants can be listed: (*P. rapae* $\cong$ *P. xylostella*) $>$ (snail $\cong$ mechanical $\cong$ bean), and recent *P. rapae* $>$ (old *P. rapae* $\cong$ fresh mechanical $\cong$ old mechanical). The hierarchy for time spent searching on damaged cabbage plants starting with the longest is *P. rapae* $>$ *P. xylostella* $>$ (snail $\cong$ mechanical $\cong$ bean).

DISCUSSION

The present study showed that the type of damage inflicted on the cabbage plants influenced the landing and the searching behavior of *C. rubecula*.

Preference for Landing. During all tests reported here the landing sites of the flying females were the damaged leaves, as was also observed in our previous study (Agelopoulos and Keller, 1994). Proof that the decision for landing is mainly governed by plant volatile chemicals related to damage is also apparent in the ability of *C. rubecula* to discriminate between plants based on the type of damage. *C. rubecula* preferred cabbages damaged by the two lepidopterans over those damaged by snails or mechanical means. The parasitoids *Cotesia glomerata* and *C. marginiventris* responded similarly when host-plants damaged

by their hosts were paired with host-plants damaged by mechanical means (Turlings et al., 1990; Steinberg et al., 1993). Although the above-mentioned preference for damaged plants by the lepidopterans over those damaged by snails or mechanical means showed that the volatile blends emitted were different, at this stage we cannot attribute the difference to the type of damage. Mechanical damage and damage caused by snails were inflicted on the plant just before the commencement of the tests, in comparison to damage by the lepidopterans, which was produced over a period of 24 hr. Further investigation is needed to understand if continuity of damage for a period of 24 hr induces the plant to produce a volatile blend different from that produced by short-term damage. *C. rubecula* could not distinguish while flying between cabbages damaged by *P. rapae* and cabbages damaged by *P. xylostella*. This can be attributed to the experience of females or to the fact that feeding by *P. rapae* induces the plant to produce more or less the same volatile chemicals as feeding by *P. xylostella*. We have to take into consideration that the females used for the tests had not been exposed to host-infested plants for 24 hr. Females of the parasitoid *Microplitis croceipes*, which had a preflight experience just before the commencement of the test with a cowpea-*Heliothis zea* complex, expressed a stronger attraction to *H. zea* than to *Spodoptera frugiperda* (nonhost species) feeding on cowpea (Zanen and Cardé, 1991). Further investigation is needed to understand if exposure of females to a host-infested plant just before the commencement of the experiment can affect their ability to distinguish between cabbages damaged by the two lepidopterans. Finally, *C. rubecula* favored recent over old damage caused by its host, indicating that when damage by larvae has stopped, the plant does not release the same volatile chemicals as when currently damaged by larvae.

Searching Behavior on Damaged Leaves. Both the time of first evaluation and the overall time spent searching on the damaged leaves was influenced by the type of damage. Damage caused by the lepidopterans was searched more intensively than damage caused by snails or mechanical means. When plants damaged by the lepidopterans were tested together, the females searched considerably longer on the leaf damaged by the host, implying that the two lepidopterans left different stimuli on the damaged leaves. The complexity of factors involved in searching behavior once a female is on a plant should not be underestimated. The stimuli encountered on a damaged leaf could be chemicals originating from the host or the plant. When there was a total absence of chemicals by the lepidopterans on plants, as in the case of mechanically damaged cabbage and beans, the females palpated the undamaged and damaged sites of the leaves, indicating that searching was induced by plant chemicals. The involvement of chemicals originating from the larvae in our experiment needs further study, as it is unknown if removing the larvae and their by-products and washing the plant with water is sufficient to remove all the chemicals related to them. It is

easy to attribute the longer searching time on leaves damaged by the host to host chemicals left on the leaves, but it is not easy to explain the fact that *C. rubecula* searched significantly longer on leaves damaged by *P. xylostella* over those damaged by mechanical means or snails. *P. xylostella* is not a host species. We suspect that the two species have some chemicals in common, as they utilize a similar range of host plants. It has been shown that frass of Lepidoptera feeding on the same host plant contains some common chemicals (Thibout et al., 1993), and it may be that this is a case of contamination by common frass chemicals. One could argue that *P. xylostella* has completely different kairomones from *P. rapae* but because infestations of both species commonly occur on the same plant, *C. rubecula* uses the kairomones of *P. xylostella* as indicators of the presence of its host nearby. We think this is unlikely, as kairomones of *P. xylostella* would not be reliable where infestation of *P. xylostella* occurs separately from that of *P. rapae* (Vet et al., 1991).

Movements between Plants. In all arrangements, both plants were visited by several wasps, and, in most cases, the downwind plant was the first to be searched. It seems that the decision to leave the downwind plant and fly to the upwind plant was induced by the absence of the host and, at the same time, by the perception of chemicals related to damage of a cabbage nearby (upwind plant). The upwind plant was searched as well. There were many cases where, after searching the upwind plant, the female wasps drifted back to the downwind plant. It seems that the decision to leave the upwind plant was induced by the absence of the host and the absence in the air of volatile chemicals related to damage. Memory of what had been encountered previously may be one of the factors involved in making the decision to fly back. The nonhost plant species, bean, was also visited and searched, implying that *C. rubecula* may spend time on damaged nonhost plant species during its search for hosts if nothing more attractive is near.

From the results of this and our previous study, we conclude that the plant is a substantial source of information to searching females of *C. rubecula*. It provides information about the presence and location of damage; if the damage is caused by Lepidoptera, other herbivores, or mechanical means; and if it is recent or old in the case of damage caused by Lepidoptera. The parasitoid species *Cotesia marginiventris* (Cresson) and *Cotesia glomerata* L. utilize the information emitted by their host-plants in a similar way (Steinberg et al., 1993; Turlings et al., 1990, 1991).

Identification of the volatile chemicals released by cabbage is needed to determine how the damage produced by various means and the duration of damage affect the volatile blend released.

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AGGREGATION PHEROMONE SYSTEM OF ADULT GREGARIOUS DESERT LOCUST SCHISTOCERCA GREGARIA (FORSKAL)

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Abstract—Six electrophysiologically active aromatic compounds, viz., anisole, benzaldehyde, veratrole, guaiacol, phenylacetonitrile, and phenol, were identified in the volatiles of older-adult male desert locust. Young adults and females of all age groups produced none or only trace quantities of these compounds. Comparison of the aggregation responses of young and older adults to the crude, older-adult, volatile extract and different synthetic blends of the six compounds showed that the aggregation pheromone system of the adult gregarious locust consists of phenylacetonitrile, guaiacol, phenol, and benzaldehyde. Like the crude volatile extract of older males, neither the synthetic blend of the six compounds nor the adult pheromone blend evoked any significant aggregation responses from nymphs. These results confirm our previous report of sexual differentiation in the production of adult aggregation pheromone in the desert locust and of the evidence of two distinct aggregation pheromone systems in the two stages of the insect.

Key Words—*Schistocerca gregaria*, Orthoptera, Acrididae, gregarious locusts, aggregation pheromones, anisole, benzaldehyde, veratrole, guaiacol, phenylacetonitrile, phenol.

INTRODUCTION

The mediation of chemical stimuli in the aggregation of gregarious locusts was first recognized by Nolte (1963) and later confirmed by Gillett (1968), who demonstrated that the grouping behavior of nymphs and adults of *Schistocerca*

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gregaria Forskal reared in visual and tactile isolation was influenced significantly by the action of an airborne factor. Nolte et al. (1970) traced the source of the aggregation stimulus to the hopper feces and identified 5-ethylguaiacol (5-ethyl-2-methoxyphenol), referred to as locustol, as the principal component of the gregarization pheromone (Nolte et al., 1973; Nolte, 1976). In a subsequent study, Fuzeau-Braesch et al. (1988) showed that airborne collections from different stages of *S. gregaria* and *Locusta migratoria* contained varying amounts of four components, three of which were identified as phenol, veratrole, and guaiacol. Bioassays of these compounds revealed that phenol, guaiacol, and the mixture of the three substances significantly increased the grouping behavior in both species, and they were considered as locust "cohesion" pheromones.

Results of our more recent studies in olfactometric bioassays suggest that the aggregation behavior of *S. gregaria* is mediated by two sets of releaser pheromone systems and that the nymphal stages of the locust respond only to nymphal volatiles, while the adults respond only to adult volatiles (Obeng-Ofori et al., 1993). Subsequently, we showed that the production of the pheromone in the adult is confined predominantly to the male (Obeng-Ofori et al., 1994) and that no such sexual differentiation occurs in the nymphs.

In continuing our studies on the pheromone system mediating the primer and releaser effects associated with locust gregarization, we have investigated the chemistry, electrophysiology, and releaser effects of airborne collections of adult stages of *S. gregaria*. In this paper we report the composition of the adult aggregation pheromone.

METHODS AND MATERIALS

Insects. Crowded desert locusts, *Schistocerca gregaria* (Forskal) (Orthoptera: Acrididae) from the ICIPE colony originating from a stock obtained from The Desert Locust Control Organisation for Eastern Africa (DLCO-EA) in Addis Ababa, Ethiopia, were used for the study. Insects (300–400) of both sexes were bred under crowded conditions in aluminum cages (50 × 50 × 50 cm). They were reared in a special room (4.5 × 4.5 m), which was well aerated by a duct system that maintained a negative pressure with a nycthemeral temperature of 30–35°C and a light–dark cycle of 12 : 12 hr. Fresh wheat shoots and bran were provided daily.

Collection of Volatiles. Volatiles from young (7–10 days after molt) and older (20–27 days after molt) adults were collected by aeration and adsorption on thoroughly cleaned charcoal (80–100 mesh, Chrompack) packed between two glass wool plugs in 6-cm-long × 8-mm-ID glass traps (Obeng-Ofori et al., 1993). Air from a compressed air cylinder was passed through a charcoal filter, over locusts contained in a 1-liter three-necked, round-bottomed flask, and

through the charcoal trap at 106 ml/min for 24 hr at 30 ± 1°C. Collections were done from: (1) 20 older adults (20–27 days after molt, 10 males and 10 females); (2) 10 older adult males and females (20–27 days after molt) separately, (3) 20 young adults (7–10 days after molt, 10 males and 10 females), and (4) 10 young adult males and females (7–10 days after molt) separately. Trapped volatiles were eluted with HPLC grade dichloromethane (Aldrich Ltd., 8 ml) and concentrated under a stream of nitrogen at 0°C to 100 μl. Five hundred nanograms of *o*-methylacetophenone were added as an internal standard for subsequent analyses.

Analyses of Volatiles. Aliquots (2 μl) of the extracts were analyzed by gas chromatography (GC) and by gas chromatography–mass spectrometry (GC-MS). GC analyses were performed on a Hewlett-Packard (HP) 5890 Series II gas chromatograph equipped with a flame ionization detector (FID) and a HP capillary column (Carbowax 20 M, 50 m × 0.2 mm ID × 0.2 μm) using nitrogen as the carrier gas at a flow rate of 0.35 ml/min. The oven temperature was initially isothermal at 60°C for 10 min, then programmed at 5°C/min to 180°C and to 220°C at 10°C/min. Chromatographic peaks were integrated using a HP 3396 integrator. GC-MS analyses were carried out on a VG Masslab 12-250 mass spectrometer (EI, 70 eV) coupled to a HP 5790 gas chromatograph.

Coupled gas chromatography–electroantennography (GC-EAD) was performed on the Carbowax 20 M, 50 m × 0.2 mm ID column and employed the same GC conditions as for analyses of volatiles. The column effluent was split 1 : 1 into two 50-cm-long deactivated fused silica columns connected to the FID and the antenna, respectively. A makeup gas (40 ml/min) was added just before the split point to accelerate the effluent through the deactivated columns. The effluents were driven from the chromatograph through a transfer line maintained at 150°C by a THC-3 temperature control unit (Syntech) and into a moistened airstream (4.0 ml/sec, 90% relative humidity, 25°C) focused onto the antennal preparation *via* a stainless steel tube (5 mm ID). The return time constant (RC) was set at 2 sec. FID and EAD signals were monitored synchronously using a program on a GC/EAD interface card (Syntech) installed in a PC (Harvard Professional Computer, American Megatrends Inc.).

Electroantennography. Electroantennograms (EAGs) were recorded from antennae of males and females of older adults. Each antenna was pulled off the head capsule so that it came off with a short segment of the antennal nerve. The scape and pedicel of the antenna were cut off, and the open base of the flagellum was inserted into the recording electrode (a glass micropipet containing locust saline) (Hoyle, 1951). Two segments of the antennal tip were also excised and the open end of the antenna inserted into the indifferent electrode. Both electrodes were appropriately connected to the universal AC/DC UNO5 amplifier (Syntech). Filter paper strips (1 × 2 cm) (Whatman No. 1) were inserted into the wide end (0.5 cm ID) of a 16-cm-long Pasteur pipet (Corning Inc.), and

test chemicals prepared in dichloromethane (HPLC grade, Aldrich Ltd.) were applied individually onto each strip at a dose of 10 μg (in 5 μl solvent). The controls were clean filter paper strips (air only) and filter papers imbued with 5 μl dichloromethane. A 200-msec pulse of air puffed through the Pasteur pipet into an airstream at 4 ml/sec delivered the test chemicals over the antennal preparations. The preparations containing the stimuli were used only once to avoid variations in EAG amplitudes arising from loss of chemicals due to their differential volatility and were applied in a randomized order. Six synthetic compounds (1–6), whose structures are shown in Figure 1, were tested on male and female antennae of older adults (N = 13). Arcsine square root transformed means of EAG amplitudes were subjected to a two-way analysis of variance by stimulus (S1) and sex (S2) (SAS Institute, 1985). Interaction S1 vs. S2 would reveal evidence of sexual differentiation in antennal responses to chemicals. Mean EAG amplitudes were compared using LSD test (α = 0.05).

Behavioral Bioassays. The method of performing behavioral bioassays of nymphs and adults of *S. gregaria* in a glass olfactometer has been described previously (Obeng-Ofori et al., 1993). Briefly, air from a compressed air cylinder, purified by passing through a charcoal filter, was split into two streams, each stream passing into a 2-liter, round-bottomed flask and then distributed into one of the two sides of the olfactometer at a flow rate of 120 ml/min/side. One flask contained the natural or synthetic locust volatiles, while the other flask acted as control. In between experiments, the connector tubings and flow meters were flushed with clean air and were also changed regularly to minimize contamination with volatiles. In addition, blank controls were run periodically, and control and treatment flasks were systematically switched so that they were alternately linked to the two sides of the chamber during tests.

The tests were carried out on fifth-instar nymphs (3–5 days after molt),

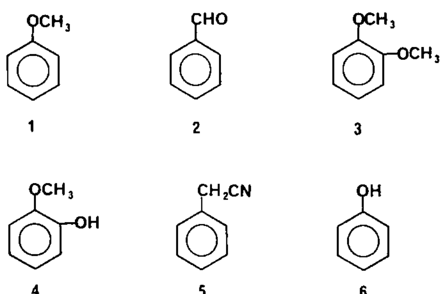

FIG. 1. Electrophysiologically active compounds found in volatiles from older adults *S. gregaria*. **1** = anisole (AS), **2** = benzaldehyde (BA), **3** = veratrole (VT), **4** = guaiacol (GA), **5** = phenylacetonitrile (PA), **6** = phenol (PN).

young adults (4–10 days after molt), and older adults (18–25 days after molt). Test solutions containing the stimuli were transferred into 3.7-ml glass vials, each containing 2 ml of light paraffin oil (Merck) to moderate the release of volatile compounds. Each vial was capped with a screw cap, which had a 1.5-mm vent. Doses were expressed as locust emission hours (LH) (1 LH = volatiles emitted by one locust for 1 hour). The test solution was held in one flask and the control (2 ml paraffin oil mixed with pure dichloromethane) in the other flask of the olfactometer. The synthetic test compounds were obtained from Aldrich Ltd., and the purity of each compound was checked by GC.

Sets of five locusts (young and older adults) of mixed sexes were bioassayed in each of the following situations: (1) control treatment with both sides free of locust odor; (2) a choice of control air column and column treated with a volatile collection of older adults (crude extract); (3) a choice of control air column and column treated with a 100:15:6:5:4:4 blend of synthetic phenylacetonitrile, benzaldehyde, veratrole, anisole, guaiacol, and phenol at release rates corresponding to 25, 50, 100, 150, 200 and 250 LH (the ratio represents the relative composition of a volatile collection from a single sample of a population of older adult males: see *Collection of Volatiles* above); (4) a choice of control air column and a column treated with each of the above-mentioned compounds presented separately in the relative concentration present in the natural blend and at the same release rates as in (3); (5) a choice of control air column and a column treated with synthetic pheromone components as shown in Table 1; and (6) as in (3) with 20-μl aliquots from solutions of individual compounds containing 0.1, 0.01, 0.001, 0.0001, and 0.00001 μmol to compare the intrinsic biological activities of the individual compounds. The aggregation responses of fifth-instar nymphs were tested in situations (2) and (3). In addition, the response of each of the sexes of locusts in the different stages to the synthetic blend was evaluated at a release rate corresponding to 150 LH.

Test insects were introduced into the bioassay arena of the olfactometer in groups of five, and after 15 min the number of insects in each section of the arena was counted. Uncommitted insects found in the middle part of the arena were treated as nonresponders. All tests were replicated five times, and locusts were used only once. The aggregation index was calculated as $100\ (T - C)/N$ where T is the number of locusts found in the treated compartment, C is the number of locusts found in the control compartment, and N is the total number of locusts tested. If this calculation resulted in a negative number, the aggregation index was assumed to be zero. Means of aggregation responses over the dose range were transformed into arcsine and submitted to a two-way analysis of variance by insect stage (F3) and volatile (F4). Interaction F3 vs. F4 would reveal aggregation responses of a particular stage to the volatiles. Mean aggregation responses over the dose range were compared using the LSD test (α = 0.05).

TABLE 1. MEANS OVER DOSE RANGE TESTED OF AGGREGATION RESPONSES OF *S. gregaria* TO VOLATILE CONSTITUENTS OF OLDER ADULTS TESTED AT CONCENTRATIONS OF 25–250 LH[a]

Treatment	Young adults	Older adults
Crude adult volatiles	66(5) a	67(7) a
PA + BA + VT + AS + GA + PN (100:15:6:5:4:4)[b]	61(7) a	64(6) a
PA	61(3) ab	62(4) ab
BA	37(3) c	37(2) c
VT	inactive	inactive
AS	inactive	inactive
GA	47(4) bc	49(4) bc
PN	46(5) c	44(5) c
BA + VT + AS + GA + PN	63(4) a	65(5) a
BA + GA + PN	63(5) a	63(4) a
VT + AS	inactive	inactive
GA + PN	59(6) ab	59(7) ab
PA + BA + GA + PN[b]	69(4) a	69(5) a
PA + GA + PN	65(4) a	67(4) a
PA + VT + AS	65(3) a	66(3) a

[a]Responses given as aggregation index. Standard errors in parentheses. Column and row means followed by the same letter are not significantly different ($P < 0.05$) by LSD test (percentage transformed to arcsine before analysis). PA = phenylacetonitrile, BA = benzaldehyde, VT = veratrole, AS = anisole, GA = guaiacol, PN = phenol.
[b]Evoked no aggregation responses from fifth instar nymphs.

RESULTS

Analysis of Volatiles. Gas chromatographic analysis of trapped volatiles from the different stages showed both qualitative and quantitative differences in the profiles (Figure 2). Trapped volatiles from young adults recorded consistently fewer peaks compared to trapped volatiles from older adults. Of the electrophysiologically active components (see below), although compound **5** was the major product in our volatile collections from older adults (comprising about 75–85% of the volatiles), it was conspicuously absent in the volatiles of young adults (Figure 2). Separate examination of the volatiles of male and female older adults showed that compounds **2**, **3**, and **5** were specific to the former (Figure 2); only trace amounts of these components were detected in trapped volatiles of older adult females in collections in which 50 or more individuals were used. Compounds **1**, **4**, and **6** were present as minor components in both sexes. The mean release rates of compounds **1–6** in 10 separate volatile collections from 10 older adult males were estimated by GC, using authentic samples of **1–6** to

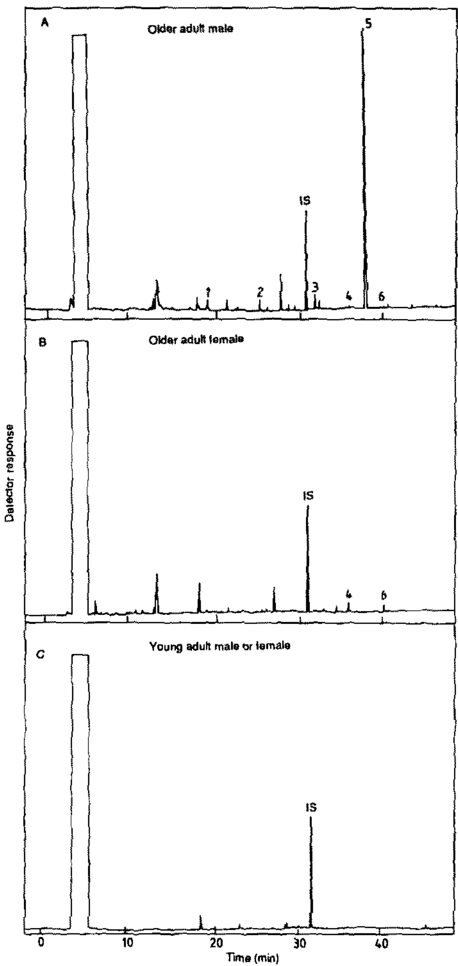

FIG. 2. Gas chromatograms of volatiles collected from older and young adult males and females of *S. gregaria* injected onto a 50-m Carbowax 20 M capillary column. The EAG-active peaks are labeled as compounds **1, 2, 3, 4, 5,** and **6**.

be ca. 0.9, 1.5, 2.0, 0.2, 50 and 0.7 ng/male/hr, respectively, and ranged between ca. 0.2–2.0, 0.3–4.5, 0.3–9.0, 0.03–0.9, 20.0–110.0, and 0.06–1.4 ng/male/hr, respectively. The **1:2:3:4:5:6** ratio calculated from GC peak areas ranged between 1:1:1:0.1:100:0.2 and 2:3:6:0.4:100:0.6.

GC-EAD analysis carried out on 10 separate collections, each from 10 older adult males, consistently gave EAD signals corresponding to GC peaks

labeled **2–6** from male and female antennae of young and older adults. Compound **5** evoked the strongest EAG response, and this may be partly related to its relative proportion in the volatiles (Figure 2). Compound **1** evoked no EAG responses at concentrations occurring in the natural extracts, but evoked weak responses when examined by GC-EAD at the natural concentration of compound **5**.

GC-MS analysis of compounds **1–6** gave mass spectra characteristic of the following, respectively: **1**, anisole (AS), *m/z* (rel. intensity) at 108(100), 65(82), 78(76), 39(36), 51(17), 79(17), 93(15), 63(12), 50(11), 38(10); **2** benzaldehyde (BA), *m/z* (rel. intensity) at 77(100), 106(88), 105(84), 51(48), 50(27), 78(17), 76(17), 52(11), 39(7); **3**, veratrole (VT), *m/z* (rel. intensity) at 138(100), 95(64), 77(54), 123(42), 65(28), 52(28), 41(22); **4**, guaiacol (GA), *m/z* (rel. intensity) at 109(100), 81(68), 124(64), 53(12), 40(10), 39 and 51(8); **5**, phenylacetonitrile (PA), *m/z* (rel. intensity) at 117(100), 90(51), 116(42), 89(34), 51(17), 63(16), 39(14), 77(10), 65(8); and **6**, phenol (PN), *m/z* (rel. intensity) at 94(100), 66(36), 39(29), 65(27), 40(18), 55 and 38(17). GC retention times of **1–6** and coinjection with authentic samples on Carbowax and methyl silicone columns confirmed the identities of the six compounds (Figure 1). Combined GC-EAD recordings using synthetic **1–6** confirmed the retention times and EAG activities observed with the natural extract.

Electroantennograms. EAG responses showed no significant differences between the responses of males and females (Figure 3). In both sexes the responses were significantly higher than the controls of air and dichloromethane. Phenylacetonitrile was most active, evoking a response ca. two to threefold greater than phenol, which evoked the weakest response. Responses evoked by guaiacol, veratrole, and benzaldehyde were not significantly different from one another, but were stronger than that for anisole.

Behavioral Bioassays. The crude volatile extract from older adults and the full blend of six electrophysiologically active components elicited high aggregation responses from both young and older adults but not from the nymphs

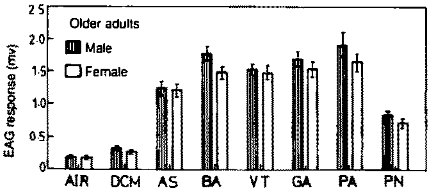

FIG. 3. Means of EAG amplitudes (mV) in response to air, dichloromethane (DCM), and 10 μg of each of the synthetic chemicals: anisole (AS), benzaldehyde (BA), veratrole (VT), guaiacol (GA), phenylacetonitrile (PA), and phenol (PN); $P < 0.05$.

(Figures 4A–D and Table 1).Aggregation responses shown by young and older adults to different blend combinations and also to the individual compounds were not significantly different (Figures 4A–D and 5A–D and Tables 1 and 2). Aggregation responses evoked by different synthetic blends, except the binary blend of veratrole and anisole (which was inactive), were not significantly different from that evoked by the crude volatile blend collected from the insects (Figures 4A–D and 5A–D and Table 1). Of individual compounds, phenylacetonitrile evoked the strongest aggregation responses from the insects, comparable to that of the crude volatile blend and those of synthetic blends. Guaiacol and phenol each elicited moderate activity, with benzaldehyde eliciting somewhat weaker activity in the two adult stages. The responses of the two adult sexes to the synthetic blend as well as to the individual components were not significantly different (Table 3).

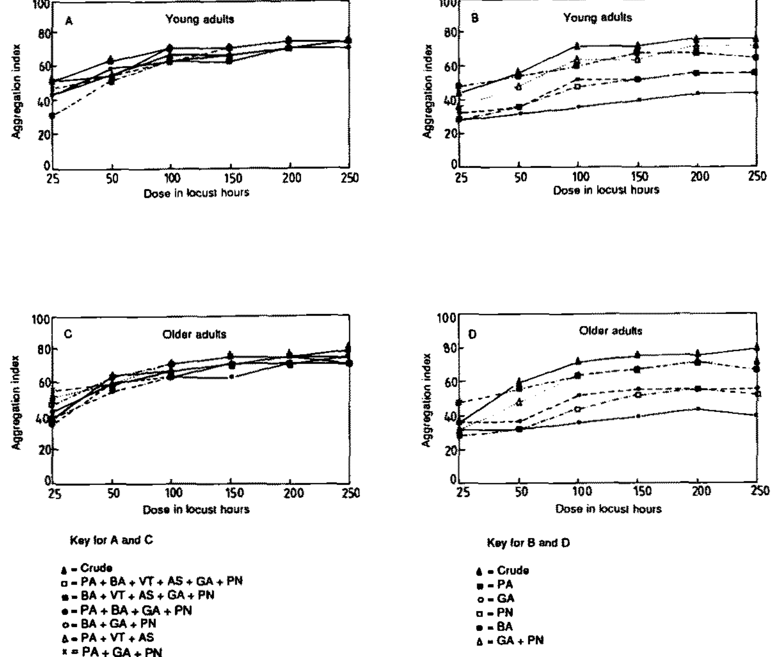

FIG. 4. Dose–response relationships for EAG-active components from adult volatiles at natural relative concentrations causing aggregation of young adults to: (A) crude volatiles and synthetic blends, (B) individual components; older adults to (C) crude volatiles and synthetic blends, (D) individual components.

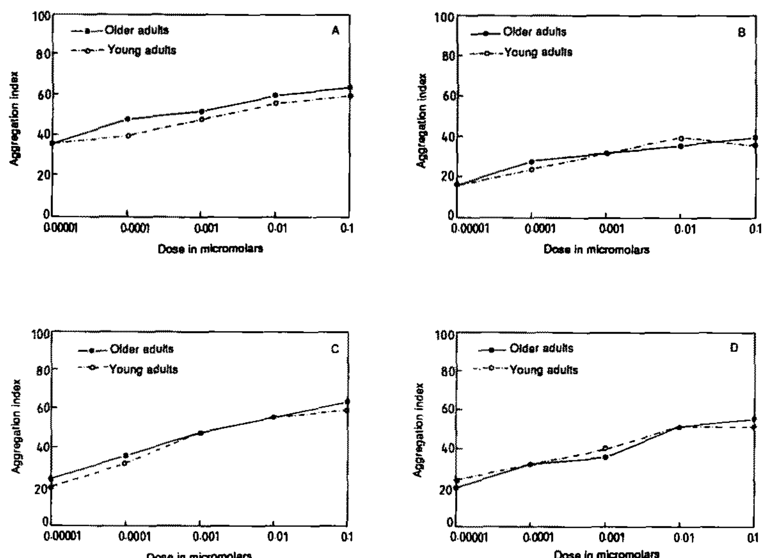

FIG. 5. Dose-response relationships in aggregation of young and older adults to individual EAG-active components from older adult volatiles at equimolar concentrations of (A) phenylacetonitrile, (B) benzaldehyde, (C) guaiacol, and (D) phenol.

TABLE 2. MEANS OVER DOSE RANGE TESTED OF AGGREGATION RESPONSES OF *S. gregaria* TO VOLATILE CONSTITUENTS OF OLDER ADULTS TESTED AT CONCENTRATIONS OF 0.00001–0.1 μM[a]

Treatment	Young adults	Older adults
Phenylacetonitrile	48(5) a	52(5) a
Benzaldehyde	30(4) b	30(4) b
Guaiacol	43(8) ab	46(7) ab
Phenol	40(6) ab	39(7) ab
Anisole	inactive	inactive
Veratrole	inactive	inactive

[a] Responses given as aggregation index. Standard errors in parentheses. Column and row means followed by the same letter are not significantly different ($P < 0.05$) by LSD test (percentage transformed to arcsine before analysis).

TABLE 3. AGGREGATION RESPONSES OF TWO SEXES OF *S. gregaria* TO VOLATILES PRODUCED BY OLDER ADULTS

	Aggregation index (%)[a]					
	Fifth-instar nymphs		Young adults		Older adults	
Treatment	Males	Females	Males	Females	Males	Females
Adult blend[b]	0 ns	0 ns	68**	64**	68**	72**
Phenylacetonitrile	0 ns	0 ns	60**	60**	64**	64**
Benzaldehyde	40*	40*	36*	32*	36*	44*
Guaiacol	60**	64**	60**	56**	56**	52**
Phenol	52**	56**	52**	60**	56**	52**
Anisole	inactive		inactive		inactive	
Veratrole	inactive		inactive		inactive	

[a] Difference from control (χ^2 test) indicated by: NS = not significant; $^*P < 0.05$, $^{**}P < 0.01$
[b] Dose of 150 LH in 2 ml paraffin oil.

DISCUSSION

Six electrophysiologically active aromatic compounds were found in the volatiles of older adult male desert locusts. These were identified as anisole, benzaldehyde, veratrole, guaiacol, phenylacetonitrile, and phenol (Figures 1 and 2). These compounds could not be detected in the volatile collections from young adults, which would account for the lack of an aggregation stimulus in this stage of the insect, which we had previously demonstrated in olfactometer bioassays (Obeng-Ofori et al., 1993, 1994). Comparison of volatiles emitted by the two sexes of the older adults (Figure 2) revealed that the compounds, particularly phenylacetonitrile, benzaldehyde, and veratrole, were associated with the males, although trace amounts of these compounds could be detected in the volatiles of older females. These findings confirm our previous olfactometric studies, which demonstrated sex differentiation in the production of aggregation pheromones in the adult gregarious desert locust (Obeng-Ofori et al., 1994).

Veratrole, guaiacol, and phenol had been previously identified as the key components of the "cohesion" pheromone for *S. gregaria* and *L. migratoria* (Fuzeau-Braesch et al., 1988). However, in our studies, these compounds were present only in minor amounts in the volatiles of older adults. To our knowledge, the other components of the blend—phenylacetonitrile, benzaldehyde and anisole—have not been reported previously in airborne collections of gregarious locusts.

Both the young and older adults were strongly induced to aggregate by the crude volatile extract and the blend of six electrophysiologically active com-

pounds in the proportion found in adult male volatiles (Figures 4A and C and Table 1). On the other hand, the nymphs were indifferent to these blends (Table 1). These results confirm our earlier report on the existence of two distinct releaser aggregation pheromone systems in the desert locust, one specific to adults and the other to nymphs (Obeng-Ofori et al., 1993).

Of the six electrophysiologically active compounds, the compositionally dominant phenylacetonitrile elicited the strongest aggregation responses from the two stages of the adult insect and was comparable to the crude older adult volatile extract (Figure 4B and D and Table 1). Guaiacol and phenol each elicited moderate activity, with benzaldehyde being somewhat less active. Anisole and veratrole individually and in a binary mixture were inactive in eliciting this behavior. Addition of this binary mixture to phenylacetonitrile or to a blend of guaiacol, phenol, and benzaldehyde did not significantly affect the aggregation responses of both young and older adults when compared to the former and latter stimuli (Figure 4A and C and Table 1).

The propensity of the insect to aggregate more or less to the same extent in response to different blends (Figure 4A and C and Table 1) is unexpected. Most surprising are the responses elicited by blends lacking in phenylacetonitrile (such as the blend of benzaldehyde, guaiacol, and phenol), which are not significantly different from those elicited by blends containing this compound, including the crude volatile collection from the insect. Since phenylacetonitrile is not only a dominant component of volatile emissions of older males (ca. 80%) but is also intrinsically the most potent in eliciting the aggregation response in adults, the fact that it may be replaced by blends of other minor components without apparent loss of overall aggregation response in our bioassay system is quite remarkable. The phenomenon of mutually replaceable components in chemical communication was first reported with respect to minor components of the sex pheromone of the cabbage looper moth, *Trichoplusia ni* (Linn et al., 1984), and its neurophysiological basis was recently investigated (Todd et al., 1992). Our understanding of the basis of the phenomenon in the desert locust must await similar detailed sensory physiological studies as well as the elucidation of the role of different components in eliciting different elements of behavior that may be involved in the aggregation process.

What then constitutes the aggregation pheromone of the adult desert locust? Three possible candidates may be considered, each of which is able to simulate the effects of the crude volatile collections from the older males of the insect: phenylacetonitrile alone, the blend of six electrophysiologically active compounds, which includes two inactive constituents, and the blend of the four compounds of varying individual activity. Since the insect perceives a blend rather than a single compound and since no significant behavioral effects could be demonstrated for anisole and veratrole, we propose that the aggregation

pheromone system of the adult gregarious desert locust is made up of a blend of phenylacetonitrile, guaiacol, phenol, and benzaldehyde.

As expected on the basis of our previous findings (Obeng-Ofori et al., 1994), the male-produced aggregation pheromone is active with respect to both sexes of the adult (Table 3). Indeed, the responses of the two sexes to the synthetic blend as well as to the active individual components are not significantly different. Moreover, no significant differences between the EAG responses of the males and females were found (Figure 3), reflecting the occurrence of the same level of receptors in the two sexes for these components as has been reported for *Locusta migratoria* (Greenwood and Chapman, 1984).

In summary, our results confirm the existence of stage differentiation in pheromone-mediated aggregation behavior of the desert locust. Additionally, they confirm the existence of sex differentiation in the biosynthesis of the adult pheromone, with the males assuming the task of producing this pheromone. Work on the primer effects of the pheromone components and other volatile constituents on adults and the identification of the nymphal aggregation pheromone system is in progress.

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CHEMICAL SUPPRESSION OF FEEDING IN LARVAL WEAKFISH (*Cynoscion regalis*) BY TROCHOPHORES OF THE SERPULID POLYCHAETE *Hydroides dianthus*

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Abstract—We studied the effect of exudates from trochophore larvae of the polychaete *Hydroides dianthus* on feeding in larval weakfish (*Cynoscion regalis*). Laboratory prey consisted of *H. dianthus* trochophores and/or comparably sized rotifers (*Brachionus plicatilis*). When experiments were conducted in filtered seawater, ingestion of rotifers was always greater than ingestion of trochophores. However, consumption of rotifers was depressed when water from *H. dianthus* cultures (= trochophore water) was the experimental medium. The same effect was noted whether we added trochophore water from polychaete cultures that were two or five days postfertilization. However, no effect was noted when we used water from rotifer cultures. We concluded that *H. dianthus* trochophores release a water-soluble compound that inhibits feeding in weakfish larvae.

Key Words—Fish larvae, chemical ecology, feeding suppression, *Cynoscion*, *Hydroides*, trochophore, rotifers, fish, polychaete.

INTRODUCTION

While most species of fish larvae are visual predators (e.g., Hunter, 1980), olfaction may play a role in the foraging behavior that precedes actual ingestion. For example, Iwai (1980) found that for many species, taste buds do not appear

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until late in development, and even then, the buds are restricted to the interior of the mouth. Thus, Iwai concluded that any chemical stimuli important in preingestion foraging must be olfactory in nature (Iwai, 1980). Similarly, Dempsey (1978) found that herring larvae showed an olfactory response when foraging for brine shrimp nauplii (*Artemia* sp.). More recent work on weakfish larvae (*Cynoscion regalis*) has shown lateral-line development during the larval stage (Connaughton et al., 1993); however, the influence of other senses has not been examined.

Weakfish larvae are planktonic, and the duration of larval development is approximately three weeks (Duffy and Epifanio, 1993). Experiments examining prey selection by larval weakfish have shown them to be selective feeders, but preferences are not dependent upon only one factor (Connaughton and Epifanio, 1993; Pryor and Epifanio, 1993). Preliminary laboratory experiments have shown that weakfish rarely ingest trochophore larvae of the serpulid polychaete *Hydroides dianthus* and that feeding on rotifers (*Brachionus plicatilis*) may be reduced in the presence of these trochophores (Connaughton, 1994). This suggests some type of chemical defense system in the trochophores. Indeed, there is evidence that larvae of several species of invertebrates may release noxious chemicals that induce predators to avoid the larvae or to spit them out (Lucas et al., 1979; Young and Bingham, 1987; Luckenbach and Orth, 1990). These types of chemical defense systems may be more common among species that lack morphological deterrents to predation (e.g., Pennington and Chia, 1984; Morgan, 1987). In the present study, we have investigated the mechanism behind the observed suppression of feeding by larval weakfish when in the presence of *H. dianthus* trochophores.

METHODS AND MATERIALS

Larval Culture. Adult weakfish, *Cynoscion regalis* (Bloch and Schneider), were collected from Delaware Bay in May and June, 1992. Gametes were removed from ripe specimens, and fertilized eggs were returned to the laboratory. Fish larvae were cultured in the laboratory at 20–21°C and 30 ppt on a 14-hr light 10-hr dark photoperiod and were fed a diet of wild zooplankton (53–253 μm) until needed for the experimental trials (Pryor and Epifanio, 1993). As previous results (Pryor and Epifanio, 1993) indicated that older larvae (≥ 11 days post-hatching) fed better than younger, older larvae (11 and 13 days post-hatching) were used in our experiments.

In all experiments, fish larvae were fed diets consisting of various combinations of trochophores (*Hydroides dianthus*) and/or rotifers (*Brachionus plicatilis*). Adult *H. dianthus* were collected from the field and maintained on a

diet of naked flagellates (*Isochrysis galbana*) and diatoms (*Chaetoceros* sp.) in the laboratory. Worms were induced to spawn by removing them from their tubes (Costello et al., 1957), and the fertilized eggs were kept overnight in filtered seawater. Trochophores were cultured in 5-liter containers of filtered seawater, and a dilute suspension of *I. galbana* was added daily. Two- and five-day-old trochophores were used in the experiments. Length and width of 50 trochophores of each age were measured using a computer-assisted image analysis system. Five-day-old trochophores had mean dimensions of 188 × 144 μm and mean values for 2-day-old trochophores were 97 × 89 μm.

Rotifers were maintained in laboratory culture and fed a diet of naked flagellates daily. Rotifers are a common prey item used in the culture of larval fish (e.g., Theilacker and McMaster, 1971) and a preferred laboratory food of larval weakfish (Pryor and Epifanio, 1993). In some of the experiments, we used two size classes of rotifers (large: 216 × 162 μm, and small: 160 × 107 μm).

Water from 2-, 3-, 4-, and/or 5-day-old trochophores was used for bioassays after chemical partitioning of the water. Initial experiments showed that both 2- and 5-day-old trochophore water suppressed feeding in larval weakfish, so we concluded that water from trochophores between these ages could be used in the analysis.

Selection Experiments. Because we had conducted a number of earlier experiments on selective feeding by weakfish larvae, we were interested in determining the effect of *H. dianthus* trochophores on this process. The experimental protocol was identical to that described in Pryor and Epifanio (1993). Trials were conducted in 40-liter cylindrical tanks. Weakfish larvae (11 days post hatching) were placed in each experimental tank on the evening before an experimental trial. This allowed the larvae to clear their guts and acclimate to the experimental tanks. In each trial, weakfish larvae were allowed to select between two prey types that were added in equal abundances (50 : 50). In selection experiments that included only rotifers, experimental tanks were filled with 40 liters of filtered seawater, 100 fish larvae were placed in each tank, and prey abundance was 10,000 prey/liter. In experiments that included polychaete trochophores, we reduced the volume to 10 liters and reduced the numbers of prey and fish larvae accordingly. We did this because of limited supplies of polychaete trochophores.

After three hours of feeding, weakfish larvae were removed and preserved on frozen blocks of 5% formaldehyde (Connaughton and Epifanio, 1993; Pryor and Epifanio, 1993). This was done to reduce regurgitation by larval fish. Subsequent analysis of gut contents allowed determination of the number of each prey type ingested by each fish. The proportion of each prey type was determined from the pooled gut contents of each tank. We used a goodness of fit test (*G* test) based on binomial proportions (Zar, 1984) and a sequential Bonferroni

technique (Rice, 1990) to test for significant deviations from the expected proportions (50:50).

Water-Solubility Experiments. All experiments were performed at 20–21°C in 1-liter, black-lined fingerbowls filled with filtered seawater. There were four treatments: (1) 5-day-old trochophore water, (2) 2-day-old trochophore water, (3) rotifer water, and (4) a filtered seawater control. There were five replicate bowls within each treatment, and 10 fish larvae per bowl.

We define trochophore water as the water in which the trochophores were cultured. Water was filtered to remove algae and trochophores before it was used. Rotifer water was prepared in a similar manner. Weakfish larvae (11 days post hatching) were placed in the appropriate bowls 2 hr before the experiment. Preliminary experiments showed 2 hr to be a sufficient time period for acclimation and gut clearance. Rotifers (mixed size classes) were then added to the bowls (10,000/liter), and weakfish larvae were allowed to feed undisturbed for 3 hr. Analysis of gut contents then proceeded as above.

Results were analyzed using nonparametric methods (Kruskal-Wallis one-way ANOVA) and an a posteriori multiple comparison test (Zar, 1984, pp. 199–201), which compared the mean number of rotifers ingested in each treatment.

Chemical Analysis of Trochophore Water. Water from 4- and 5-day-old trochophore cultures was fractionated to further isolate the active metabolites. Two different methods were used. In the first, 5-day-old trochophore water was fractionated by reverse-phase vacuum liquid chromatography (VLC, C-2 Kieselguhr, 10–40 μm). Trochophore water was filtered twice through the sorbent. The resulting eluent constituted the aqueous fraction. Next, the sorbent was eluted with methanol and chloroform to yield an organic fraction. Solvents from the organic fraction were evaporated in vacuo. An aqueous fraction of filtered seawater put through the VLC served as a control for potential sorbent effects. The second method involved a liquid–liquid partitioning between 4-day-old trochophore seawater and chloroform. Both the aqueous and the organic fractions were evaporated in vacuo to remove the residual organic solvent.

Bioassays to establish the effect of the fractionated trochophore water were conducted on 13-day post hatching weakfish larvae. There were six treatments: (1) filtered seawater, (2) filtered seawater passed through the VLC column, (3) trochophore organic fraction removed from the VLC sorbent, (4) trochophore seawater (aqueous fraction) passed through the VLC sorbent, (5) trochophore organic fraction removed by chloroform partitioning, and (6) trochophore seawater (aqueous fraction) after chloroform partitioning. Each treatment had five replicate bowls with 10 fish larvae per bowl. Larvae were acclimated to each treatment for 2 h prior to the experiment. Experimental duration was 3 h and the number of rotifers consumed in each treatment was determined by gut analysis.

RESULTS

Just as in earlier experiments (Connaughton and Epifanio, 1993; Pryor and Epifanio, 1993), selection experiments showed that weakfish larvae prefer large to small rotifers (Table 1). In this treatment the larval fish consumed over 3600 rotifers (approximately 48 rotifers/larvae/hr). When weakfish larvae were given a choice between 2- and 5-day-old trochophores, they consumed a total of 38 prey items (approximately 0.51 trochophores/larvae/hr). In treatments where weakfish larvae chose between rotifers and trochophores, the total ingestion of trochophores was always less than 20 and total ingestion of rotifers never exceeded 306 (approximately 4 rotifers/larvae/hr).

In the water-solubility experiment, there was a significant effect of *H. dianthus* treatments on the mean number of rotifers ingested by weakfish larvae ($P = 0.002$). Feeding in both trochophore water treatments was lower than feeding in the rotifer water or seawater control (Figure 1).

The results of the experiments using organic and aqueous water fractions show a significant decrease in the number of rotifers eaten in both organic fractions as well as the aqueous VLC fraction (Figure 2). There was no difference in the number of rotifers eaten between the aqueous and organic VLC treatments. Treatments containing the organic fraction extracted with chloroform were significantly lower than their aqueous counterparts. They were also significantly lower than either VLC fraction. There was no difference between the filtered seawater control, the VLC seawater control, and the aqueous fraction from the liquid–liquid extraction (Figure 2).

TABLE 1. *Cynoscion regalis*: RESULTS OF PAIRWISE PREY SELECTION USING 11 DAY POST HATCHING WEAKFISH LARVAE[a]

Treatment	Feeding ratio
LR/SR	**3248/363
LH/SH	18/20
LR/LH	2/1
LR/SH	**306/1
SR/LH	14/13
SR/SH	**84/14

[a] Treatment is the paired prey combination administered to the larvae. Feeding ratio is the number of each prey item from the gut contents, paired respectively with treatment ratios. An asterisk denotes a significant difference from the underlying (50:50) ratio and is adjacent to the preferred item. LR = large rotifers; SR = small rotifers; LH = large *H. dianthus* trochophores; SH = small *H. dianthus* trochophores.

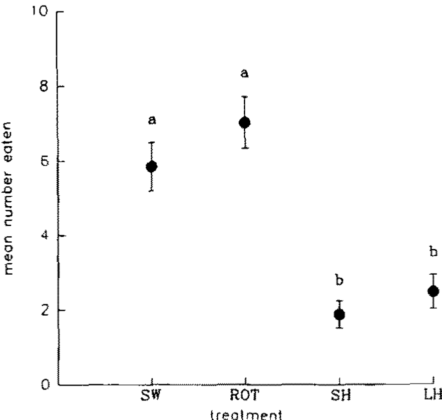

FIG. 1. *Cynoscion regalis:* number of rotifers consumed in four different water treatments. Values presented are the mean (± standard error). SW = filtered seawater control; ROT = rotifer water; SH = small (2-day-old) *H. dianthus* water; LH = large (5-day-old) *H. dianthus* water. Data points with the same letter are not significantly different.

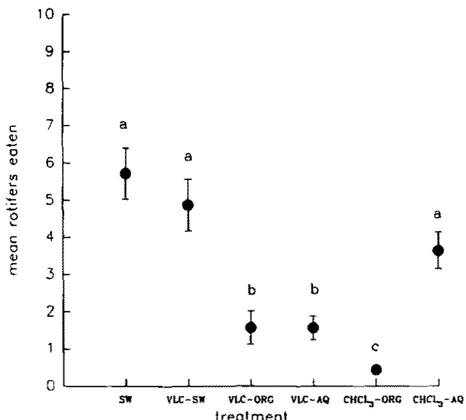

FIG. 2. *Cynoscion regalis:* number of rotifers eaten in the different treatments after separation of the water using vacuum liquid chromatography (VLC) and liquid–liquid partitioning ($CHCL_3$–seawater). Values presented are the mean (± standard error). SW = filtered seawater control; VLC-SW = filtered seawater through VLC column; VLC-ORG = fraction of *H. dianthus* absorbed onto VLC sorbent; VLC-AQ = fraction of *H. dianthus* water passing through VLC sorbent; $CHCL_3$-ORG = organic fraction from liquid–liquid partition; $CHCL_3$-AQ = aqueous fraction from liquid–liquid partition. Data points with the same letter are not significantly different.

DISCUSSION

The outcome of our selection experiment gives a clear indication that the trochophore larvae of *H. dianthus* are unpalatable to weakfish, regardless of the age of the trochophores. This could be interpreted as the result of either noxious taste, which would require physical contact between the prey organism and the sensory organ, or noxious odor, which in the marine environment implies some waterborne chemical. The results of our second experiment support the latter hypothesis because they show that ingestion of rotifers is depressed by the mere presence of water in which trochophores had been cultured, again regardless of the age of the trochophores. While the trochophore water may have also contained the exudates of the algae that were used to feed the trochophores, an identical algal diet was used in rotifer culture, and rotifer water caused no suppression of feeding in weakfish larvae.

While feeding by larval weakfish was always significantly lower in the trochophore water treatments than in the controls, a greater suppression of feeding was seen in the selection experiments when rotifers were paired with large rather than small trochophores. Large trochophores are similar in size to the small rotifers and may have been initially consumed by weakfish larvae. This consumption of the large trochophores may have reinforced the aversion resulting in inhibition of further feeding. However, since we were unable to determine the order in which weakfish larvae consumed the prey items, we do not know if feeding suppression by the water-soluble chemical is enhanced by contact.

Analysis of the gut contents of field-caught specimens indicates that polychaete larvae are an important item within the diet of larval weakfish, at least in Delaware Bay, USA (Goshorn and Epifanio, 1991). However, *H. dianthus* trochophores are small, lack spines or setae (Scheltema et al., 1981), and have a relatively slow swimming speed (mean speed = 2.5 mm/sec) (Connaughton, 1994). Thus a chemical defense system may be particularly important to this species. In a similar vein, Luckenbach and Orth (1990) presented evidence that suggested a chemical deterrent in the larvae of *Pinnotheres maculata*, a zoea form with poorly developed spines. In a related study, larvae of the starfish *Acanthaster planci* were found to have saponins, which deterred predation by larval fish (Lucas et al., 1979), with the amount of deterrence dependent upon the degree of hunger of the fish. Furthermore, tadpole larvae of the ascidian *Ecteinascidia turbinata* also contain metabolites that make them unpalatable to fish predators (Young and Bingham, 1987).

Initial fractionation of the trochophore water indicated that VLC on a C-2 sorbent would not sufficiently separate the active metabolites. However, a chloroform–seawater liquid–liquid partition shows that the active metabolites are present in the organic fraction. The exact nature of the active compound remains unknown.

The results of our study indicate that feeding in weakfish larvae can be suppressed by a waterborne chemical(s) released from *H. dianthus* trochophores. As spawning of adult *Hydroides* coincides with larval period for weakfish in the Delaware Bay (Costello et al., 1957), the presence of the trochophores could influence feeding of larval weakfish in the field by suppressing the consumption of other prey items.

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SIMULTANEOUS EFFECTS OF FERULIC AND *p*-COUMARIC ACIDS ON CUCUMBER LEAF EXPANSION IN SPLIT-ROOT EXPERIMENTS[1]

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Abstract—Experiments were conducted to determine how plant responses to mixtures of allelochemicals may change as the proportion of roots in contact with allelochemicals is modified. Thirteen-day-old cucumber seedlings were treated with ferulic and/or *p*-coumaric acid in a split-root nutrient culture system. Leaf areas were determined just prior to treatment and at harvest, 24 hr after treatment. Ferulic acid was more inhibitory to cucumber leaf expansion than *p*-coumaric acid. The effects of ferulic and *p*-coumaric acids on leaf expansion were additive. For individual acids, mixtures of ferulic and *p*-coumaric acids in the same container and combinations of ferulic and *p*-coumaric acids in separate containers, the inhibition of leaf expansion was directly related to the product of the concentration of the acid(s) and the proportion of roots treated with the acid(s). Pretreatment with 0.2 or 0.4 mM ferulic acid and subsequent treatment with 0.5 mM ferulic acid failed to show evidence of acclimation by cucumber seedlings.

Key Words—Allelopathy, ferulic acid, *p*-coumaric acid, phenolic acid mixtures, *Cucumis sativus*, split-root treatments, leaf expansion.

INTRODUCTION

Many potentially allelopathic compounds have been identified in a wide variety of soil types from numerous ecosystems (Whitehead, 1964; Guenzi and McCalla,

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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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1966; Shindo et al., 1978; Whitehead et al., 1982, 1983; Kuiters and Denneman, 1987). Of these compounds, the widely studied phenolic acids are believed to be among the most important. Actual concentrations of individual phenolic acids in soils have been estimated to be below the level required for inhibition of plant growth and development (Whitehead et al., 1981). This has led to doubts concerning the contribution of phenolics to allelopathy under field conditions. However, it has been suggested that noninhibitory concentrations of individual phenolic acids could, in combination, have inhibitory effects on germination and plant growth (Einhellig, 1987). The combined action of allelopathics in a mixture may be additive, antagonistic, or synergistic (Einhellig, 1987; Blum et al., 1989; Lyu et al., 1990; Gerig and Blum, 1991). Unless compounds are completely antagonistic, all three types of combined actions could result in mixtures having a greater inhibitory or stimulatory effect on plant growth than would be predicted by the concentration of any one allelochemical. Gerig and Blum (1991) have found that the amounts of individual phenolic acids in four-compound mixtures required for an inhibitory effect were small enough to be in the concentration range actually found in soil extracts.

In addition to the composition of these mixtures, the distribution of the source of such mixtures in soils is also important. Patrick (1971) and Patrick et al. (1964) noted the uneven distribution in soils of organic fragments, a primary source of allelopathic compounds. Such uneven distribution suggested that only a portion of any root system may be in contact with allelopathic agents and that this proportion would change with plant growth and development. Very few studies have examined how allelopathic effects are influenced by the proportion of a plant's root system in contact with allelochemicals. In split-root studies using cucumber seedlings, it has been shown that the impact of ferulic acid on leaf expansion (Klein and Blum, 1990) and on the uptake of phosphorus, potassium, and water (Lyu and Blum, 1990) was related to the proportion of the roots in contact with the acid. How plant responses to mixtures of allelochemicals may change as the proportion of roots in contact with allelochemicals is modified has not yet been examined. This study investigates this topic, using cucumber as the bioassay species and ferulic and *p*-coumaric acids as two representative allelopathic compounds.

METHODS AND MATERIALS

Plant Material. Cucumber seeds (*Cucumis sativus* L. cv. Early Green Cluster) were germinated in trays of vermiculite in the dark at 30°C for two days. The seedlings were then exposed to 11 hr of light (levels given below) and 12 hr of dark before transplanting them into jars containing 110 ml of Hoagland's solution (pH 5.5) (Hoagland and Arnon, 1950). The seedlings were suspended

by foam collars placed in holes in the lids of the jars. The jars were wrapped in aluminum foil to exclude light. Seedlings were grown for an additional 10 days under light banks (140 $\mu E/m^2/sec$; 12-hr photoperiod) at room temperature (21–30°C) (Blum and Dalton, 1985). Deionized water was added daily to replace water lost via evapotranspiration. Nutrient solutions were changed five days after transplanting.

Individual Acid Experiments. One day 13, seedling roots were suspended in 700-ml containers that enclosed two smaller containers (Klein and Blum, 1990). The seedling root mass was placed entirely within one of the small containers or was split (approx. 1:2; see data analysis for details) between the two small containers. The small containers were filled with 100 ml of Hoagland's solution or with one of four concentrations (0.2, 0.4, 0.6, 0.8 mM) of ferulic (FER) or *p*-coumaric (PCO) acid in Hoagland's solution (pH 5.5). Starting one day prior to treatment, length and width measurements of the leaves were recorded daily. Seedlings were harvested to obtain dry weights of roots in each small container 24 hr after treatment.

The experiment using FER was run separately from the experiment using PCO (N = 52 in each experiment); there was a one-week delay between the treatment dates of the two experiments.

Only one container (100 ml) was used when the entire root system was treated since the nutrients supplied were more than adequate for normal growth (Blum and Dalton, 1985). Preliminary experiments also showed that the use of one or two containers did not modify leaf area expansion, indicating that root injury associated with the process of splitting roots into the small containers was minimal.

Mixture Experiment. The general procedures of this experiment were the same as the previous experiments. However, in this case, various fractions (see data analysis for details) of the root were treated with either 0.50 mM FER, 0.64 mM PCO, or a mixture with 0.25 mM FER and 0.32 mM PCO. All of these phenolic acid solutions contained Hoagland's solution (pH 5.5). The remaining proportion of roots not placed in one of these treatment solutions was placed in Hoagland's solution. Also included in this experiment was a group of seedlings that had their root mass split (approx. 1:2 or 2:1) between 0.50 mM FER and 0.64 mM PCO in Hoagland's solution. The total number of seedlings used in this experiment was 48.

Potential Acclimation. To test the possibility that cucumbers could acclimate to phenolic acids, seedlings were pretreated with one of three pretreatment concentrations (0, 0.2, or 0.4 mM) of FER prior to treating with 0.5 mM FER (pH 5.5). Seedlings were pretreated on days 9 and 11 by changing all solutions and were treated on day 13 in the split root system as previously described.

Data Analysis. Leaf length (L; millimeters) and width (W; millimeters) measurements were used to calculate leaf areas using the following formula:

leaf area = $-1.457 + [0.00769 * (L * W)]$ (Blum and Dalton, 1985). Absolute and relative rates of leaf expansion (*AGR* and *RGR*, respectively) were then calculated as follows (Radford, 1967):

$$AGR = \text{leaf area on day}_{x+1} - \text{leaf area on day}_x$$

$$RGR = \ln(\text{leaf area on day}_{x+1}) - \ln(\text{leaf area on day}_x)$$

The units for *AGR* and *RGR* are square centimeters per day and square centimeters/square centimeter per day, respectively. Leaf area of cucumber seedlings has been shown to be directly related to dry weight (Blum and Dalton, 1985).

Dry weights of roots in each of the small containers were used to calculate the actual proportion of roots per container. These calculated values were used in all analyses. Root dry weight has been shown to be directly related to root length (Blum and Rebbeck, 1989).

Data were analyzed using the Statistical Analysis System (SAS) programs for analysis of variance and regressions (SAS Institute, Inc., 1988).

RESULTS

Individual Acid Experiments. At a given concentration, FER had a greater inhibitory effect than PCO on cucumber leaf expansion. For both FER and PCO, the inhibition of leaf expansion was directly related to the product of the concentration of the phenolic acid and the proportion of roots treated with the phenolic acid. The models for FER effects on absolute (*AGR*) and relative (*RGR*) growth rates were as follows:

$$AGR = 42.15 - 29.28\ (\text{CONC} * \text{PFER}) \qquad P = 0.0001 \qquad r^2 = 0.76$$

$$RGR = 0.378 - 0.241\ (\text{CONC} * \text{PFER}) \qquad P = 0.0001 \qquad r^2 = 0.77$$

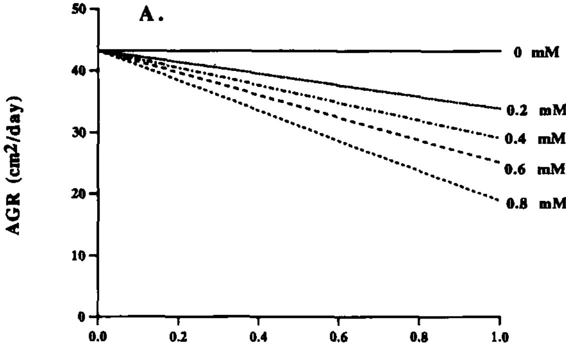
where CONC is the concentration of FER (millimolar) and PFER is the proportion of roots treated with FER. The models for the effects of PCO on *AGR* and *RGR* were as follows:

$$AGR = 39.36 - 22.96\ (\text{CONC} * \text{PPCO}) \qquad P = 0.0001 \qquad r^2 = 0.68$$

$$RGR = 0.406 - 0.196\ (\text{CONC} * \text{PPCO}) \qquad P = 0.0001 \qquad r^2 = 0.58$$

where CONC is the concentration of PCO (millimolar) and PPCO is the proportion of roots treated with PCO. *AGR* regression lines for each of the compounds and their concentrations used in this experiment are shown on Figure 1. Figure 2 shows the percent reductions of *AGR* from the control values of 41.86 (FER) and 40.25 (PCO) cm^2/day for the whole-root treatment groups.

Mixture Experiment. Analysis using Duncan's multiple-range test found no significant difference between the 0.50 mM FER and the 0.64 mM PCO treat-



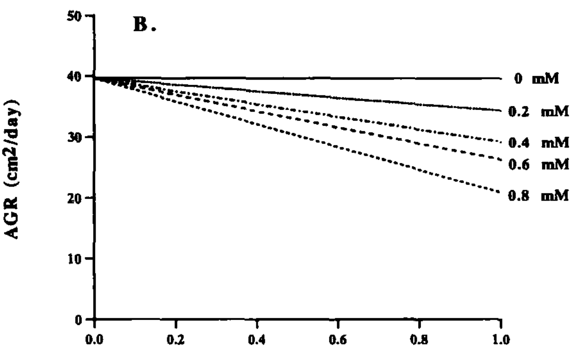


FIG. 1. Regression lines for absolute rates of leaf expansion (*AGR*) of cucumber seedlings for which various proportions of roots were treated with 0, 0.2, 0.4, 0.6, or 0.8 mM of (A) ferulic acid or (B) *p*-coumaric acid. The units given for proportion of treated roots are provided merely as reference points and do not represent actual experimental units. See text for regression equations.

ment effects on *AGR* and *RGR*, indicating that these chosen concentrations provided statistically equivalent doses. This analysis also found no significant difference for *AGR* and *RGR* between these two treatments and the mixture (0.25 mM FER + 0.32 mM PCO) treatment, indicating that the combined action of FER and PCO was additive. *AGR* and *RGR* of seedlings whose roots were treated with FER and PCO in separate containers were not significantly different

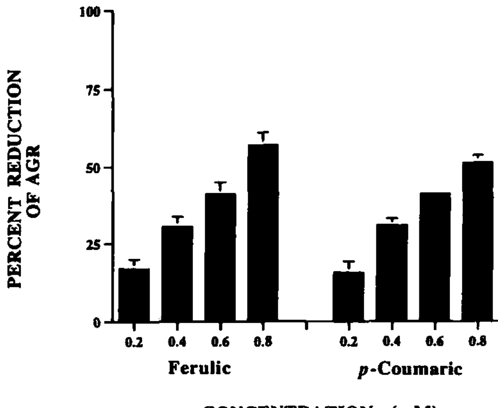


FIG. 2. Percent reduction of absolute rates of cucumber leaf expansion (*AGR*) from control values of 41.86 (ferulic) and 40.25 (*p*-coumaric) cm^2/day when entire root system was treated with 0.2, 0.4, 0.6, or 0.8 mM ferulic or *p*-coumaric acid. The bars denote the standard error of the mean with four replicates.

from the *AGR* and *RGR* of seedlings whose roots were treated with a combination of FER and PCO in the same container. A preliminary experiment (data not presented) using 0.5 mM FER and 0.5 mM PCO treatments in separate containers ($N = 50$) also resulted in additive effects of FER and PCO on cucumber leaf expansion.

As observed with the individual phenolic acids, the inhibition of leaf expansion was directly related to the product of the concentration of the phenolic acids and the proportion of the roots treated with the phenolic acids. Thus, a single model could be used to describe the effects of combinations and mixtures of phenolic acids on leaf expansion (Figure 3). The models for *AGR* and *RGR* were as follows:

$$AGR = 42.81 - 35.26(\text{CONCF} * \text{PFER}) - 27.94(\text{CONCP} * \text{PPCO})$$

$$P = 0.0001 \qquad r^2 = 0.69$$

$$RGR = 0.402 - 0.272(\text{CONCF} * \text{PFER}) - 0.227(\text{CONCP} * \text{PPCO})$$

$$P = 0.0001 \qquad r^2 = 0.70$$

where CONCF is the concentration of FER (millimolar), CONCP is the concentration of PCO (millimolar), PFER is the proportion of roots treated with FER, and PPCO is the proportion of roots treated with PCO.

Potential Acclimation. Pretreatment of cucumber seedling roots with 0.2 mM or 0.4 mM FER had no significant effect on the subsequent response of

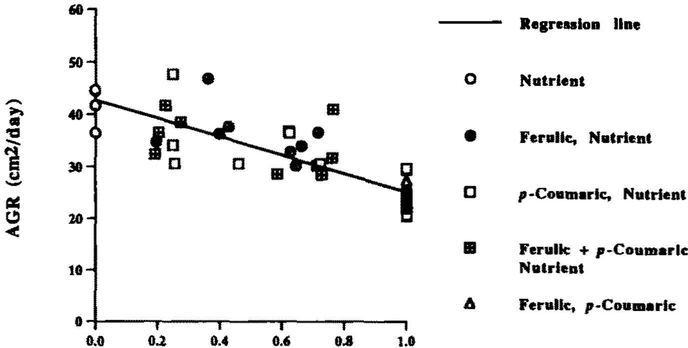


FIG. 3. Regression line and data points (N = 48) for absolute rates of leaf expansion (*AGR*) of cucumber seedlings for which various proportions of roots were treated with equivalent doses of ferulic and/or *p*-coumaric acids. See text for regression equations.

AGR and *RGR* to treatment with 0.5 mM FER. This suggested that, under the conditions of the experiment, *AGR* and *RGR* of cucumber seedlings did not acclimate to FER treatment.

DISCUSSION

The results of this study are consistent with those of Klein and Blum (1990), who found that the inhibition of cucumber leaf expansion was directly related to the proportion of roots exposed to ferulic acid (FER). During a given experiment, Klein and Blum (1990) used only one concentration of FER. In this study, we used several concentrations of ferulic and *p*-coumaric (PCO) acids and found that at all concentrations of both phenolic acids, inhibition of leaf expansion was directly related to the proportion of roots treated. Klein and Blum's (1990) single treatment experiments had a treatment period of 48 hr. We chose a 24-hr treatment period to minimize root growth (which could change the proportion of roots/container) and to avoid possible confounding of results by the recovery of cucumber seedlings during the second 24-hr period (Blum and Rebbeck, 1989; Klein and Blum, 1990).

The applicability of this study's findings to natural systems was assessed by investigating the potential for acclimation. Low concentrations of allelopathics like phenolic acids are likely to be constantly present in nature. If these background concentrations cause acclimation in seedlings, then the exposure to higher concentrations would not have the effects seen in these experiments.

Previous experiments by Blum and Dalton (1985) suggested that cucumber seedlings do not acclimate to FER. We suspected that, perhaps, the pretreatment concentrations used by Blum and Dalton (1985) were too low (0.1 and 0.2 mM) and/or that the subsequent treatment was too high (1.0 mM) for acclimation to be detected. However, our pretreatment concentrations of 0.2 and 0.4 mM and treatment concentration of 0.5 mM FER also failed to show evidence of acclimation.

In nature a plant does not encounter individual compounds, but rather a complex mixture of different compounds, many of which may be allelopathic. The differential uptake of phenolic acids in mixtures shown by Shann and Blum (1987) and Lyu et al. (1990) suggested that compounds may interfere with each other when acting on the same roots; this may not occur if the same compounds simultaneously act on different portions of a plant's root system. This study, however, found no significant difference between exposing the same roots to a mixture of FER and PCO in a single container and simultaneously exposing different roots to FER and PCO in separate containers.

For this mixture experiment, 0.50 mM FER and 0.64 mM PCO concentrations were chosen as "equivalent doses," based on the results of the individual acid experiments. These were the concentrations necessary to cause approximately 35% reduction in the treatment groups when the entire root system was treated with FER or PCO. Because preliminary experiments and other studies using FER and PCO mixtures indicated that the combined action of FER and PCO on cucumber leaf expansion are additive, the chosen concentrations of FER and PCO were cut in half to have a mixture whose "active dose" was theoretically equivalent to the 0.50 mM FER and the 0.64 mM PCO treatments. The results indicated that 0.50 mM FER and 0.64 mM PCO provided equivalent doses and that the combined action of FER and PCO were additive. Additive effects of FER and PCO have also been reported by Blum et al. (1985a,b), Lyu et al. (1990), and Gerig and Blum (1991).

A unique aspect of this study is the characterization of the relationship between concentration and the proportion of roots exposed to phenolic acid(s). The inhibition of leaf expansion was directly related to the product of the concentration of the phenolic acid(s) and the proportion of roots treated with the acid(s). This means that doubling the amount of roots exposed to phenolic acid(s) has the same effect on leaf expansion as doubling the concentration of the acid(s) to which the root portion is exposed. This relationship was found with individual acids (FER and PCO), mixtures of FER and PCO in the same container, and combinations of FER and PCO in separate containers. These results suggest that the inhibition of cucumber leaf expansion by phenolic acids occurs via a localized phenomenon at the root level.

This study clearly indicates that determining the role of phenolic acids in allelopathic interactions requires more than bulk soil determination of single-

compound concentrations. In nature, the action of mixtures is likely to be of more importance than single compounds. If all phenolic acids work in an additive manner similar to that observed with FER and PCO in this study, the level of inhibition will depend on the total "active dose" and the total proportion of roots exposed to the acid(s). This suggests that the distribution of roots and allelochemicals in nature may substantially influence the magnitude of allelopathic interactions. In addition to total active dose and the proportion of roots in contact with phenolic acid mixtures, soil pH (Balke, 1985; Blum et al., 1985b, 1989), nutrition (Stowe and Osborn, 1980; Hall et al., 1982, 1983), temperature (Glass, 1976; Bhowmik and Doll, 1983; Einhellig and Eckrich, 1984), etc., must also be considered.

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CHEMICAL COMPOSITION OF NORTH AMERICAN BEE PROPOLIS AND BIOLOGICAL ACTIVITY TOWARDS LARVAE OF GREATER WAX MOTH (*Lepidoptera: Pyralidae*)

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Abstract—Bee propolis is a sticky amalgamation of plant resins collected by honeybees (*Apis mellifera* L.) and used in the hive for filling cracks and repairing combs. Propolis contains a diversity of compounds of plant origin, and is reported to have medicinal, antimicrobial, insecticidal, and phytotoxic properties. We examined the physical and chemical composition of North American samples of bee propolis from several sites in North America and tested for bioactivity against larvae of the greater wax moth (*Galleria mellonella* L.), a common apiary pest. The amount of methanol-extractable resin in samples from Ohio and Georgia ranged from 24% to 79% by weight. Propolis collected from hives in Ohio was more chemically diverse (over 30 compounds detected by paper chromatography) than material from south Georgia (fewer than 10 major compounds) and contained a lower proportion of methanol-insoluble beeswax. The paper chromatographic surveys revealed little variation in the chemical profile of specific hives over a six-month period and no differences between propolis from adjacent hives. Four flavonoids were identified from propolis collected in Ohio: kaempferol, galangin, 3,3′-dimethoxyquercetin and 3-methoxykaempferol. When mixed into artificial diet, fractionated propolis reduced larval growth of the greater wax moth, but not dramatically. An array of phenolics reported from propolis (caffeic acid, chrysin, ferulic acid, galangin, kaempferol, and quercetin) were bioassayed individually for effects on larvae, but none reduced larval growth at the

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concentrations tested, suggesting that wax moths are tolerant of some phenolics in their diet.

Key Words—*Galleria mellonella*, *Apis mellifera*, Pyralidae, Apidae, Lepidoptera, Hymenoptera, greater wax moth, honeybee, propolis, plant resins, phenolics.

INTRODUCTION

Propolis is a gluelike substance that honeybees (*Apis mellifera* Linnaeus) collect from plant exudates and use in the hive to fill cracks and repair comb. It is reported to have bacteriostatic properties that help control the microflora in the hive, and antibacterial, antifungal, antiviral, and phytoinhibitory properties of propolis and its constituents have been demonstrated by a number of workers (Ghisalberti, 1979; Pepeljnjak et al., 1985; Serkedjieva, 1992). Over 70 compounds, mostly flavonoids, have been reported from propolis in Europe (Bankova et al., 1982, 1983; Ghisalberti, 1979; Maciejewicz et al., 1985; Nenov et al., 1983; Popravdo et al., 1982), but the composition and biological activity of propolis in North America is largely unstudied (but see Lindenfelser, 1967). Recently, propolis mixed into artificial diet was found to reduce growth and survival of greater wax moth larvae (*Galleria mellonella* L. Pyralidae: Lepidoptera), (Eischen and Dietz, 1987), which feed on honeycomb and pollen and are pests of apiaries. Eischen and Dietz (1987) suggested that the biological activity of propolis might provide new opportunities for the biological control of the greater wax moth, since the use of insecticides in active hives is problematic. This project was undertaken to characterize the chemical composition of bee propolis from several apiaries in the eastern United States and to evaluate the effects of propolis fractions on survival and growth of wax moth larvae. Several phenolics commonly found in European propolis (caffeic acid, chrysin, ferulic acid, galangin, kaempferol, and quercetin) were also assayed for activity against greater wax moth larvae.

METHODS AND MATERIALS

Source of Propolis. Propolis of Italian honeybees (*A. mellifera ligustica* Spinola) was collected at three sites in the United States: western Ohio, north Georgia (Athens), and south Georgia (Claxton). In western Ohio, hives were surrounded by predominantly deciduous forest; in north Georgia by a variety of deciduous trees and conifers, and in south Georgia, hives were located in pine forest.

The following characteristics of propolis from the three sites were compared: (1) relative amounts of methanol-soluble resin and insoluble residue

(beeswax), (2) the proportions of material in different solvent fractions, and (3) the chemical profile of the fraction richest in phenolics and other ultraviolet-absorbing compounds (ethyl acetate). Seasonal and between-hive variation in chemical composition at one site (north Georgia) was evaluated by comparing paper chromatograms of the ethyl acetate fraction of monthly samples from three adjacent colonies (ca. 2 m apart). At this site, propolis was sampled from June 1987 to May 1988 excluding winter months (September through February) when hive activity was low. Propolis was collected by scraping the lid and upper edges of the topmost super of multiple hives at each site and stored at −4°C until analysis.

Fractionation of Propolis and Chromatographic Survey. Frozen propolis (three Ohio samples and three Georgia samples) was ground into a coarse powder and extracted in four volumes of 80% methanol (24 hr each). The insoluble residue that remained (mostly beeswax) was removed by filtering and dried at room temperature. The methanol-soluble portion of propolis was fractionated as follows: extract was evaporated in vacuo to a near-aqueous solution (aqueous methanol) and partitioned with petroleum ether (petroleum ether I) followed by ethyl acetate. The ethyl acetate fraction was then partitioned with petroleum ether (petroleum ether II). The percent composition of resin and wax fractions of Ohio (three samples) and north Georgia (two samples) propolis were compared using Student's t test ($P < 0.05$). One sample from south Georgia was also fractionated, but was not included in the analysis because the extraction procedure was not replicated.

For bioassays, the ethyl acetate fraction of one sample of Ohio propolis was further fractionated on a Sephadex LH 20-100 flash column eluted with a methanol gradient (50%–100%). Fractions (100 ml) were collected as they eluted and combined into four major fractions (designated A, B, C, and D in order of elution) based on the similarity of ultraviolet-absorbing compounds on two-dimensional paper chromatograms.

After preliminary surveys of chromatograms of the four major fractions (aqueous methanol, petroleum ether I, petroleum ether II, and ethyl acetate) from each site, efforts to identify compounds were restricted to the ethyl acetate fraction, which contained the majority of ultraviolet-absorbing compounds. Compounds in these fractions were isolated using two-dimensional descending paper chromatography (2D-PC) developed in two solvent systems: TBA (*t*-butyl alcohol–acetic acid–H_2O, 3:1:1, v/v, long dimension) and 15% acetic acid (glacial acetic acid–H_2O, 15:85, v/v, short dimension). Chromatograms were observed under long-wave ultraviolet light (365 nm) with and without ammonia fuming.

Isolation and Identification of Compounds. Compounds were isolated using 2D-PC developed in a variety of solvent systems, including water, Forestal's solvent system (HOAc–H_2O–HCl, 30:10:3) or the BAW system (*n*-butanol–

HOAc-H_2O, 4:1:5) (Markham, 1982; Mabry et al., 1970). Compound purity was verified by thin-layer chromatography (polyamide developed in chloroform–methanol–methyl ethyl ketone–acetone, 15:10:5:1, v/v (Stahl, 1969). Flavonoid identifications were based on spectral characteristics (following Markham, 1982; Mabry et al., 1970; Jay et al., 1975), R_f values from two solvent systems (one-dimensional paper chromatograms in TBA and 15% acetic acid), and co-chromatography with standards (3,3′-dimethoxyquercetin and 3-methoxykaempferol supplied by T. Mabry).

Bioassay of Propolis Fractions and Selected Flavonoids. Greater wax moths used in the bioassays were less than three generations from the wild (collected from Wilbanks Apiary, Claxton, Georgia). Larvae were reared on a pollen–honeycomb diet (63:37) at 27°C in partial darkness as described by Eischen and Dietz (1987). Feeding assays were initiated by placing unhatched eggs in glass jars (approximately 120 ml) ventilated by 1-cm screened holes in the lid. Larvae were allowed to feed until the prepupal stage, then all jars were frozen and larvae were retrieved from the diet.

Diet for the assays was prepared by hand mixing propolis fractions (10% propolis g/g dissolved in 70 ml ethanol) into the pollen–honeycomb diet, then allowing the solvent to evaporate by spreading the diet in shallow pans over low heat (approximately 40°C) for several days. To ensure complete evaporation of solvent, diet was then ground into pea-sized chunks and lyophilized for 48 hr. Two control diets, one treated with ethanol and another with nothing added, were prepared simultaneously and handled the same as the treatment diets. After lyophilization, dried diet was weighed directly into glass jars and rehydrated with distilled water.

In the first bioassay, only the methanol-insoluble residue (beeswax) and aqueous methanol fraction were tested. In the second bioassay, the methanol-soluble fractions (petroleum ether I, petroleum ether II, and the four component fractions of the ethyl acetate fraction A, B, C, D) were tested. The following commercially available phenolics were also included in the second bioassay: kaempferol (0.05%), quercetin (0.05, 0.25, 0.5%), chrysin (0.05, 0.25, 0.5%), galangin (0.05%), caffeic acid (0.05%), and ferulic acid (0.05%). All were obtained from Aldrich Chemical Co., Milwaukee, Wisconsin, except for kaempferol, which was purchased from Sigma Chemical Co., St. Louis, Missouri. Each diet treatment was replicated in 15–20 jars containing 10–15 larvae per jar. Significant diet differences were detected by ANOVA on larval dry weights (log transformed to correct for unequal variances) and Dunnett's test (Steel and Torrie, 1960) used to compare treatment means to the solvent control. Larval survival was not analyzed because only the pooled survival across all replicates per diet treatment had been recorded.

RESULTS

Composition of Propolis—Geographic and Seasonal Variation The amount of methanol-soluble resin in propolis from different sites ranged from a low of 23.3% in south Georgia to 78.3% in Ohio. The remainder of the propolis consisted of methanol-insoluble residue (mostly beeswax). The proportion of insoluble residue was significantly higher in propolis from north Georgia than in samples from Ohio, in which the ethyl acetate fraction constituted a higher percentage of the gross weight (Table 1). In contrast, in samples from north Georgia, the petroleum ether II fraction was the largest fraction.

Judging from the paper chromatographic profiles of extracts, propolis collected in south Georgia appeared to have low chemical diversity, with fewer than 10 major compounds visible. Chromatograms of Ohio propolis typically contained more than 30 ultraviolet absorbant spots. We detected little qualitative differences within sites, nor was there evidence of seasonal or between-hive variation in the monthly samples taken from adjacent hives at the north Georgia site.

Isolation and Identification of Compounds. Eleven compounds with flavonoid like ultraviolet spectra were isolated from the ethyl acetate fraction of Ohio propolis. Four flavonoid aglycones were identified as kaempferol, galangin, 3,3'-dimethoxyquercetin, and 3-methoxykaempferol from their spectral characteristics and cochromatography with authentic standards. All but the latter have been reported from European propolis. The R_f values and changes in absorbance maxima after addition of shift reagents for four of the remaining compounds suggested that they were also methoxylated flavonoids, but their

TABLE 1. PERCENT OF EXTRACTABLE RESIN AND INSOLUBLE RESIDUE (WAX) IN FRACTIONATED BEE PROPOLIS COLLECTED FROM HIVES AT THREE GEOGRAPHIC LOCATIONS[a]

	Ohio (N = 3)	North Georgia (N = 2)	South Georgia (N = 1)
Insoluble residue	25.1 ± 7.1a	55.3 ± 6.8b	76.0
Extractable resin			
Aqueous methanol	0.80 ± 0.7a	1.7 ± 0.4a	1.1
Petroleum ether I	0.93 ± 0.4a	4.4 ± 0.6a	0.1
Petroleum ether II	8.0 ± 12.9a	37.6 ± 4.8a	0.1
Ethyl acetate	62.7 ± 8.2a	14.4 ± 3.9b	22.0

[a] Means with different letters are significantly different between Ohio and north Georgia sites, Student's t test ($P > 0.05$). N = number of propolis samples extracted.

structures were not elucidated. We did not detect several compounds commonly reported in European propolis, including ferulic acid, caffeic acid and cinnamic acid, based on cochromatography with authentic standards.

Effects of Propolis Constituents on Wax Moth Larvae. Neither the aqueous methanol fraction nor the methanol-insoluble residue significantly reduced growth of greater wax moth larvae compared to their respective control treatments (Table 2); in fact, the aqueous methanol fraction significantly increased larval weight. In the second bioassay, the propolis fractions (ethyl acetate A, B, C, D, petroleum ether I and II) reduced larval weight compared to the solvent control, but differences were not significant, perhaps due to the high variance in the solvent control weights (Table 3). Several of the selected phenolics, ferulic acid (0.05%), quercetin (0.05%), and chrysin (0.25% and 0.5%), positively affected larval weight, but not significantly.

DISCUSSION

The gross composition of North American propolis (percent beeswax and methanol-soluble resin) is variable, but within the range reported for European propolis (Ghisalberti, 1979). The proportion of beeswax to plant resin in propolis likely depends on the availability of plant resins and the specific use to which it is applied within the hive. Propolis used to repair honeycomb is often supplemented with larger quantities of wax to give it a firmer composition, while propolis applied in a thin coat to the surface of comb usually contains little or no wax (Meyer, 1956). Bees may also incorporate more wax into propolis during periods when resins are scarce or difficult to collect (Meyer, 1956). The low proportion of resin in propolis collected from south Georgia in this study may reflect a low availability of collectable resins in pine forests (Popravdo, 1977).

The chromatographic surveys of Ohio and north Georgia propolis revealed

TABLE 2. SURVIVAL AND DRY WEIGHT OF GREATER WAX MOTH LARVAE FED DIETS CONTAINING INSOLUBLE RESIDUE (WAX) AND AQUEOUS FRACTION FROM PROPOLIS

Treatment	Survival (%)[a]	Dry weight (mg)	N[b]
Diet (control)	86	84.1 ± 4.2	19
Diet + insoluble residue	91	79.0 ± 4.9	20
Diet + solvent (control)	69	43.1 ± 4.5	15
Diet + aqueous fraction	81	62.1 ± 6.3	17

[a] Percent survival from first instar to end of experiment (all replicates pooled).
[b] N = number of replicate jars, each replicate consisting of 10–15 larvae.

TABLE 3. SURVIVAL AND DRY WEIGHT OF GREATER WAX MOTH LARVAE FED DIETS CONTAINING FRACTIONATED PROPOLIS AND SOME SELECTED PHENOLIC COMPOUNDS

Treatment	Dry weight (mg)[a]	Survival (%)[b]	N
Untreated diet	46.3 ± 8.7[c]	100	14
Solvent control	23.2 ± 7.8	81	14
Ethyl acetate A	12.0 ± 1.8	57	19
Ethyl acetate B	8.3 ± 1.5	36	17
Ethyl acetate C	11.1 ± 2.0	90	18
Ethyl acetate D	12.7 ± 2.2	98	18
Petroleum ether I	9.3 ± 1.4	98	18
Petroleum ether II	9.2 ± 1.4	77	17
Ferulic acid 0.05%	37.5 ± 7.0	100	13
Quercetin 0.05%	32.8 ± 6.2	99	15
Quercetin 0.25%	18.1 ± 4.3	72	15
Quercetin 0.5%	17.8 ± 4.0	72	14
Chrysin 0.05%	14.5 ± 2.9	72	14
Chrysin 0.25%	31.5 ± 20.5	53	14
Chrysin 0.5%	31.2 ± 8.7	73	12
Caffeic acid 0.05%	30.2 ± 4.1	96	13
Kaempferol 0.05%	21.0 ± 6.5	79	13
Galangin 0.05%	16.4 ± 2.9	84	13

[a] Mean dry weight ± SE.
[b] Percent survival of larvae to prepupal stage (pooled replicates).
[c] Mean is significantly different from solvent control, Dunnett's test ($P > 0.05$)

a chemical diversity similar to that of European samples (Ghisalberti, 1979). Only one of the four compounds identified in our study (3-methylkaempferol) is newly reported for propolis. This compound occurs naturally in *Populus* and *Aesculus* (horsechestnut) (Harborne et al., 1975), two sources of resin utilized by bees. Kaempferol is a common flavonoid that has been isolated from many plants, including *Betula*, *Alnus*, *Populus*, and *Salix*, which are also propolis resin sources (Ghisalberti, 1979). Galangin occurs in *Populus* and *Pinus* (Harborne et al., 1975) and is common in European propolis (Ghisalberti, 1979), and 3,3-dimethoxyquercetin has also been reported from European propolis (Schneidewind et al., 1975). We did not detect quercetin in Ohio propolis, although it is quite widespread in the plant kingdom and has been found in numerous studies of propolis from other regions (Bankova et al., 1983; Ghisalberti, 1979; Nenov et al., 1983).

Several of the methanol-soluble resin fractions from propolis retarded growth of greater wax moth larvae, but not as dramatically as crude propolis extract (Eischen and Dietz, 1987). It is possible that fractionation of constituents may

have disrupted synergistic or cumulative toxic effects in crude extract, or the propolis used in this study had a different chemical composition. We cannot discount the possibility of loss of activity from autooxidation of compounds during the diet-drying process, although the level of heat used was low. The more oxidation-sensitive constituents in propolis are likely to be oxidized under natural hive conditions before extraction. The larval bioassays on the honey–beeswax–pollen diet exhibited high within-treatment variation, perhaps indicating incomplete mixing of diet components. The heterogeneity of the diet may have contributed to the retention of solvent, but the persistence of these effects after lyophilization suggests that additional factors were involved.

To summarize, we found little evidence of seasonal or hive-to-hive variation in the specific constituents of propolis, but significant differences between geographic locations. The assays of propolis fractions and individual compounds indicate that greater wax moth larvae are able to tolerate phenolics in their diet and may respond positively to some, although many plant phenolics are toxic or antifeedant towards insects (Levin, 1976; Shaver and Lukefahr, 1969; Isman and Duffey, 1983). This may be due to the low concentrations used in our assays (0.05–0.50%), since equivalent concentrations of quercetin and rutin do not inhibit tobacco bollworm, tobacco budworm, or pink bollworm growth (Shaver and Lukefahr, 1969). Dietary phenolics have also been reported to improve the performance of some insects (Bernays and Woodhead, 1982; Kato, 1978; McFarland and Distler, 1982). Although individual phenolics and fractionated propolis had little effect on greater wax moth larvae in our study, the extent of geographic variation in propolis composition and the possibility that toxicity decreases during fractionation makes it difficult to generalize about the biological activity of material from other sites. The degree of tolerance of greater wax moth larvae to dietary phenolics and the chemical variation in propolis from different geographic locations must be considered concurrently when evaluating the effect of propolis on natural greater wax moth infestations.

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Book Review

The Scent of Orchids. Olfactory and Chemical Investigations. Roman Kaiser. Basel, Amsterdam: Elsevier Science Publishers, 1993. 264 pp. U.S. $175.00, ISBN 0-444-89841-7. German edition: Editiones Roche, Dfl. 280.00. ISBN 3-907946-87-1. Available from Elsevier Science Publishers B.V. Molenwerf 1, B.O. Box 211, 1000 AE Amsterdam, The Netherlands or for USA/ Canada: Elsevier Science Publishing, Co., Inc., P.O. Box 882, Madison Square Station, New York, NY 10159, USA.

Orchids are very fascinating not only to biologists but to the general public due to the variety of exotic flower shapes and colors. These often are complimented with aesthetic and unusual odors that lend to the complex of more than 25,000 species known worldwide. Investigations of the pollination biology alone have brought to light numerous methods of attracting insects or other animals that function in the transfer of pollinaria. Many of these attracting methods correlate with the exciting and exotic appearance of orchid flowers. Besides the normal reward with nectar or collectable perfumes for the males of South American Euglossine bees, orchids are masters of deception, giving to visitors the illusion of sex partners, sleeping places, oviposition sites, or just pretending to be a good but empty (nectarless) restaurant. In all cases, the different odors are the most important attracting signals. Detailed and consistent investigations of the chemical compounds are only known for some European species of the genus *Ophrys* (which attract male Hymenoptera by visually, olfactorially, and mechanically faking the conspecific female) or for many species of the Euglossine-pollinated genera (Catasetinae, etc.).

This book by the Swiss odor chemist Roman Kaiser from the Givaudan-Roure Research Center in Dübendorf near Zürich, is an excellent enlargement of this field. It is interesting not only from a scientific point of view but also for its beautiful illustrations. There is, on one hand, an extensive list of detailed chemical compounds found in 158 different orchid species from 62 genera. On the other hand, it is illustrated with more than 170 excellent color prints of predominantly tropical species. The aim of this book is to convey an impression of the enormous variety in scent and chemical compounds as well as the visual appearance of orchid flowers.

The book has three main parts: an introduction to the world of orchid scents, an interdisciplinary discussion of orchid scents, and the chemistry of orchid scents. Part one gives an introduction to orchids and plant scents in

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general. Very helpful is the chapter "Trapping and investigation of orchid scents," which introduces techniques, mainly gas chromatography, and methods of compound identification illustrated with informative figures. The chapter "Scent and pollination principles" gives a short survey on pollination types in orchids and the role of scents so far investigated. Part two describes results of scent identification in connection with the distribution of species: "Orchids of the American tropics" (pp. 49–122), "Orchids of the African tropics" (pp. 123–143), "Orchids of the Indo-Australian tropics and subtropics" (pp. 144–175), and "Some European orchids" (pp. 176–181). Part three contains comprehensive tables of the analytical composition of individual orchid scents. In these tables, the sequence of individual components corresponds with the order in which they are eluted from a DB-Wax capillary column. Since the composition of the scent of any given species can vary considerably from one plant to another, figures are not generally provided for concentrations below 1%. Apart from those cases in which additional information was thought to be of interest, such components are indicated in the table as minor compounds. These lists are the most interesting elements of the book from a scientific point of view.

These numerous results can be the beginning of further investigations of other interesting orchid species. In only a few cases is the biological meaning of these different chemical compounds really known. That means that these extensive lists of chemical compounds are at the moment more than a demonstration of the astonishing variety of scents in orchids. A comparative analysis of the different compounds has not been undertaken.

Finally, here is a book of excellent value, which will become the reference text on orchid scents. The interdisciplinary concept of the beautifully color-illustrated book will guarantee that a broad spectrum of readers will find it appealing: scientists, naturalists, as well as hobbyists engaged in the fragrance field, pollination biology, or just in the beauty of orchids. Unfortunately the price (US $175) will likely prevent a wider distribution of the book.

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QUANTIFICATION OF CANTHARIDIN IN CANTHARIPHILOUS CERATOPOGONIDAE (DIPTERA), ANTHOMYIIDAE (DIPTERA) AND CANTHARIDIN-PRODUCING OEDEMERIDAE (COLEOPTERA)

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Abstract—Cantharidin contents were determined in several canthariphilous insects by means of quantitative gas chromatography. Usually the ceratopogonids *Atrichopogon oedemerarum* and *A. trifasciatus* caught in the field contained low concentrations of cantharidin, with concentrations in males, in most cases, being lower than in females. When fed in the laboratory with synthetic cantharidin, these species concentrated cantharidin by as much as 100-fold (males) and 40-fold (females). Accumulation in the different body tagmata (head, thorax, abdomen) of these species is similar. Maximal concentrations of cantharidin in tissues of *Atrichopogon* are comparable to those known from oedemerid and meloid beetles. In *A. trifasciatus* about 90% of total cantharidin content is bound in tissues. Investigations using the canthariphilous anthomyiid fly *Anthomyia pluvialis* and three cantharidin-producing oedemerid species revealed the same pattern of distribution in different body tagmata as in *Atrichopogon*.

Key Words—Cantharidin, pharamacophagy, Diptera, Ceratopogonidae, *Atrichopogon oedemerarum*, *A. trifasciatus*, Anthomyiidae, *Anthomyia pluvialis*, Coleoptera, Oedmeridae, *Oedemera* spp., quantitative analysis.

INTRODUCTION

The terpenoid cantharidin plays an important role in the chemical defense of blister beetles (Meloidae) (Carrell and Eisner, 1974) and false blister beetles

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(Oedemeridae) (Carrel et al., 1986). These beetles are the only known natural sources of cantharidin. It is synthesized by males and transferred during copulation to females in several species of meloid beetles (Sierra et al., 1976; McCormick and Carrel, 1987). This nuptial gift chemically protects females after copulation and later the female will incorporate most of her cantharidin in the eggs. Thus, the eggs, the most vulnerable phase of an insect's life cycle, are effectively protected against predators (Eisner, 1988).

Although many arthropods are deterred by this substance (Carrel and Eisner, 1974), some groups of insects are attracted to cantharidin-containing beetles or to traces of synthetic cantharidin. These are termed "canthariphilous" insects (Görnitz, 1937). Eisner (1988) reported that canthariphilous pyrochorid beetles use the terpenoid in a similar way to meloids. This also seems to occur in anthicid beetles (Schütz and Dettner, 1992). It appears that cantharidin uptake is a case of pharmacophagy similar to that described in pyrrolizidine alkaloid-sequestering insects (Boppré, 1986). Although cantharidin content in meloid beetles has been analyzed in a few species (e.g., McCormick and Carrel, 1987), there is no quantitative information available about takeup and enrichment of this substance in most canthariphilous insects. This information may be a key to our understanding of why canthariphilous insects are searching for, and perhaps feeding on, this rare substance. Our study is the first to investigate these aspects in canthariphilous ceratopogonid flies (gnats) of the genus *Atrichopogon*. First, we investigated whether flies in the field contain cantharidin. Secondly, synthetic cantharidin was offered to *Atrichopogon* in the laboratory in order to obtain information about enrichment capacities in body tissues. For this purpose, bodies were dissected and analyzed to discover potential regions of storage. We also provide quantitative data for the canthariphilous fly *Anthomyia pluvialis* (Diptera: Anthomyiidae) as well as analyzing some cantharidin-synthesizing oedemerid beetles, which are potential sources of cantharidin in Upper Franconia (Germany), the area where field studies took place.

METHODS AND MATERIALS

Cantharidin Traps

Folded filter paper disks (5.5 cm diam.) were impregnated with 300 μg cantharidin in acetone, placed at the bottom of capped rectangular plastic boxes (10 × 10 × 6 cm) and covered with a gauze cone. Two sides of the plastic box had a hole (3.5 cm diam.) covered with plastic gauze to facilitate escape of volatilized cantharidin. A hole in each gauze bore a rectangular plastic tube

(1 cm diam.) allowing the entry of attracted insects. The traps were mounted on poles or placed on vegetation at a height of 1 m.

Insects

Ceratopogonidae. Atrichopogon oedemerarum Storå and *Atrichopogon trifasciatus* Kieffer were caught in the field from May to August 1990/1991 in Upper Franconia (Bavaria, Germany) using cantharidin traps. One group of flies had no access to the bait substance in the trap or in the laboratory. Although they might have gained some cantharidin from natural sources before trapping, we name this group cantharidin-unfed (C-unfed). A second group of field-captured flies had free access to synthetic cantharidin (C-fed) in the laboratory for at least two days. The cantharidin source consisted of a filter paper disk wetted with water that had previously been impregnated with 300 μg cantharidin dissolved in acetone.

Only *A. trifasciatus* laid eggs under laboratory conditions. Two samples, each of 50 eggs, were extracted and analyzed by quantitative gas chromatography. One sample was from a field-captured female with no access to cantharidin in the laboratory and the other was from a female fed with *A. oedemerarum*, which had had access to the terpenoid in the laboratory. Many larvae were obtained from other eggs of *A. trifasciatus*, but very few imagines were reared.

Anthomyiidae. Anthomyia pluvialis L. was trapped with cantharidin baits in May 1990 in Wiesbaden (Hessia, Germany). F_1 imagines from females with or without access to synthetic cantharidin were reared in the same year.

Oedemeridae. Oedemera femorata (Scop.), *Oedemera flavipes* (Fabr.), *Oedemera* c.f. *lurida* (Marsh.) beetles were caught in the field using insect nets in Upper Franconia. Some eggs were laid by *O. femorata* and *O. flavipes* in the laboratory and analyzed by quantitative gas chromatography.

Predator–Prey Interactions

The deterrent function of cantharidin was tested using the empidid fly *Platypalpus* sp. as predator and *A. oedemerarum* as prey. Each prey item had a different cantharidin content, depending on different exposure times to the synthetic substance in the laboratory. A first test was conducted with four live *A. oedemerarum* females and a single *Platypalpus*. The *A. oedemerarum* contained small amounts of cantharidin, since they were exposed to cantharidin-impregnated dry filter paper disks for six days prior to the experiment. These disks were much less attractive to the gnats than water-wetted disks. A second test as carried out in which, in contrast to the first experiment, the prey (three

live *A. oedemerarum* females) had 18 days of access to water-wetted disks containing the bait substance.

Microscale Extraction

Since ceratopogonids are quite small (70–500 μg fresh weight, body length 1.2–2 mm), a special extraction procedure for single specimens had to be developed, modifying a method for quantification of cantharidin in human serum described by Steyn and Hundt (1988). Whole specimens or single body regions (head, thorax, abdomen) of insects were treated by hydrolysis with 20–50 μl 6 N hydrochloric acid at 120°C for 4 hr in fused glass capillaries in order to dissolve all body structures and to free bound cantharidin. Afterwards, the samples were centrifuged for 10 min at 2000*g*. Then equivalent amounts of chloroform (20–50 μl in ceratopogonids, 100–300 μl in anthomyiid flies and oedemerid beetles) were added and, after closing the capillaries, samples were vigorously shaken on a vortex mixer for 30 sec. This was followed by centrifugation as described above. Finally, the capillaries were opened, and the organic phase was filtered, transferred to a vial, and supernated with about 1 ml water to minimize evaporation of chloroform. Samples were stored at −20°C until analysis.

Relation of Bound Canatharidin to Free Cantharidin

Two samples were investigated, one of five males and one of five females of *A. trifasciatus* that had access to cantharidin in the laboratory (males, 30 days; females, two days). Insect bodies were homogenized in 250 μl water and 50 μl ethanol. Six samples of 30 μl each were taken from homogenized males and females, but only three were hydrolyzed. The extraction procedure was the same in all samples. Two extraction modes were used, one after hydrolysis and one with no hydrolysis, in order to test how much cantharidin is bound in canthariphilous ceratopogonids.

Quantitative Gas Chromatography

A Carlo Erba Mega HRGC 5160 gas chromatograph with on-column injector, equipped with a OV-1701 fused silica capillary column (Chrompack, 12 m × 0.32 mm ID, 0.25 μm phase thickness) was used for analyses. The analytical column was connected to a deactivated precolumn (1.5–0.3 m). Chromatographic conditions were as follows: initial temperature 55°C for 2 min, followed by a temperature increase of 15°C/min up to 200°C, then with 20°C/min to 260°C for 5 min. Temperature of the detector (FID) was 325°C. Helium was used as carrier gas (50 kPa; 2.5 ml at 100°C). Hydrogen pressure was 60 kPa and air pressure 150 kPa. Amounts of cantharidin in the samples were deter-

mined by means of calibration curves. Mann-Whitney U tests were used to compare the amounts and concentrations of cantharidin in C-fed or C-unfed specimen and females or males.

RESULTS

Content and Distribution of Total Cantharidin in Canthariphilous Ceratopogonid and Anthomyiid Flies

A. oedemerarum *(Ceratopogonidae).* Figure 1 shows the average cantharidin content in males and females from the field (C-unfed) and in specimens that had free access to cantharidin in the laboratory (C-fed). In both cases, females contained significantly more cantharidin than males (C-unfed: $U = 123$, $P \leq 0.012$; C-fed $U = 27$, $P \leq 0.039$). Whereas some specimens from the

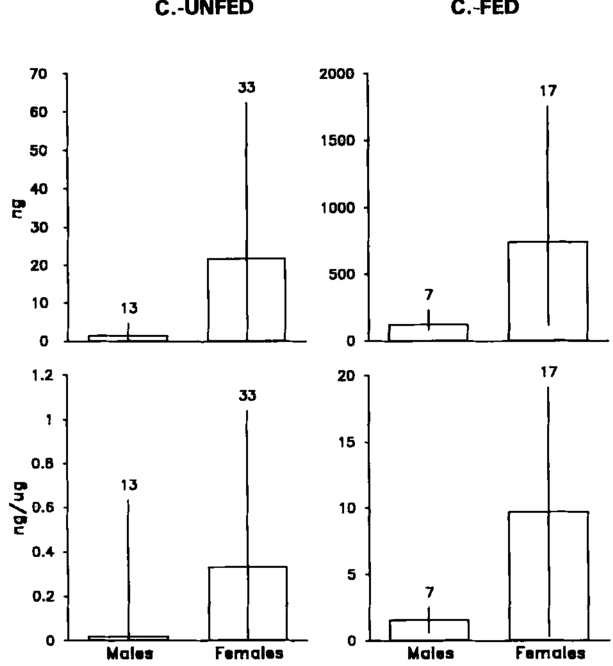

FIG. 1. Average content of cantharidin in males and females of *A. oedemerarum*. C.-unfed.: field-caught specimens with no access to synthetic cantharidin; C.-fed: field-caught specimens which had access to synthetic cantharidin in the laboratory. Absolute amounts in nanograms (ng), concentration in ng/μg body weight. Bars: standard deviation (above: N analyzed specimens.)

field had no detectable cantharidin, all C-fed *A. oedemerarum* contained considerable amounts of the terpenoid. Compared to unfed flies, females increase cantharidin in their bodies by about 43-fold (29-fold in terms of concentration) and males by about 96-fold (74-fold, respectively) if given access to synthetic cantharidin in the laboratory. Absolute values are greater in C-unfed (15-fold) and C-fed (6-fold) females than in males. One can conclude that either the eagerness or the capability to take up cantharidin is more developed in females. Quantitative results given in Table 1 show that cantharidin is not concentrated in any particular part of the body (e.g., abdomen containing reproductive organs).

A. trifasciatus *(Ceratopogonidae)*. This species (Figure 2) is quite different from *A. oedemerarum*, since differences in cantharidin content between sexes cannot be established in specimens caught in the field (C-unfed). Mean values from C-fed specimens showed that females contained on average threefold more cantharidin. Comparison of data from males and females showed a significant difference ($U = 50$; $P \leq 0.0274$). Compared to field-caught flies which had no access to synthetic cantharidin, the C-fed group contained three- to fourfold (males) and nine- to 11-fold (females) more of the substance. The difference between all C-fed and C-unfed flies is similar to that for *A. oedemerarum* (amount: $U = 132$, $P \leq 0.0000$; concentration: $U = 118$, $P \leq 0.0000$).

As in *A. oedemerarum*, analysis of body parts does not show a difference in the distribution of cantharidin. There is, however, a trend in both sexes caught in the field to have the highest concentration in the head and the greatest amount in the thorax (Figure 3, left side). Concentration seems to be more evenly distributed in C-fed specimens (Figure 3, right side). Interestingly, the pattern of distribution and the degree of enrichment did not change in a female with access to cantharidin for 261 days (Figure 3, right side, thick line).

Extraction and analysis of 50 eggs from a field-captured female showed a total cantharidin content of 8.6 ng, while the similar-sized egg clutch of a female that could prey on C-fed *A. oedemerarum* in the laboratory contained 110.5 ng. Eleven analyzed larvae of larval stage 1 (2-3.6 ng cantharidin) and two F_1 imagines (male: 7.13 ng; female: 11.7 ng) stored some cantharidin.

Anthomyia pluvialis *(Anthomyiidae)*. Although this fly belongs to a different family, quantitative analysis reveals a similar pattern to that found in *A. trifasciatus*. There is no significant difference between the sexes in both C-unfed and C-fed anthomyiids (Figure 4), but after cantharidin exposure, males contained on average twofold, females eightfold more of the terpenoid.

Analysis of body parts of C-unfed flies showed that specimens of both sexes had the greatest amount in the thorax (Table 1), which is the heaviest body part. No cantharidin could be detected in extracts of F_1 adults either from C-fed or C-unfed females.

TABLE 1. DESCRIPTIVE STATISTICS OF ANALYSIS OF BODY TAGMATA[a]

	Males					Females				
	N	Mean ± SD (ng)		Mean ± SD (ng/μg)		*N*	Mean ± SD (ng)		Mean ± SD (ng/μg)	
Atrichopogon oedemerarum										
C-fed										
Head	2	21.2	7.3	3.8	0.8	4	15.8	18.6	2.6	3.1
Thorax	2	53.7	17.3	1.1	0.1	5	379.7	502.9	7.8	10
Abdomen	3	24	15.5	0.4	0.2	6	473.2	514.3	11.2	12.2
Anthomyia pluvialis										
C-unfed										
Head	6	33.0	23.2	0.08	0.04	6	29.9	4.2	0.15	0.1
Thorax		183.3	182.2	0.07	0.05	4	295.3	214.6	0.12	0.1
Abdomen	6	47.5	22.4	0.06	0.02	4	166.2	112	0.05	0.04
C-fed										
Head	3	84.1	113.7	0.26	0.31	6	769.2	346.9	1.2	0.9
Thorax	3	1235.4	1927	0.53	0.76	5	4120.0	1381	1.2	1.2
Abdomen	3	381.2	597.4	0.18	0.13	5	2000.0	666.6	1.2	1.5
Oedemera femorata										
Head	3	359	170	0.96	0.39	3	1515	382	2.7	0.3
Thorax	2	3662	2677	0.57	0.31	2	14577	6311	2.2	0.2
Abdomen	3	831	1438	0.76	0.43	3	11205	1415	1.8	0.6
Oedemera flavipes										
Head	2	1297	7.83	1.90	0.57	2	1089	450	1.68	0.08
Thorax	2	22370	14170	3.13	1.16	1	11880	—	1.46	—
Abdomen	2	3850	528	1.76	0.08	2	6685	2809	1.52	0.63

[a] C-fed, C-unfed: Specimen had or had no access to synthetic cantharidin in the laboratory; *N*: number of analyzed specimens; (ng) amount of cantharidin in nanograms; (ng/μg) concentration in ng cantharidin/μg body weight. (Results of *A. trifasciatus*, see Figure 3).

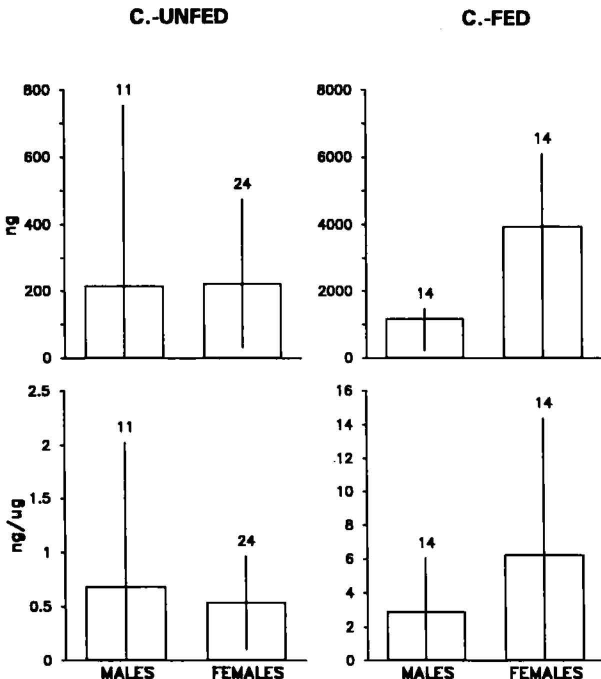

FIG. 2. Average content of cantharidin in males and females of *A. trifasciatus*. C.-unfed.: field-caught specimens with no access to synthetic cantharidin; C-fed.: field-caught specimens which had access to synthetic cantharidin in the laboratory. Absolute amounts in nanograms (ng), concentration in ng/μg body weight. Bars: standard deviation (above: *N* analyzed specimens).

Content and Distribution of Total Cantharidin in Cantharidin-Producing Oedemerid Beetles

False blister beetles are the only known natural sources of cantharidin in Upper Franconia, the area where our field studies were carried out. Meloid beetles have previously been recorded in this area (Weidner, 1990), but they are now absent. Oedemerids were sometimes found on flowers of Apiaceae and Asteraceae. These beetles were not attracted to synthetic cantharidin in the laboratory, thus the total amount of detected cantharidin is probably synthesized by the beetles.

Oedemera femorata. Three females and one male were analyzed. In females, three- to fourfold more cantharidin was detected than in the male (Figure 5). The pattern of distribution in different body parts (Table 1) is similar to *Atri-*

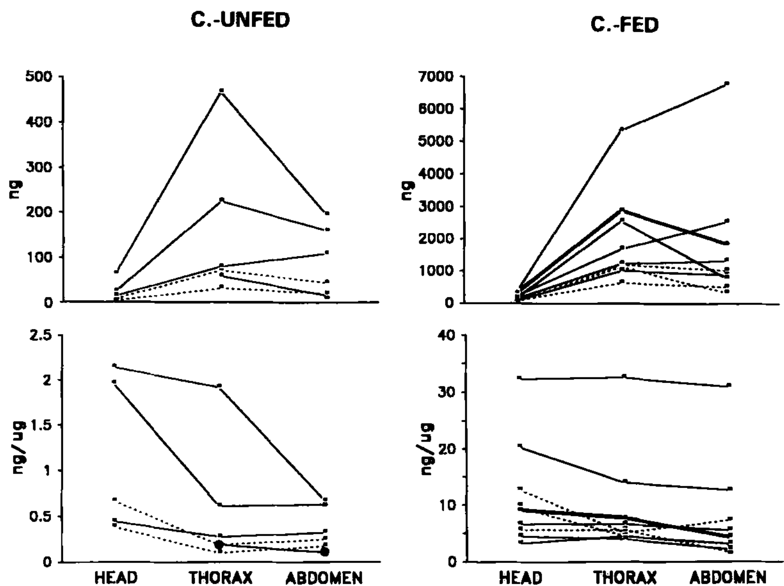

FIG. 3. Distribution of cantharidin in head, thorax, and abdomen of single field-caught *A. trifasciatus* specimens with no access to (C.-unfed.) or access to synthetic cantharidin (C.-fed). Absolute amounts in nanograms (ng), concentration in ng/μg body weight. Females: black lines (points: thorax, abdomen of a female; thick line: female after 261 days with cantharidin exposition); males: dotted lines.

chopogon and *Anthomyia*. There are no prominent cantharidin pools in head, thorax, or abdomen that would indicate storage organs for the terpenoid. First analysis of eggs (*Oedemera femorata*, *O. flavipes*) revealed considerable amounts of the terpenoid (up to 300 ng/egg).

Oedemera flavipes *and* Oedemera *c.f.* lurida. Analytical results for these two species confirm those found in *Oedemera femorata*. There are no significant differences between body parts (Table 1).

Table 2 shows analytical results from all species based on differences in cantharidin content. It is obvious that, within the group of cantharidin consumers, *A. oedemerarum* has the greatest difference between females and males caught in the field and that both sexes have the highest uptake of synthetic cantharidin in the laboratory. Although the sample size is too small to draw general conclusions, it may be significant that female *Oedemera femorata* had several times more cantharidin than the single analyzed male, whereas in *Oedemera flavipes* males contained a little more of the terpenoid than females.

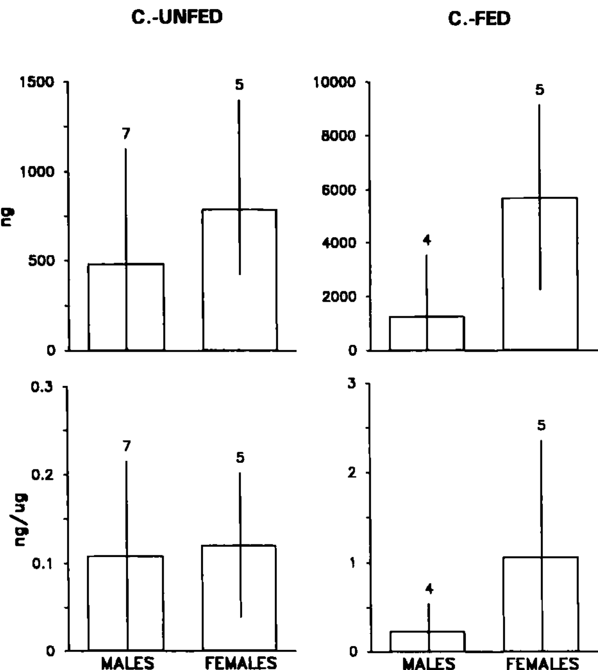

FIG. 4. Average content of cantharidin in males and females of *Anthomyia pluvialis*. C.-unfed: field-caught specimens with no access to synthetic cantharidin; C.-fed: field-caught specimens which had access to synthetic cantharidin in the laboratory. Absolute amounts in nanograms (ng), concentration in ng/μg body weight. Bars: standard deviation (above: N analyzed specimens).

Relation of Bound Cantharidin to Free Cantharidin in A. trifasciatus

Only 6–10% of the cantharidin found in hydrolyzed samples could be detected in samples that were extracted without hydrolysis (Table 3). Lower values for females are due to different exposure times to synthetic cantharidin. While males were exposed for a period of 35 days, females were only exposed for two days. Despite this fact, proportions of bound and free cantharidin are quite similar in both sexes.

Predator–Prey Interactions

In the first test, all *A. oedemerarum* females with low cantharidin content were killed by *Platypalpus*. Attacks lasted for 60–120 seconds, and afterwards the empidid was engaged in cleaning of mouthparts and legs. When females

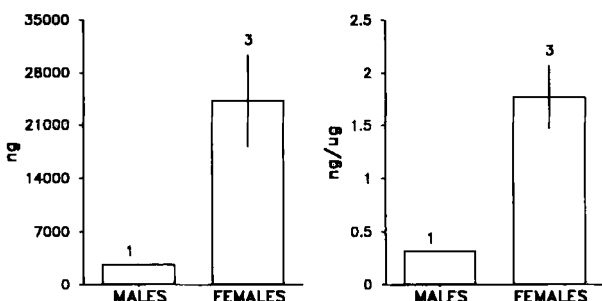

FIG 5. Average content of cantharidin in females and one male of *Oedemera femorata* caught in the field. Absolute amounts in nanograms (ng), concentration in ng/μg body weight. Bars: standard deviation (above: *N* analyzed specimens).

TABLE 2. RATIOS OF MEAN TOTAL CANTHARIDIN CONTENT AND MEAN CONCENTRATION OF CANTHARIDIN IN HYDROLYZED EXTRACTS OF INVESTIGATED SPECIMENS[a]

Species	*N*(Ff/Mf), difference	*N*(Fc/Mc), difference	*N*(Fc/Ff), difference	*N*(Mc/Mf), difference
Cantharidin Consumers				
A. oedemerarum (*N*)	33/13	17/7	17/33	7/13
Content (ng)	13	6	43	96
Conc. (ng/μg)	15	3.2	29	74
A. trifasciatus (*N*)	24/11	14/14	14/24	14/11
Content (ng)	1.1	3.2	11.2	3.6
Conc. (ng/μg)	0.8	2.1	8.5	3.2
Anth. pluvialis (*N*)	5/7	5/4	5/5	4/7
Content (ng)	1.6	4.4	7.2	2.7
Conc. (ng/μg)	1.1	4.6	8.8	2.1
Cantharidin Producers				
Oedem. femorata (*N*)	3/2	none	none	none
Content (ng)	4			
Conc. (ng/μg)	3			
Oedem. flavipes (*N*)	2/2	none	none	none
Content (ng)	0.8			
Conc. (ng/μg)	0.6			

[a] Difference: *x*-fold difference between average values of compared groups; *N*(F/M): *N* females and males either from the field (f, or C-unfed) or cantharin-fed specimens (c).

TABLE 3. RELATION OF FREE AND BOUND CANTHARIDIN IN SAMPLES FROM FIVE HOMOGENIZED MALES AND FIVE HOMOGENIZED FEMALES OF *A. trifasciatus* AFTER FEEDING ON SYNTHETIC CANTHARIDIN[a]

Sex	No hydrolysis		Hydrolysis		Free Cantharidin
	ng	ng/μg	ng	ng/μg	(%)
Males	30.2 ± 2.17	0.293 ± 0.0074	308.3 ± 8.33	1.00 ± 0.024	10
Females	12.7 ± 1.79	0.017 ± 0.0021	203.6 ± 7.97	0.27 ± 0.008	6.24

[a] Values represent average ± SD for three samples each.

with considerable amounts of the terpenoid (about 1000 ng) were offered to *Platypalpus* in a second test, only two attacks were observed. The deterrent effect could be observed after each attack once the predator had pierced the integument of the gnat with its biting mouthparts. The initial attack lasted only about 5 sec before the predator realized the unpalatability of the prey. Afterwards the empidid cleaned its mouthparts vigorously. All *A. oedemerarum* used in this experiment survived.

DISCUSSION

If one compares the quantitative data from cantharidin producers and consumers listed in Table 4, the following can be stated: (1) Absolute cantharidin amounts are different in natural cantharidin sources such as meloid and oedemerid beetles due to their differences in size, but they are similar in concentration in the tissues. Only one study (McCormick and Carrel, 1987) took age of individual beetles and transfer of the terpenoid during copulation into account. This may be the reason why most data from other studies vary considerably within one sex. (2) Some females of *Atrichopogon* caught in the field concentrated cantharidin in the same order of magnitude as oedemerids. Cantharidin-fed *Atrichopogon* even exceeded these values. This indicates that the ability to enrich and store cantharidin in the body is at least similar in these cantharidin consumers.

The terpenoid content is nearly evenly distributed in head, thorax, and abdomen of the cantharidin producers and consumers analyzed in our study. In *A. trifasciatus*, this distribution pattern did not change with time, as it was the same in a female after 260 days in the laboratory with free access to cantharidin. Hemolymph is, therefore, unlikely to be the main storage site for cantharidin because most hemolymph is localized in the abdomen. This is the only tagma

TABLE 4. RANGE OF CANTHARIDIN CONTENT IN CANTHARIDIN-PRODUCING MELOID AND OEDEMERID BEETLES AND IN CANTHARIPHILOUS ANTHOMYIID AND CERATOPOGONID FLIES[a]

Species	Sex	Cantharidin content: Relative	Cantharidin content: Total	Ref.[b]
I. Cantharidin producers				
Family: Meloidae		%	mg	
Epicauta fabricii	F	0.5–4.0	0.14–0.75	1
(LeConte)	M	0.6–12.7	0.06–3.38	1
E. murina	F	0.4–2.8	0.16–0.89	1
(LeConte)	M	1.8–3.8	0.35–1.06	1
E. pennsylvanica	F	<0.1–2.2	0.01–0.29	1
(De Geer)	M	<0.1–2.7	0.03–0.68	1
E. maculata	F	0.3–3.1	0.07–0.50	1
(Say)	M	2.9–9.1	0.44–1.89	1
E. sericans	F	0.5–2.9	0.14–0.79	1
LeConte	M	0.7–11.3	0.13–4.52	1
E. immaculata	F	2.0–8.0	2.18–8.49	1
(Say)	M	3.9–8.5	1.43–11.13	1
E. lemniscata (F.)	?	0.9–5.4	n.i.	2
E. pestifera	Fv	n.i.	≈0.1	3
	Mv	n.i.	≈3.4	3
	Fp	n.i.	≈3.3	3
	Mp	n.i.	≈0.8	3
	F		0.035	4
E. vittata	?	n.i.	0.31–1.45	5
Zonitis atripennis	F	<0.1–0.5	0.01–0.13	6
(Say)	M	0.3–0.4	0.03–0.07	1
Lindsleya	F	0.2–0.6	0.07–0.19	1
sphaericollis (Say)	M	0.6–1.8	0.10–0.39	1
Meloe laevis	F	<0.1–0.4	0.08–1.50	1
Leach	M	0.1–0.9	0.09–1.50	1
M. niger Kirby	?	0.12*	0.88	6
M. proscarabeus L.	?	0.19*	2.0	7
Lytta polita	FV	n.i.	≈0.4	3
	Mv	n.i.	≈0.5	3
	Fp	n.i.	≈3.5	3
	Mp	n.i.	≈0.1	3
L. vesicatoria	Ff	≈1.0	≈0.84	8
(L.)	Mf	≈1.8	≈0.68	8
Family Oedemeridae			μg	
Oxocopis thoracica	F	0.34*	35.2	9
(Fabr.)	M	0.23*	15.5	9

TABLE 4. CONTINUED

Species	Sex	Cantharidin content: Relative	Cantharidin content: Total	Ref.[b]
Family Oedemeridae			μg	
Heliocis repanda	F	0.72*	7.4	9
(Horn)	M	0.29*	2.1	9
Oedemera femorata	F	0.15–0.21*	21–31	10
(Scop.)	M	0.03–0.09*	2.8–9.6	10
Oe. flavipes	F	0.16*	22.0	10
(Fabr.)	M	0.21–0.34*	16.5–38.5	10
Oe. c.f. *lurida* (Marsh.)	F	0.23*	12.4	10
II. Cantharidin consumers				
Family Anthomyiidae		‰	ng	
Anthomyia pluvialis L.	Ff	0.04–0.22*	385–1395	10
	Mf	0.02–0.34*	49–1537	10
	Fc	0.1–3.33*	219–9350	10
	Mc	0.02–0.71*	41–4746	10
Family Ceratopogonidae		‰	ng	
Atrichopogon oedemerarum Storå	Ff	0.00–3.58*	0–204	10
	Mf	0.00–0.11*	0–10	10
	Fc	0.11–31.8*	22–2573	10
	Mc	0.67–3.24*	71–253	10
A. trifasciatus	Ff	0.02–1.63*	7–724	10
Kieffer	Mf	0.00–4.8*	0–1525	10
	Fc	0.12–31.7*	12–12558	10
	Mc	0.04–12.6*	7–2377	10
A. lucorum	Ff	0.00–1.12*	0–166	10
(Meigen)	Mf	0.03*	3.59	10
	Fc	1.78–7.52*	240–1777	10
A. brunnipes	Ff	0.00–1.40*	0–430	10
Meigen	Mf	0.23–0.36*	32–35	10
	Fc	0.35–19.5*	42–2225	10
	Mc	3.72–4.26*	472–543	10

[a] F, Female; M, Male; v, virgin; p, mated specimens; f, insects caught in the field (C-unfed); c, C-fed insects; relative amounts: dry weight, with * fresh weight (cantharidin-consumers: in 0/00); total amounts in mg, μg and ng; n.i.: no information available.

[b] References: (1) Capinera et al., 1985; (2) Ray et al., 1979; (3) McCormick and Carrel, 1987; (4) Carrel et al., 1985; (5) Eisner et al., 1990; (6) Mayer and Johansen, 1977; (7) Dixon et al., 1963; (8) Sierra et al., 1976; (9) Carrel et al., 1986; (10) own results.

with reduced sclerotization and thus variable in size. It contains the esophageal diverticulum, mid-gut, stomach, hind-gut, malpighian vessels, and sex organs. Morphological studies of Gad (1951) and Megahed (1956) and our own observations showed that the sclerotized head capsule of *Atrichopogon* contains the brain, muscles of the mouth parts, and two pumps (cibarial and pharyngeal). The thorax is strongly sclerotized and predominantly filled with flight muscles.

Perhaps cantharidin can be stored in different tissues of a cantharidin-tolerating organism. Since the main proportion (about 90%, Table 2) of cantharidin is bound in *A. trifasciatus*, this seems to be the most common way of detoxifying and storing this compound. Unfortunately no data are available on the corresponding relationship between bound and free cantharidin in meloids and oedemerids. The existence of binding sites for the terpenoid in all investigated tissues was established for mice (Graziano et al., 1988). Cantharidin is highly toxic for these mammals (LD_{50}: 1 mg/kg). In addition certain analogs of this substance can also cause similar toxic effects. Binding of cantharidin seems to be a general mechanism causing either toxic effects in nontolerant organisms (mammals, most insects, plants) or no harmful effects in cantharidin-tolerant organisms (certain insects, frogs, some birds, hedgehogs). The eagerness to gain cantharidin may be very strong: canthariphilous ceratopogonids can be attracted to certain analogs of cantharidin (Frenzel et al., 1992).

In comparing *Atrichopogon* containing natural cantharidin (C-unfed) with C-fed specimens, we noted that *A. oedemerarum* had the highest cantharidin enrichment of all analyzed insects when synthetic cantharidin was provided (males about 100-fold, females about 40-fold). However, males caught in the field contained about 15-fold less cantharidin than females. If the greed for cantharidin is equal in the sexes, this would mean that males are not as successful in gaining the substance from natural sources as females or that they might have transferred it during copulation.

The situation is completely different in *A. trifasciatus*, since males and females collected in the field do not differ in their cantharidin content. Thus, they seem to have similar success in obtaining this substance. The total amount of cantharidin from specimens collected in the field is substantially greater than in *A. oedemerarum*. Although no significant differences in cantharidin content occurred between the sexes, females did, on the average, have three times more of this substance than males. It would be of interest to increase sample sizes to provide a better test of this relationship.

First quantitative results of eggs analyzed from *A. trifasciatus* indicate that there may be a transfer of cantharidin that has been accumulated by the female into the eggs. Moreover, cantharidin could be detected in larvae and F_1 imagines. The biological significance of this finding, e.g., as a deterrent, is unknown.

Although *Anthomyia pluvialis* belongs to a different taxonomic group (Brachycera: Anthomyiidae) and has, in contrast to biting ceratopogonids, sucking

mouthparts, quantitative results are similar to those found in *A. trifasciatus*. Since anthomyiid flies cannot attack living meloids or oedemerids, they may take up cantharidin from dead insects. This has been reported in the case of dead meloid beetles by Havelka (1978). No cantharidin could be detected in eggs or adults of the laboratory-reared F_1 generation. Thus, it does not seem that the terpenoid is transferred to following generations in *A. pluvialis*.

Although absolute amounts of cantharidin in the false blister beetle *Oedemera femorata* are considerably higher than in C-fed *Atrichopogon* or *Anthomyia*, concentration is of the same order of magnitude or even greater in C-fed *Atrichopogon* (see Figures 1 and 2). General differences in concentration and distribution of cantharidin in investigated consumer and producers (Oedemeridae) of cantharidin could not be established.

The high cantharidin amounts indicate that these beetles, which occur sympatrically with *Atrichopogon* in Upper Franconia, could be the natural source of cantharidin for this insectivore. Estimated hemolymph and thus cantharidin uptake by *Atrichopogon* feeding on *Oedemera* is in the same order of magnitude as cantharidin content found in *Atrichopogon* caught in the field. Besides feeding on adult beetles (personal observation), *Atrichopogon* may also feed on eggs of oedemerid beetles that contain considerable amounts of cantharidin (unpublished data).

The function of attraction to and feeding on cantharidin in *Atrichopogon* is still not completely understood. The terpenoid most likely serves as a kairomone for canthariphilous gnats while they are searching for cantharidin-containing hosts. This should be especially important for females, since males are supposed to feed only on nectar (Downes 1971). However, males were also found in cantharidin traps which indicates that cantharidin may also function in bringing the sexes together. This is true in *A. trifasciatus*, since a lot of copulations were observed in or near the trap and in the laboratory. However, copulation was never observed in several thousand trapped *A. oedemerarum*. Possibly this species needs certain unknown additional factors to induce mating.

Cantharidin content as a criterion of sexual selection in copulating *A. trifasciatus* is less probable. In this male-choice system, mating seems to be an aggressive act without any prior mate testing, such as is known from pyrochorids (Eisner, 1988). While the male fly copulates, the female often tries to attack him.

Cantharidin might serve as a chemical defense against predators. This could be demonstrated with the empidid fly *Platypalpus* sp. preying on *A. oedemerarum*. If the prey could accumulate cantharidin in high amounts by feeding on synthetic cantharidin, the predator was prevented from killing it.

Transfer of cantharidin from the female to the eggs could only be studied in *A. trifasciatus* and *Anthomyia pluvialis*, because only these canthariphilous species could be reared in the laboratory. While the eggs and F_1 imagines of

Anthomyia contained no cantharidin, it could be detected as trace amounts in eggs of *A. trifasciatus*. Whether or not this is sufficient for an effective chemical defense against egg predators is not known. Cantharidin is further transferred from the eggs to the larvae and to the imago. This would indicate that canthariphilous insects such as ceratopogonids use cantharidin in the same way as oedemerids and meloids: as a substance with potential deterrent effects, important enough to be transferred to the following generation.

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Olea europaea CHEMICALS REPELLENT TO *Dacus Oleae* FEMALES

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Abstract—The egg dispersion strategy of the olive fruit fly *Dacus oleae*, which is dependent on chemicals from the fruit, was investigated. In particular, the exact role of *o*-diphenolic compounds, such as the typical olive glucosides, oleuropein and demethyloleuropein, and their derivatives was clarified. It appears that the strong chemotactile repulsive effect exerted by the water fraction of crushed olives is due mainly to (*E*)-2-hexenal. Several compounds, such as β-3,4-dihydroxyphenylethanol and other oleuropein derivatives, which exert a strong chemotactile repulsion, were newly identified or confirmed either in fresh olive juice or in olive mill waste water. This result confirms that the small droplets of olive juice, regurgitated just after egg laying by the *D. oleae* female all around the oviposition hole, actually prevent other females from ovipositing on the same fruit.

Key Words—*Olea europaea*, chemotactile repellents, *Bactrocera* (*Dacus*) *oleae*, Diptera, Tephritidae, oviposition behaviour, olive *o*-diphenolic compounds.

INTRODUCTION

Four types of interaction affecting olive fruit fly host acceptance are generally acknowledged (Cirio, 1971; Vita and Cirio, 1977; Girolami and Strapazzon, 1982): (1) olfactory attraction, (2) chemotactile attraction, (3) olfactory repul-

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sion, and (4) chemotactile repulsion. In a previous paper we reported our findings on items 1 and 3 (Scarpati et al., 1993). In this paper we report the results of our research on item 4.

Several authors have claimed that the oviposition mechanism of the olive fruit fly, *D. oleae*, is regulated by chemical factors contained in the fruit of the host plant, in particular *o*-diphenolic substances related to the metabolism of oleuropein **1** (Cirio, 1971; Vita and Barbera, 1976; Girolami et al., 1981). Oleuropein has also been shown to act as an antibiotic that selects the symbiotic bacterial flora required for larval development (Mazzini and Vita, 1981). It is conceivable that oleuropein plays a fundamental role in the olive–fruit fly relationship, as it is contained both in the leaves and in undamaged ripening fruits in considerable amounts, about 30 g/kg (Panizzi et al., 1960).

The aqueous phase obtained in the olive crushing process for oil production, the so-called olive-mill wastewater (WW), is known to have a repellent action. This action, which can be observed in the field by spraying plants with WW, brings about a marked reduction in the number of ovipositions (Cirio and Vita, 1976; Fiume and Vita, 1977; Vita et al., 1977). This repellency, however, disappears within a few days, and the authors postulate chemical alteration of the WW components under the effect of light and oxygen (Vita and Cirio, 1977; Cirio and Vita, 1980). Vita and Barbera (1976) ascribe the WW activity to β-3,4-dihydroxyphenylethanol **2**, (DHPE), resulting from enzymatic hydrolysis of oleuropein.

In olive-oil producer countries the disposal of WW, which amounts in Italy to some 1.5 million tonnes per year, represents a serious problem owing to its toxic nature. A practical use of WW in the control of the olive fly, a true pest of the Mediterranean area, could be possible if a suitable method could be found for stabilizing the repellent action of the WW over time. A preliminary step in this direction would be to acquire a knowledge of all the main chemicals contained in olive juice showing repellent properties for the *D. oleae* female.

It would be equally important to identify the main chemicals repellent to *D. oleae* females in already oviposited olives, which are responsible for the strong egg dispersion in the fruits. Vita and Barbera (1976) attribute to **2** the chemotactile repellent action of the drupe marking by the *D. oleae* female, at the end of oviposition. On this topic, we recently suggested (Scarpati et al., 1993) that olfactory repulsion may be caused by the emission of (*E*)-2-hexenal **3** and hexanal **4** from the oviposition hole, as we identified these aldehydes in the headspace (HS) of the olive pulp; furthermore we demonstrated a clear oviposition deterrency for **3** and **4**, and for **3** also an olfactory repulsion (Scarpati et al., 1993).

In view of the scanty and often contradictory evidence available on the various topics mentioned, we have reinvestigated the chemical composition of

both WW and fresh olive juice, making a contemporary control of the repulsion activity of identified compounds.

METHODS AND MATERIALS

General Experimental Materials. Analytical thin layer chromatography (TLC) was performed on E. Merck Kieselgel 60 F-254 plates, with 0.2 mm film thickness. Spots were visualized either by UV light or by spraying with $FeCl_3$ (3% in EtOH) or phosphomolybdic acid (10% in EtOH). Preparative TLC (PTLC) was performed on E. Merck Kieselgel 60 F-254 plates, with 2 mm film thickness. Column chromatography (CC) was carried out with a Duramat 80 IP31 apparatus at a medium pressure (1–3 bars) on a Merck Lobar Lichroprep PR-8 (310-25, 40-63 μm) column. HPLC was performed using a Perkin Elmer series 10 apparatus with a Supelco Lichrospher RP-18 column, and a LKB mod. 2138 Uvicord detector. GC was performed with a HP 5890 apparatus with a FID detector, using a Supelco SPB-1 capillary column, with N_2 (2 ml/min) as carrier gas. UV spectra were run with a Varian Cory 210 spectrophotometer and ^1H NMR spectra with a Varian Gemini 200 instrument. For enzymatic hydrolysis, almond β-glucosidase (Fluka, 49290) was used. Pyrocathecol **11** (Aldrich, 13501-1), and tyrosol **10** (Fluka, 56105) were used. Olives (Itrana or Leccino variety) were collected from July to December in the Lazio region of Italy.

Isolation of Glucosides 1, 5 and 6 From Whole Olives. Five hundred grams of recently picked ripening olives was extracted for 1 h with boiling MeOH (600 ml). TLC ($CHCl_3$/MeOH 8:2) revealed the presence in unripe and ripening olives (Itrana or Leccino variety) of oleuropein **1,** near small amounts of ligstroside **5;** in completely ripe olives (Leccino variety) the only glucoside present was demethyloleuropein **6.** After evaporation of the solvent under vacuum, **1** and **6** were purified by CC (MeOH/H_2O 1:1), while **5** was purified by PTLC ($CHCl_3$/MeOH 8:2) (Bianco et al., 1993). Pure glucosides were used both for bioassays and transformation into the respective hydrolysis products. The purified hydrolysis products (see below) were used either for the bioassays or as chromatographic standards.

Aglycones 7 and 8. β-Glucosidase (150 mg) was added to a solution of **1** (700 mg) in 35 ml of H_2O under stirring at 35°C in the presence of 35 ml of $CHCl_3$. After 1 hr, the reaction was complete. The $CHCl_3$ layer was separated, dried with anhydrous Na_2SO_4, and evaporated under vacuum. The aglycone **7** was purified by PTLC ($CHCl_3$/MeOH 95:5). Compound **8** was obtained from **6** with the same procedure and purified according to Scarpati and Lo Scalzo, (1993).

DHPE 2 and Secoiridoid 9. One gram of **1** was treated under stirring at

room temperature with 5 ml of 1 N H_2SO_4. After 24 hr monitoring on TLC ($CHCl_3$/MeOH 8:2), hydrolysis of **1** was found to be complete, with formation of **2** and **9**. The solution was neutralized to pH 7.5 and extracted with ethyl acetate to remove **2**. The organic solution, dried with Na_2SO_4, was evaporated under vacuum. Crude DHPE was purified by CC (MeOH/H_2O 6:4). The neutral solution was acidified with 6 N HCl and the acid secoiridoid **9** was extracted with Et_2O. Et_2O solution was dried with anhydrous Na_2SO_4 and evaporated under vacuum. Crude secoiridoid **9** was purified by CC (MeOH/H_2O 1:1).

Laboratory Olive Juice. The homogenate prepared at 4°C from 500 g of recently picked, previous stoned, ripening olives (Itrana variety), was centrifuged at the same temperature. The cold aqueous layer, 150 ml, defined as LWW, was separated from the oil and immediately worked up.

One hundred milliliters was extracted with cold solvents, hexane, chloroform, and ethyl acetate in that sequence. Hexane removes traces of emulsified oil and comparatively nonpolar compounds, such as the oleanolic acid present in large quantities on the olive skins. The chloroform essentially extracts oleuropein aglycone **7**, ethyl acetate **2** and **9**. After removal of the solvent under vacuum, each fraction was diluted in water to the same volume of starting LWW and subjected to analytical chromatography. TLC was performed on $CHCl_3$/MeOH 8:2 and 85:15.

The content of the components was determined by HPLC (gradient elution from 10 to 90% MeOH in Na_2HPO_4/CH_3COOH, 0.05M, pH 3.2, in 30 min, 1 ml/min, detection at 254 nm) by comparison with standards of known concentration.

The remaining crude LWW (50 ml) was immediately used for repellency tests.

The dry residue accounted for 7–8% of the juice; ash accounted for 2% of the dry residue.

Olive Mill Wastewaters. The WW collected in various olive mills in the Lazio region were subjected to bioassay. The content of the components was determined as described for LWW.

(E)-*2-Hexenal and Hexanal from LWW HS.* The headspace volatiles from 100 ml of LWW, prepared from ripening olives as previously described, were analyzed using graphitized carbon absorption, thermal desorption, and GC-MS analysis, according to Scarpati et al. (1993).

(E)-*2-Hexenal Content.* Five milliliters of freshly prepared LWW was diluted to 50 ml with water, with 2 ml of an internal standard solution (0.1% octanal in EtOH/H_2O 1:1) added, and the resulting solution was passed through a small column (10 × 30 mm) of Lichroprep RP-18, for aldehydes absorption. Elution was achieved with 30 ml of CH_2Cl_2, which removes the (*E*)-2-hexenal and the internal standard. After concentrating the CH_2Cl_2 solution to about 0.2 ml through a Vigreux column, quantitative GC determination was performed.

Chemotactile Repulsion Test. An ovipositing gravid female insect (7–15 days old) was placed in a glass test tube (length 16 cm, ID 2.5 cm) containing a filter paper strip (12 × 0.5 cm) placed lengthwise and impregnated with 0.2 ml of the test solution. The test tube was sealed with a cotton wool swab soaked in water, to avoid water evaporation from the sample. The experiment was carried out at 22–25°C. The behavior of the insect was then carefully observed whenever the tarsi of its claws came in contact with the strip of paper. If it crossed the paper without showing any signs of distress, this was considered as one acceptance. Otherwise, if the fly backed off quickly or actually jumped away, it was considered as a refusal. Jumping is a sign of extreme distress caused by the test compound [e.g., fresh LWW or (*E*)-2-hexenal]. After refusal the insect stops and rubs its front legs together. It sometimes remains motionless for some minutes, as though paralyzed (e.g., pyrocatechol).

A total of 10 contacts for each insect was considered as one replication. The test was then repeated for the same compound, changing both the insect and the test tube with the sample. The percent repellency response was defined, as the ratio of refusals vs. the total number of contacts (×100).

In the tables are indicated the mean values of percent repellency responses and the standard errors (±SE). Significant differences ($P < 0.05$) within each sample are represented by different lowercase letters, as indicated by Duncan's (1955) multiple-range test.

In blanks, the insect was exposed to exactly the same procedure, the paper strip being soaked with 0.2 ml of distilled water, or 15% 1,2-propandiol in water. The observed repellency values were 17–20%.

RESULTS AND DISCUSSION

Chemical Investigations. We previously prepared the pure samples of olive glucosides and their hydrolysis products, before beginning the research on active compounds of WW and LWW.

The glucosides **1, 5,** and **6** (Figure 1), present only in whole olives (Scarpati and Lo Scalzo, 1993), were isolated by extracting fresh fruits with boiling methanol, in order to deactivate the enzymes released by the injured mesocarp. These enzymes produce rapid alteration in olive chemicals, as shown by LWW analysis, in which native glucosides disappear, while their derivatives **2, 7, 8, 9,** and **10** (Figure 1) are detectable.

The only *o*-diphenolic compound detectable in WW of various origins was DHPE **2,** at a concentration in the region of 0.05%, much lower than oleuropein content in olives (3%).

For the first time we identified secoiridoid **9** in WW of various origin present at a concentration of about 0.30%. It seems to be the oleuropein subunit that is most stable to the enzyme action.

FIG. 1. Nonvolatile chemicals from whole and milled olives.

In order to ensure complete identification of the active components of olive juice, the latter was suitably prepared under controlled laboratory conditions in order to reduce the deep decomposition occurring in WW during the long olive-mill process. The aqueous phase (LWW) of crude olive juice was subsequently extracted with hexane, chloroform, and ethyl acetate. The various fractions were then analyzed.

The main chemicals of LWW from unripe and ripening olives, identified and determined by chromatographic comparison with authentic samples, were **2** (0.10–1.15% w/v), **9** (0.15–0.20%), and the oleuropein aglycone **7** (0.25–0.30%) (Panizzi et al., 1960), near small amounts of tyrosol **10**, a derivative of ligstroside **5** (0.05%). The glucosides **1** and **5** were not detectable.

In LWW from ripe olives only one new *o*-diphenolic component, **8**, was

identified (Scarpati and Lo Scalzo, 1993) as a derivative of demethyloleuropein **6** (5–6% in whole olives).

Analysis of LWW headspace using a previously described method (Scarpati et al., 1993) reveals, as main components, the unsaturated aldehyde **3** together with smaller amounts of the hexanal **4.** The (*E*)-2-hexenal, **3,** was found in a freshly prepared LWW at a concentration of 0.1%. However, the concentration decreases with time as a result of the decomposition and volatility of **3.** It derives from linolenic acid, present in olive oil from 0.2 to 1.5% (Ullmann's Encyclopedia, 1987), through the action of peroxidases on the triglycerides at the time of cell decompartmentalization (Hatanaka et al., 1986).

Laboratory Bioassays. We first developed a suitable test of chemotactile repellency. As the repellency values found for distilled water, used as blank, were 17–20%, we set the lower threshold of repellency response to 40%. We then applied the test to DHPE, obtained and purified from acid hydrolysis of oleuropein, the repellency of which had already been demonstrated by Vita and Barbera (1976). Table 1 shows how a repellency of between 41 and 75% increases as the concentration of **2** in water increases from 0.10 to 0.30%. The results show that the lower threshold level for DHPE activity is at 0.10% concentration.

TABLE 1. CHEMOTACTILE REPELLENCY (% RESPONSE) TO *Dacus oleae* FEMALES OF DHPE 2 AND SECOIRIDOID 9

Reps[a]	Compound	Conc. (% w/v)	Repellency (%)[b] (±SE)
25	DHPE[c]	0.30[d]	75a (±3.52)
15	DHPE[c]	0.25	68b (±2.72)
10	DHPE[c]	0.20	62c (±0.97)
10	DHPE[c]	0.15	57d (±1.15)
25	DHPE[c]	0.10	41e (±2.09)
Blank			
40	H_2O		17f (±2.12)
20	Secoiridoid[c]	0.47[d]	66b (±2.45)
20	Secoiridoid[c]	0.30	57cd (±5.21)
10	Secoiridoid[c]	0.15	27g (±2.88)
Blank			
30	Solvent[e]		19f (±3.41)

[a] Replicates.
[b] Means within tests with different lettes are significantly different, $P < 0.05$, Duncan's multiple range test (Duncan, 1955).
[c] Compunds prepared by oleuropein hydrolysis.
[d] 19.5 mM solutions.
[e] 1,2-Propandiol/H_2O (15% v/v).

We have found that WW samples of various origins displayed a repellency of about 75%, while the DHPE concentration was in the region of 0.05% (see Methods and Materials). It can thus be inferred that the repellency of WW cannot be ascribed to DHPE alone.

A repellent action was found also for **9** by testing solutions of pure **9** in 1,2-propandiol 15% in H_2O, because of its partial solubility in H_2O (Table 1). 1,2-Propandiol 15% gave the same value of repellency as distilled water. As **9** is found in WW at a concentration of about 0.30%, which corresponds to a 57% repellency contribution, and since no synergism between **2** and **9** was detected, it must be concluded that their presence is not sufficient to justify the repellency displayed by the WW. The repellency of LWW, in which the decomposition of active compounds was limited, was obviously more strongly expressed (nearly 90%) and remained appreciable up to 1:4 dilution (42% repellency, Table 2).

Repellency tests were extended to the whole olive and LWW main components, using solutions at the same molar concentration (19.5 mM) as the most active DHPE solution tested (0.30%). Oleuropein and its aglycone **7** displayed repellency values of 77 and 75%, respectively (Table 3). Girolami et al. (1974) claimed that **1** and **7** were inactive. They were led to this conclusion by comparing the activities of solutions of the samples at concentrations equal by weight. The new *o*-diphenolic compound from ripe olive LWW, **8**, (Scarpati and Lo Scalzo, 1993), was found to be equally active.

The hypothesis that the *o*-diphenolic group present in **1**, **2**, **7**, and **8** is the main cause of their repellency is confirmed by the strongly repulsive, and even paralyzing, activity displayed by pyrocatechol **11** at the same molar concentra-

TABLE 2. LABORATORY OLIVE JUICE (LWW) CHEMOTACTILE REPELLENCY PERCENT RESPONSE

Reps.[a]	Sample	Repellency (%)[b] (±SE)
20	LWW	90a (±1.78)
	LWW : H_2O	
15	1:1	73b (±2.42)
15	1:2	57c (±1.78)
15	1:3	50d (±2.23)
20	1:4	42e (±0.87)
Blank		
50	H_2O	18f (±1.04)

[a] Replicates.
[b] Means within tests with different letters are significantly different, $P < 0.05$, Duncan's multiple range test (Duncan, 1955).

TABLE 3. CHEMOTACTILE REPELLENCY PERCENT RESPONSE TO *Dacus oleae* FEMALES OF NONVOLATILE OLIVE CHEMICALS

Reps[a] and Compound	Conc. (% w/v)[b]	Repellency (%)[c] (±SE)
Orthodiphenolic		
15 Oleuropein **1**	1.00	77a (±2.32)
10 Oleuropein aglycone **7**	0.70	75a (±2.51)
10 Compound **8**	0.60	73a (±1.85)
15 Demethyloleuropein **6**	1.00	25b (±1.76)
10 Pyrocathecol **11**[d]	0.21	91c (±2.86)
Monophenolic		
10 Tyrosol **10**	0.27	26d (±2.76)
10 Ligstroside **5**	1.00	28d (±2.09)
Nonphenolic		
20 Secoiridoid **9**	0.47	66e (±2.45)
10 Sodium salt of **9**	0.51	64e (±2.78)
Blank		
30 H_2O		19f (±2.09)
20 Solvent[e]		20f (±1.67)

[a] Replicates.
[b] 19.5 mM solutions.
[c] Means within tests with different letters are significantly different, $P < 0.05$, Duncan's multiple range test (Duncan, 1955).
[d] Pyrocathecol is not present in olives.
[e] 1,2-propandiol/H_2O (15%v/v) used for compounds **7**, **8** and **9**, slightly soluble in water.

tion (19.5 mM). Its repellency had already been reported by Girolami et al. (1981). Further confirmation comes from the fact that monophenolic compounds, such as tyrosol **10** and its oleuropein analog derivative, ligstroside **5** (Gariboldi et al., 1985), found in early unripe olives (Bianco et al., 1993), displayed no appreciable activity (26–28%, Table 3).

Both the repellency displayed by secoiridoid **9** and the absence of repellency in demethyloleuropein **6**, which differs from oleuropein only in the free carboxylic function, constitute exceptions to previous hypothesis. As **6** is the only *o*-diphenolic substance contained in ripe olives (Scarpati and Lo Scalzo, 1993), the latter should be, and in fact are, highly receptive to *D. oleae* oviposition (Vita and Barbera, 1976).

Other phenolic compounds, which other authors (Bolice and Cera, 1984) claim to have identified in WW, but which have not been confirmed by our studies—syringic, veratric, vanillic, and *p*-hydroxyphenylacetic acids—were found to be inactive (30–40%).

Our new important finding is that an aqueous solution of (*E*)-2-hexenal (50 ppm) displayed a very high repellency value (86%). It may thus be inferred that

TABLE 4. CHEMOTACTILE REPELLENCY PERCENT RESPONSE TO *Dacus oleae* FEMALES OF OLIVE JUICE VOLATILES AND RELATED COMPOUNDS

Reps[a]	Compound	Conc. (ppm v/v)	Repellency (%)[b] (±SE)
LWW headspace aldehydes			
17	(*E*)-2-Hexenal	50[c]	86a (±3.76)
16	(*E*)-2-Hexenal	35	70b (±1.23)
10	(*E*)-2-Hexenal	30	60c (±2.34)
10	(*E*)-2-Hexenal	20	46d (±0.98)
10	(*E*)-2-Hexenal	10	22e (±1.45)
20	Hexanal	50[c]	57c (±3.85)
12	Hexanal	35	47d (±2.87)
12	Hexanal	20	32f (±1.87)
10	Hexanal	10	19g (±2.65)
Aldehydes with related structure			
9	(*E*)-2-Heptenal	57[c]	58c (±2.00)
8	(*E*)-2-Heptenal	50	57c (±1.76)
8	(*E*)-2-Heptenal	40	50h (±1.65)
10	(*E*)-2-Heptenal	20	20g (±1.76)
10	Octanal	64[c]	48h (±3.65)
12	Octanal	50	42i (±1.87)
9	Octanal	35	38l (±2.76)
9	Octanal	25	30f (±1.65)
Blank			
40	H_2O		19g (±2.09)

[a] Replicates.
[b] Means with tests with different letters are significantly different, $P < 0.05$, Duncan's multiple range test (Duncan, 1955).
[c] 0.5 mM solutions.

the very high repellency of freshly prepared LWW, containing **3** at a 0.1% concentration, can be essentially ascribed to **3**. Hexanal and other aldehydes of related structure, such as (*E*)-2-heptenal or octanal, at the same molar concentration (0.5 mM), were found to be less active (Table 4).

In conclusion, we identified two groups of substances that are mainly responsible for olive juice repellency: (1) a group derived principally from the catabolism of oleuropein **1**, i.e., DHPE **2**, aglycone **7**, secoridoid **9**, and the demethyloleuropein aglycone **8**; and (2) a group of volatiles derived from the hydroperoxidation of polyunsaturated acids: (*E*)-2-hexenal and hexanal. Both groups are characterized by loss of activity, the first group due to oxidation and the second due to decomposition and volatility.

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ABSORPTION AND RELEASE OF PHEROMONE OF *Epiphyas postvittana* (LEPIDOPTERA: TORTRICIDAE) BY APPLE LEAVES

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Abstract—The absorption and release of the pheromone of *Epiphyas postvittana* (Lepidoptera: Tortricidae), *E* 11-14:OAc and *E,E* 9,11-14:OAc (95:5) by apple leaves was studied using electroantennograms (EAG) and sticky traps baited with pheromone-treated leaves. Leaves exposed to an airstream containing pheromone reached a constant level of pheromone release within 3 min. Release occurred over a period greater than 24 hr, following removal of leaves from the pheromone-saturated environment. Pheromone-treated leaves were effective as lures in sticky traps for at least three nights, although the average catch per night decrease logarithmically with time. In the field, pheromone was detected by EAG on leaves harvested from up to 25 cm away from a central point source of pheromone. The shape of a surface representing equal pheromone re-release from leaves around a central point source was defined by interpolation from a three-dimensional transect. Leaves harvested from 5 cm under the dispensers showed the highest pheromone release rate. Leaves downwind of the dispensers also had higher releases of pheromone. In a treated orchard, significantly higher EAG measurements were recorded in the rows of trees that contained dispensers, compared to grass interrows or untreated trees. The implications of foliar pheromone adsorption and release on atmospheric concentrations and insect behavior require further investigation.

Key Words—*Epiphyas postvittana*, Lepidoptera, Tortricidae, electroantennogram, pheromone, dispenser, apple, mating disruption, atmospheric concentration.

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INTRODUCTION

Considerable progress has been made in using lepidopterous sex pheromones for pest control by disrupting male mating behavior (Jutsum and Gordon, 1989; Ridgway et al., 1990; Suckling, 1993). Successful use of this method requires the release of large amounts of pheromones over the target area. The mechanism of disruption is still the subject of conjecture (Cardé, 1990). Information about the actual pheromone concentration required and its spacial and temporal distribution should help to understand how disruption operates.

Earlier models describing pheromone distribution suggested the presence of a slow-moving pheromone cloud with the average pheromone concentration decreasing with distance from the pheromone source (Wright, 1958; Bossert and Wilson, 1963; Wilson et al., 1969). In such an environment, pheromone molecules reaching male moth antennae elicit upwind flight towards the odor source, e.g., a calling female, or a synthetic pheromone lure (Kennedy et al., 1980). However, higher concentrations have been shown to result in arrestment (Kennedy, 1978) according to the "threshold hypothesis," which defined a middle range of behaviorally active pheromone concentrations (Roelofs, 1978). In addition, there is evidence that fluctuating rather than continuous pheromone stimulation is necessary for sustained upwind flight, providing that the peak-to-trough amplitudes reaching the antennae are sufficiently high (Kennedy et al., 1980, 1987; Kennedy, 1982; Willis and Baker, 1984; Baker et al., 1985; Baker and Haynes, 1989). Baker et al., (1985) also showed that the lack of upwind flight in a volume of uniform permeation was correlated to high, continuous antennal activity, measured by electroantennogram (EAG), which contrasts with the turbulent cloud in which upwind flight is achieved.

The notion of a uniform pheromone cloud was challenged by the measurements of Murlis and Jones (1981), using negative ions to simulate the distribution of pheromone. The discontinuous and filamentous structure of plumes was confirmed by additional experiments by Murlis (1986) and Murlis et al. (1992). However, these experiments were performed using ions as a model for pheromones. Furthermore, the absence of the natural habitat such as plant foliage, may influence the distribution of pheromone in several ways. Buffering effect of foliage in shelter belts is clearly important in reducing wind, thereby decreasing the dilution of the released pheromone. Within the boundaries of the treated area, adsorption and release of the pheromone released from leaves could contribute to a uniform distribution of the pheromone (Wall et al., 1981; Noldus et al., 1991). Adsorption and release of pheromone by leaves could affect the atmospheric concentration of pheromones and enhance its effect on mating disruption.

One technique showing promise for assisting in the determination of atmospheric pheromone in real time and at low concentration is the EAG (Baker and

Haynes, 1989; Sauer et al., 1992; Bengtsson *et al.*, 1994; Suckling et al., 1994). We wondered whether apple foliage could function as a pheromone buffer system for *Epiphyas postvittana* (Walker). This paper reports laboratory and field experiments to elucidate the nature of the possible relationship between pheromone and apple foliage over time and space. We investigated whether the EAG technique could show pheromone uptake and release from apple leaves, as a function of time of exposure, time since exposure, and distance from the point of exposure, as well as a behavioral corroboration of the effect on male moths.

METHODS AND MATERIALS

Insects

All experiments were carried out with antennae from laboratory-reared male light-brown apple moth, *E. postvittana*. After eclosion, males were kept in a refrigerator at 12°C for a maximum of 2 to 4 days before antennae were used.

Pheromones

The natural blend of pheromone for this species is a 95:5 ratio of (*E*)-11-tetradecenyl acetate (*E*11–14:OAc) and 5 μg (*E*,*E*)-9,11-tetradecadienyl acetate (*E*,*E*9,11–14:OAc) (Bellas et al., 1983). Rubber septa (Arthur H. Thomas) were used as lures in pheromone traps and as calibration sources for the EAG, and contained 100 μg *E*11–14:OAc and 5 μg *E*,*E*9,11–14:OAc (Bellas et al., 1983).

Pheromone dispensers were obtained from Shin-Etsu Chemical Co., Tokyo, and contained 54.9 mg of (*E*11–14:OAc), 2.5 mg of (*E*,*E*9,11-tetradecadienyl acetate (*E*,*E*9,11–14:OAc), and 19.7 mg of (*Z*)-11-tetradecenyl acetate (*Z*11–14:OAc), as well as 16.8 mg of other substances, such as stabilizers. These dispensers were used here as they have been used extensively in over 350 ha of trials of mating disruption of this species (Suckling and Shaw, 1991; Suckling et al., 1990, 1991; unpublished data) and are the most likely dispenser to be of commercial significance. The disadvantage of this blend, containing *Z*11–14:OAc, relates to its role as an inhibitor of trap catch when present with the pheromone (Rumbo et al., 1993). These dispensers release the pheromone at 7–15 μg/hr at 12–20°C, after ten weeks in the field (unpublished data).

Electroantennogram apparatus

The EAG apparatus consists of a Perspex antenna holder placed inside a glass chamber, which isolates the antenna from ambient air (Sauer et al., 1992). The ends of the antenna are held in wells filled with Ringer's solution, containing silver–silver chloride electrodes. A suction pump generated a steady airstream

through the chamber past the antenna. The EAG elicited by odors reaching the antenna was amplified, filtered, and stored on a 386SX portable computer operating with an A-D card, sampling at 18.2 Hz. The glassware used in these experiments was acetone-washed, heated to 170°C for at least 16 hr, and cooled before reuse.

The ground potential of the antenna (the electrical potential of the antenna in clean air) was measured with a charcoal filter attached to the inlet of the glass chamber. The EAG in ambient air was measured after removing the charcoal filter. Calibration was achieved using a rubber septa standard (above), with a defined volume of air (15 ml) blown across the septa and added to the main air stream. The measurement of atmospheric pheromone was always preceded by three calibration pulses from the rubber septa standard. EAG peak heights were measured with computer software (Sauer, 1991).

The antennal response to pheromones was always normalized to the response to the rubber septa standard (calibration pulse) by dividing the measured response by the mean voltage generated by accompanying calibration pulses. This unitless value is defined as the normalized EAG amplitude. For some experiments, the EAG response to leaf volatiles from untreated leaves had to be subtracted from the total response to treated leaves. This value was defined as the differential EAG amplitude and has been presented on a log scale to better represent real concentrations, since the dose–response is log-linear. There was a 10-fold increase in pheromone concentration for a change in differential EAG of ca. 0.25 (unpublished data). Preliminary experiments showed that antennae varied in the degree of baseline sensitivity to host-plant or environmental volatiles and pheromone in orchard air. Single antennae were therefore used for all related measurements, but absolute concentrations could not be calculated without knowing the intercept of the dose–response for an individual antenna.

Laboratory Experiments

Uptake of Pheromone by Leaves. Apple leaves of approximately uniform surface area (ca. 42 cm^2, cv. Granny Smith) were picked from an orchard where there were no pheromone dispensers (untreated area). The EAG elicited by each leaf was measured to determine the antennal response to the leaf volatiles. The petioles were then placed in a vase of water and the leaves held 15 cm downwind from two Shin Etsu pheromone dispensers mounted on the outlet side of a small fan, simulating field conditions of leaves in pheromone-treated environments. Single leaves were exposed in this way for either 3 sec, 10 sec, 30 sec, 1 min, 3 min, 10 min, or 30 min, and then put in a clean glass "cold trap" (95 ml). The cold trap was connected to the EAG apparatus, and five to seven EAG measurements of the pheromone release rate were taken using 1-sec air pulses into the cold trap within 30 sec of removal from the airstream. These EAGs

were normalized to the related calibration pulses (see above). A normalized pretreatment measurement (on the same leaf) was subtracted giving the differential EAG amplitude. Preliminary experiments indicated little difference between untreated leaves; therefore, only one leaf was measured on each occasion as a reference standard.

Release of Pheromone from Apple Leaves. Branches (20 cm length) with at least four leaves (as above) were exposed in a pheromone-saturated environment for at least 24 hr. The branches were held in a plastic bag (6-liter volume) containing two pheromone dispensers and a small fan, to ensure an even pheromone concentration in the entire bag. After removal from the pheromone-saturated bag, the branch stems were placed in water, with the leaves in a clean airstream (a fume hood with lowered shield, giving an airspeed of ca. 0.5 m/sec). Pheromone rerelease rate was measured with the EAG after time intervals of 0.25, 10, 30, 135, 360, and 1440 min.

Field Experiments

Trapping with Saturated Leaves. This experiment aimed to test whether trap catches could be made in an untreated area, with pheromone-treated leaves as lures. Traps were placed in apple trees at the Lincoln University Biological Husbandry Unit, Canterbury, New Zealand, between April 2 and April 22, 1993. Standard delta traps (Suckling and Shaw, 1990) with sticky bases were baited with leaves that had received pheromone exposure in a plastic bag, with a fan circulating the air around two Shin Etsu dispensers and around a branch with the base in a vase. Leaves were exposed in this manner to pheromone for > 3 days before use in traps, and catch was monitored nightly for the same leaves for three nights ($N = 10$ traps/treatment). Traps baited with clean leaves were used as controls ($N = 5$), while traps baited with standard rubber septa were also operated ($N = 5$) to indicate flight activity. Traps were prepared about 3 hr before dusk and hung at ca. 1.5 m height with a minimum distance of 5 m between traps. They were inspected and rerandomized daily. After three days in the traps, the leaves were replaced by new, freshly treated leaves, and the cycle repeated. All treatments were present simultaneously. The mean trap catch per trap and night for each leaf age (1, 2, or 3 days after pheromone exposure) was calculated.

Dispersion of Pheromone from a Point Source. This experiment was designed to determine the dissemination of pheromone released from dispensers, into nearby apple foliage in the field. Two weeks before the EAG measurements commenced, two dispensers, which had been aged for 10 weeks in the field, were placed in a tree (cv. Red Delicious) grown on the Lincoln canopy system (Dunn and Stolp, 1987). In this canopy, branches are trained horizontally and upright shoots from the base of lateral branches provide a uniform array of leaves in all dimensions.

Leaves of similar size (ca. 20 cm^2) were picked after two weeks on x, y, and z axes (50, 25, 12, 6, and 0 cm) from the two central dispensers, and immediately placed individually in a glass cold trap. The differential EAG amplitude was measured as described above, in which the EAG response elicited by an untreated leaf alone was subtracted from the EAG measured for a leaf post-treatment. An isosphere showing the location of equal response around the two Shin Etsu dispensers (indicated by a differential EAG amplitude of 0.2) was generated by mathematical interpolation of the measured data based on the inverse square root of distance, the Pythagorean function expected to explain changes in concentration with distance.

Measurement of EAG in an Orchard. EAG measurements were made on transects across an untreated and a pheromone-treated apple orchard. The trees (cv. Royal Gala) had been planted in 1983 (3.0 × 4.5 m), in north–south oriented rows. Trees were ca. 4.5 m high. Four Shin Etsu pheromone dispensers were placed in a cluster on every second tree, in every second row, resulting in 185 point sources/ha. Dispensers were placed at 1.2–1.6 m above ground, 1 m to the east of the trunk, 11 weeks prior to EAG measurement. The EAG apparatus was placed on a tripod at 1.4 m above ground and was moved in a transect across rows, with measurements taken at four types of sites (shown in Figure 6, p. 1838): (A) 1 m from the trunk of a treated tree, near the dispensers; (B) grass rows, 1 m east of a dispenser; (C) 4.5 m east of A in a row of trees without dispensers; and (D) grass rows, 1 m east of C.

Relative EAG amplitude (mean of three orchard air measurements) was determined at each site (N = 12–22), in a transect across sites A–D. In the untreated block (without dispensers), transects measurements were made in the corresponding positions (N = 13–16). Additional measurements (N = 6–12 per cultivar) were made in untreated blocks containing cv. Royal Gala, Braeburn, Cox's Orange Pippin, and Red Delicious trees, in order to determine the effect of fruit cultivar on EAG response. The subscripts t and u refer to treated and untreated, respectively, in the results.

RESULTS

Laboratory Experiments

Uptake of Pheromone by Leaves. The rate of uptake of pheromone in an airstream by apple leaves was very rapid, with the asymptote reached after ca. 3 min (Figure 1). The rate of uptake was described by the equation:

$$\text{EAG response} = a - a/e^t$$

where a is the asymptote ($r^2 = 94\%$, 5 df). These measurements are an indirect assessment of the process of pheromone uptake, given that our EAG results

reflect the rate of release of pheromone from the leaf, within 30 sec of removal of the leaf from the pheromone airstream. Additional time of exposure beyond 3 min did not lead to a higher pheromone release from the measured leaves, indicating that equilibrium for the application method was reached within 3 min.

The measurements of the EAG elicited by pheromone-treated leaves indicate the rate of rerelease of pheromone from the leaf surface, not the amount of pheromone actually absorbed into the leaf. Hence we do not know the degree of saturation of the leaves with pheromone, only that the leaf is able to release pheromone in proportion to the length of exposure. In our experiments we took the release as a measure of the uptake of pheromone by the treated leaves.

Release of Pheromone from Leaves. The release of pheromone from leaves was slower than the rate of uptake, with a declining rate of release detectable over several hours (Figure 2). Pheromone was still detected after 24 hr, at which

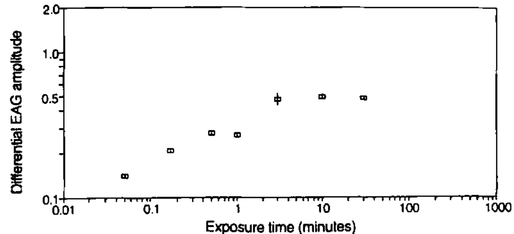

FIG. 1. Changes in differential EAG amplitude (±SE) over time measured with EAG, following increasing periods of exposure of leaves to a steady airstream containing pheromone of *Epiphyas postvittana*. The rate of uptake of pheromone by single apple leaf was very rapid, with the asymptote reached after about 3 min.

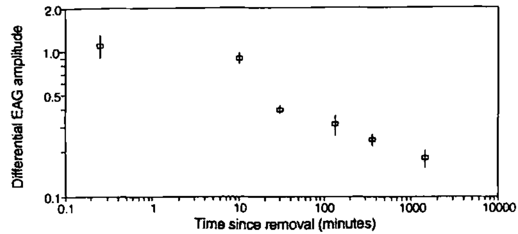

FIG. 2. Changes in differential EAG amplitude (±SE) over time measured with EAG, following removal of leaves from saturated environment of pheromone of *Epiphyas postvittana*. Measurement were made on four replicate leaves, using an untreated leaf as a reference for background plant volatile response. The release rate of pheromone initially declined rapidly, but pheromone was still detected after 24 h.

point the experiment was terminated. The differential EAG dropped from 1.1 to 0.2 in 24 hr, with half the EAG amplitude (0.65) reached after 20 min.

The different method of exposure in this experiment probably accounts partly for the high initial EAG amplitude reached by leaves kept in a pheromone saturated bag (Figure 2), compared to the amplitude reached by leaves exposed in a current of pheromone-treated air (Figure 1). The difference in the differential EAG amplitude between 30 min of exposure in an airstream, and after 24 hr in the bag was approximately twofold. The initial rapid loss of pheromone (shown by the higher EAG response in Figure 2) led to differential EAGs after 20 min, which were similar to those of the first experiment (Figure 1). The most probable sites of pheromone adsorption with leaves are the surface waxes (Wall et al., 1981) due to their similar chemical attributes to pheromones (e.g., lipophilicity) (Fernandes et al., 1964; Baker, 1982; Jeffree, 1986). The waxes would function as pheromone reservoirs lacking a rate controlling mechanism, but probably following a rate of release proportional to $t^{-1/2}$ (Zeoli et al., 1981).

Field Experiments

Trap Catch. The catch of males in traps baited with leaves (Figure 3), gives behavioral corroboration of the EAG results. The mean catch per trap per night declined logarithmically over three nights, from 0.24 males per trap per night for traps baited with leaves removed from the saturated environment 3 hr before dusk, when flight and capture occurred. No moths were caught with untreated leaves, while the mean catch with rubber septa was 5.55 males per trap per night. Catch with freshly treated leaves releasing the pheromone plus

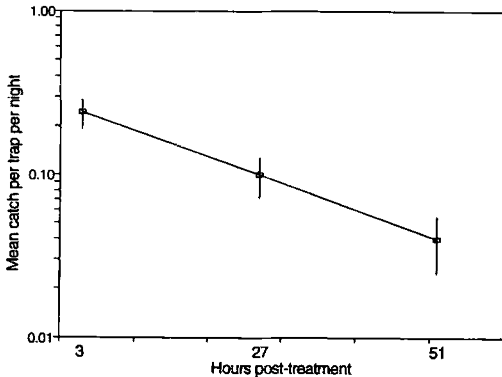

FIG. 3. Changes in trap catch of male *Epiphyas postvittana* (±SE) over time, following removal of leaves from a saturated environment of pheromone. Nearby traps baited with rubber septa caught a mean of 5.5 males per trap per night during this period.

inhibitor was therefore about 4% of catches with rubber septa lures releasing the pheromone. Since moth responses to traps baited with rubber septa are easily disrupted (Suckling and Shaw, 1992), this suggests that the attractiveness of leaves would be relatively insignificant in a mating disruption environment. This does not preclude their role in habituation or adaptation mechanisms of disruption. Traps were not replaced with new traps daily in this experiment, as it was considered that biologically significant quantities of pheromone released from the leaves would not be adsorbed and released from the traps, in contrast with leaves that had been exposed to pheromone in a saturated environment.

This experiment demonstrated that *E. postvittana* males were attracted to the pheromone blend rereleased from the leaves, despite the fact that a blend containing Z11–14:OAc has been reported to function as an inhibitor in Australia (Bartell, 1982; Rumbo et al., 1993) and New Zealand (Suckling and Rumbo, unpublished data), reducing catch by ca. three fold. Dispensers like those used in the current study (containing 30% Z11–14:OAc) were also successful at trapping moths, when placed in traps in untreated areas (Suckling, unpublished results).

Dispersion of Pheromone from a Point Source. This experiment shows the ability of apple leaves to function as a pheromone buffer system, under field conditions (Figure 4). The pheromone dispersed from the dispenser and was adsorbed onto the surrounding plants, which thus became pheromone dispensers of their own. The rate of pheromone release by leaves collected near two dispensers was a function of distance from the dispensers in the horizontal and vertical planes.

The differential normalized EAG amplitude (±SE) elicited by leaves was plotted against the different distances from a central pheromone point source.

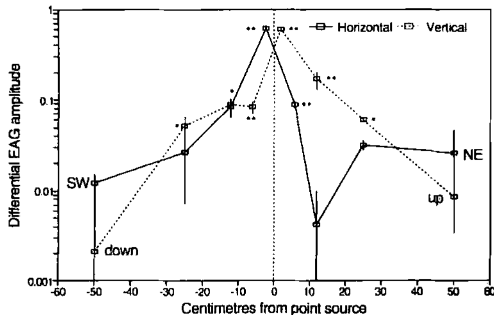

FIG. 4. Horizontal and vertical changes in differential EAG amplitude (±SE) elicited by leaves at different distances from a central pheromone point source of two *Epiphyas postvittana* pheromone dispensers placed in a three-dimensional array of apple leaves. (*$P < 0.05$; **$P < 0.01$).

The measurements used only one horizontal axis and the vertical axis, in order to sustain a good antennal response for the whole transect (Figure 4). Pheromone was detected above the background odor of untreated leaves (differential EAG) up to 25 cm away from the central point source. All differential EAG amplitudes were significantly higher than the untreated leaf ($P < 0.05$), except for SW 25 cm and NE 12.5 cm (Figure 4). Pheromone could not be detected on leaves 50 cm from the center. This is probably due to the interference of plant or environmental volatiles with the pheromone reception, which reduces the resolution of the EAG system. The leaves sampled varied in size (range 13–68 cm^2) and age, but we failed to find any consistent relation between leaf size or age and the level of EAG response to individual leaves.

Data from a second set of measurements (which covered a smaller range of distances, but across three dimensions) were used to estimate the three-dimensional shape of the surface of an isosphere of constant pheromone concentration surrounding the point source (Figure 5).

The isospheres of equal pheromone concentration (grey ribbons, differential EAG = 2) achieved by interpolation of the measured data deviate from the (ideal) sphere (white ribbon), which indicated equidistant points on the x, y, and z axes around the central point source. Higher pheromone release was measured on the leaves beneath and to the south of the pheromone source.

Pheromone molecules presumably are dispersed downward once released from the dispensers, possibly due to the influence of downdrafts. Pheromone release rates are greater at warm temperatures, so the spatial pattern is probably more strongly influenced by warmer winds. Temperature may also affect the absorptivity of the leaves.

EAG Measurements on a Transect through an Orchard. There were significant differences in the EAG responses recorded at different sites. In the tree rows (site A_t, a treated site located 1 m from the trunk of a tree with dispenser), the mean EAG was 0.367 (±0.026). This normalized EAG amplitude was significantly different from those in all other sites ($P < 0.001$) and was higher than in the untreated orchard. The average pheromone concentration in the grass rows site, B_t (1 m east of a dispenser) reached an average value of 0.252 (±0.009), which was not significantly different from sites C_t and D_t. In the next tree row (site C_t tree row with no dispenser, 4.5 m East of A_t), an average normalized EAG of 0.265 (±0.010) was measured. This was significantly different from site D_t ($P < 0.05$), but not significantly different from site B_t. The lowest normalized EAG response of 0.228 (±0.009) was measured in site D_t, the grass row, 1 m East of C_t. Measurements made in the corresponding sites in an untreated block (C_u and D_u), showed a significantly lower EAG amplitude than in the pheromone-treated sites. The normalized EAG amplitude in the grass row (site D_u) was 0.224 (±0.004), the average normalized EAG in the tree row (site C_u) 0.217 (±0.007). There was no significant difference between the

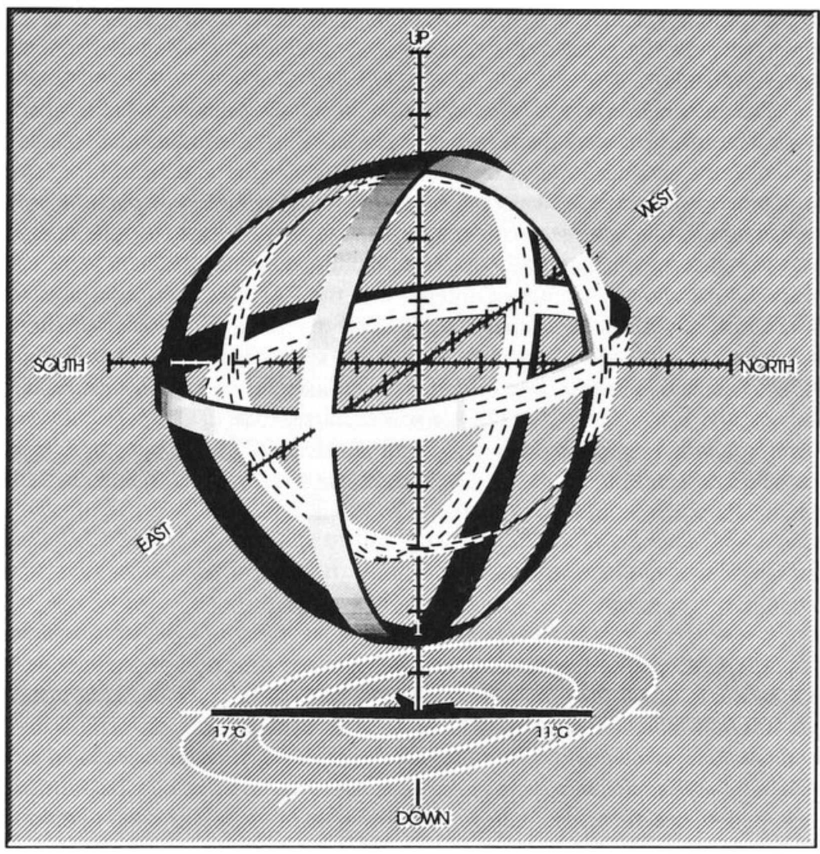

FIG. 5. Predicted isosphere of differential EAG response of 0.2 surrounding two *Epiphyas postvittana* pheromone dispensers placed in a volume of apple leaves. White bands represent a perfect sphere at 15 cm radius, while grey bands represent the derived EAG responses.

untreated tree (site D_u) and (1) untreated grass-row (site C_u) or (2) the treated grass row site D_t. The treated site C_t was significantly higher than the corresponding untreated site, C_u. The measurements in the untreated block also showed much lower variance than sites in the pheromone-treated block.

With increasing distance, the pheromone concentration decreases, and the buffering effect of the plant surfaces become predominant. This can explain the increased EAG amplitude measured at site C_t (tree row without dispenser). The EAG signal at this site was significantly higher than at site B_t (grass row, $P < 0.05$), although further away from the dispenser. The increased response at site

C_t was probably achieved by the release of pheromone from the leaves of the apple trees. In the grass rows, there was no additional pheromone from the leaves available.

The antennal responses elicited in these locations cannot be due to pheromone molecules but were the response to plant or other environmental volatiles, for which the antenna is much less sensitive. The smaller variance of the EAG amplitude in the untreated sites indicates that the distribution of plant or environmental volatiles was also less variable than the distribution of the pheromone.

The measurements made in untreated blocks containing different apple cultivars did not show a significant difference in the EAG baseline. Receptors for plant or other volatiles are generally described to have a wider range of stimulants than the pheromone receptors, which are very specific (Den Otter et al., 1978). Discrimination between different environmental volatiles in the field is not possible using the EAG. The same EAG amplitude in different cultivars, therefore, would not necessarily require an identical volatile composition, since the odorants contribution to the EAG could conceivably be different, but still achieve the same amplitude (although this seems unlikely).

DISCUSSION

The EAG device has several disadvantages for detection of pheromone in low concentrations. It is probably the most sensitive detector available, being ca. 100-fold more sensitive to pheromone than standard gas chromatographic methods (Bengtsson et al., 1994). The measurements are instantaneous and give insight into the rapid changes in pheromone concentration that may be of behavioral importance for mate finding. The low cost of sampling is an additional advantage. Due to the fast response and the mobility of the device, results can be achieved that are not otherwise possible (e.g., transects).

The main disadvantage of the EAG is the difficulty in the estimation of absolute pheromone concentrations. Although the antenna is a sensitive pheromone detector, the antennogram is nonspecific due to the large number of receptors for plant volatiles on the antenna. For *Lobesia botrana* Hb. (Sauer et al., 1992) and *Cydia nigricana* F. (Bengtsson et al., 1994), the low level of baseline response presented much less a problem in estimating atmospheric pheromone concentrations. Individual light-brown apple moths vary in responsiveness to pheromone and environmental volatiles (unpublished data). Here, the use of differential EAG measurements circumvented this problem. However, the variation in sensitivity to environmental odor and pheromone stimuli between insects requires that all related measurements be taken with a single antenna. Since antennae only lasted up to ca. 2 hr, this limited the length of our experiments.

A second major disadvantage of the EAG for field measurements of pheromone is that the antennal response is essentially sigmoid. Depending on the pheromone concentration present in the odor "background" in the field, equal changes in the antennal electrical potential could indicate different changes in pheromone concentration. Therefore the baseline potential of the antenna in clean air and untreated orchards always needs to be measured (unless a reliable "environmental odor" calibration can be developed). Hence measurement of absolute pheromone concentrations is not possible with the EAG alone, although this can be achieved with additional EAG–gas chromatography measurements (Bengtsson et al., 1994).

Apple leaves can take up and release the pheromone of *E. postvittana*, according to both electroantennogram and behavioral studies. These findings agree with several previous studies. Wall et al. (1981) showed that pheromones rereleased from pea leaves are attractive to male *Cydia nigricana*. Noldus et al. (1991) reported that pheromone absorbed onto Brussels sprouts attracts *Mamestra brassicae* and their parasitoids. Karg et al. (1990) showed that the pheromone concentration and structure of the pheromone cloud in treated vineyards was highly dependent on the state of the vegetation in the target area. In summer, with fully developed vegetation, the pheromone concentration was higher and the pheromone cloud more evenly distributed. Pheromone concentration increased rapidly after application of pheromone dispensers. However, the removal of the dispensers was followed by a fast initial drop in the pheromone concentration due to the lack of pheromone release from the dispensers, followed by a rather slow process of decline of the remaining pheromone concentration due to the rerelease of the leaves. The more rapid uptake of *E. postvittana* pheromone, compared to rate of release from apple foliage shows the same pattern in grapes (Karg et al., 1990).

In this study, the portable EAG system enables us to measure the pheromone rerelease of single pre-exposed leaves as well as the pheromone concentration within a treated orchard, thus giving a description of the actual spatial distribution within these areas. The distribution of pheromone in the target areas is important in the identification of the mechanisms of mating disruption of *E. postvittana*.

The primary mechanism(s) of mating disruption are still unknown, but two are most likely in this case: false trail following and adaptation/habituation, although other mechanisms are possible (Bartell, 1982; Cardé, 1990). Polyethylene dispensers identical to those used here were found to be attractive in traps (Suckling, unpublished data), and false trails from these dispensers cannot be completely discounted. Peripheral adaptation seems unlikely to be a major factor, with the recovery of the receptors in clean air within seconds (Rumbo, 1981). In contrast, habituation at the CNS level (Bartell and Lawrence, 1973) is probably very important in *E. postvittana*. Disruption using only a partial

(nonattractive) blend (Suckling and Clearwater, 1990) speaks for adaptation, habituation, or combinations of these as the mechanism for successful mating disruption.

The comparatively low quantities of pheromone released from a single leaf in our experiments suggests that leaves probably cannot create false trails. However, leaves could function to multiply the number of plumes of varying strength (reducing with the inverse square of the distance from the dispenser), with the result of a complex pheromone cloud, rather than trails. The total contribution from leaves releasing pheromone in a treated orchard could be sufficient to assist adaptation of the sensory organs or habituation of the insects (Baker et al., 1988). In situations with a rather homogeneous pheromone cloud, upwind flight ceases to take place (Kennedy, 1978), so it is plausible that the foliage may have a role as a spatial buffer in influencing atmospheric pheromone concentrations, and hence disruption, in addition to providing wind resistance.

The pheromone quantity depended on location in the treated area (e.g., tree row or grass row, Figure 6.) Pheromone is present, at least intermittently, in the whole treated area, and a male moth in this area would perceive pheromone. The temporal resolution of the antenna becomes slower with higher concentration and sustained stimulation (Baker et al., 1985). The fusion of the antennal signal does not have to be complete, as Baker et al. (1985) originally stated, but smoothing of the signal can be sufficient (Baker and Haynes, 1989) to end upwind flight.

Pheromone release from foliage depends on the temperature and the pher-

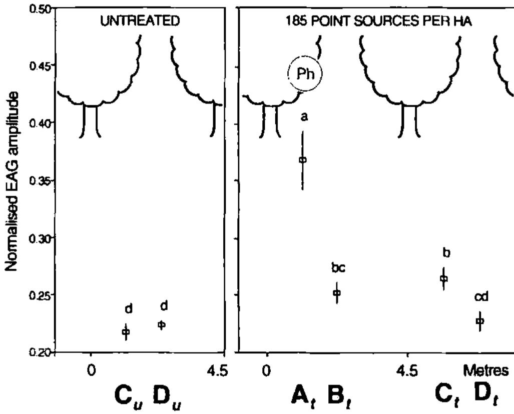

FIG. 6. Differences in mean normalized EAG amplitude (±SE) in treated and untreated blocks with samples taken in tree and grass rows. Sample sites (capital letters) are defined in the text. Ph = four pheromone dispensers. Data points separated by different lowercase letters were significantly different according to a t test ($P < 0.05$).

omone concentration gradient between plant waxes and ambient air and can be described as a pheromone reservoir lacking a rate-controlling mechanism. It is most likely that such a leaf system has different release characteristics than the polyethylene dispensers, with greater influence from wind speed on release rate. These different release characteristics could conceivably have an averaging effect on the pheromone concentration, for example, by partly compensating for increased wind speed through increased pheromone release under higher winds.

Our results clearly show that adsorption and release of pheromone of *E. postvittana* and apple foliage occurs in the treated area. This effect may be a significant influence on the distribution over time and space of the pheromone and hence, on efficacy and potential mechanisms of disruption. Orchards that lack foliage canopy due to either seasonal effects or tree management, are more likely to experience difficulty in achieving successful disruption. Foliage can be important to atmospheric pheromone concentrations in orchards in either of two ways. Foliage can reduce wind speed from physical resistance or impact on concentrations through acting as a sink and source, due to the adsorption and release of pheromone from foliage described here. The effect of foliage on quantity of atmospheric pheromone has been demonstrated here, although it remains unclear whether this effect is large enough to have a significant impact on the efficacy of behavioral disruption. Foliage also contributes to reduced signal variance, but the role of release of pheromone from apple leaves on this effect also requires further elucidation.

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SCENT, SEX, AND THE SELF-CALIBRATING RAT

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Abstract—We propose that one mechanism whereby male rats, *Rattus norvegicus*, might gauge the reproductive condition of a female is by calibrating a stable odor with an odor that fluctuates through her reproductive cycle. We provide behavioral and histological evidence in support of such a self-calibration model. Male rats sniffed frequently at various body zones of females, and the proportion of sniffs deployed to each zone varied with the females' reproductive condition and relatedness. The females' haunches received more sniffs than any other part of their bodies, irrespective of their relatedness or reproductive condition. Furthermore, males tended to sniff the haunch after sniffing the forequarters, as part of a sequence of sniffing along a female from forequarters to hindquarters. Histology of the skin sebaceous glands indicated that the secretory activity of glands in the haunch, but not those in the forequarters, changed during estrus. Therefore, male rats had the opportunity to judge a female's reproductive status by calibrating the odor of her haunch against that of her forequarters. Self-calibration could represent a means of accommodating differences in odor within individuals.

Key Words—*Rattus norvegicus*, rat, sniffing behavior, self-calibration, sebaceous glands, odor selection.

INTRODUCTION

Odors have great social significance for laboratory rats, both directly (Brown, 1985a) and as primers (Brown, 1985b). Whole-body odors (Brown, 1985a), and skin (Mykytowycz and Goodrich, 1974; Stoddart, 1980; Albone, 1984; Mader-

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son, 1985; Flood, 1985; Rivard and Klemm, 1989) are likely to be important in rat chemical communication.

One obvious source of skin odor is the secretions of sebaceous glands, and the rat haunch is well endowed with sebaceous tissue (Ebling, 1963, 1974; Ebling and Skinner, 1967; 1975; Thody and Shuster, 1975). Ebling (1977) described changes with the estrus cycle in the size and productivity of sebaceous glands on the haunch of female laboratory rats, suggesting that they played a role, through odor production, in signaling reproductive state. However, whether sebaceous glands in other parts of the body fluctuate in synchrony with the haunch glands is not known. In order to receive a signal encoded in an odor rats must smell that odor. Although body sniffing is a major component of rat social behavior in the wild, there is no evidence of its functions in the literature. Mutual sniffing at the nose has been referred to as recognition sniffing by Calhoun (1962) and Barnett (1975).

There are currently three main explanations of how mammals might encode a signal in an odor. First, the presence of one compound might provide the critical odor. Second, ratios of a small number of chemicals within an odor might signify characteristics such as the sex, age, or reproductive status of the emitter (White and Chambers, 1989; White et al., 1989). Third, information might be encoded by a pattern in the relative abundance of a spectrum of odorous compounds (a chemical image) (Albone, 1984).

A signal encoded in an odor must be translated in order to complete the communication process. Information might be translated with reference to a "lexicon," the interpretation of which might be learned (involving a scent memory) or innate (naive wild rats are frightened of the scent of foxes) (Vernet-Maury, 1980). Alternatively, the scent might be matched to another by associative learning (Gosling, 1990; Reece Engel, 1990).

Gosling (1990) has provided compelling arguments why scent-matching is an efficient mechanism for olfactory communication in other contexts. Here we propose a new model whereby odors are interpreted by calibrating a fluctuating signal with a stable signal, and we term such assessment self-calibration. In support of the self-calibration model, we have studied how male rats assess a female's reproductive status by calibrating odors from different zones of her body, the secretions of only one of which are sensitive to fluctuations in circulating sex hormones. We present behavioral evidence that males compare different zones of a female's body and histological evidence that secretion in only one of these zones (the haunch) fluctuates with estrus condition.

METHODS AND MATERIALS

Experimental Animals (Behavioral Observations). F_1 crosses of wild-trapped male *Rattus norvegicus* and female Wistar and D.A. rats were used for behavioral observations and semiochemical analysis. These animals were wild-

type in appearance but sufficiently tractable to facilitate observation and handling.

At 3–4 weeks of age, rats from six litters (23 females and 21 males) were separated into single-sex cages (30 × 44 × 23 cm hanging PVC with wire grid tops) but kept in sibling groups under standard animal house conditions. Rats were tested 8–24 weeks after separation.

Females' phase of estrus were determined by vaginal epithelial cell counts (Zarrow et al., 1964). Scores (1–5) for the abundance of leukocytes (L) and nucleated (N) and cornified (C) cells were used to determine an index of estrous (E) calculated using the formula $E = C/(C + N + L)$. Females were categorised as estrous if $E > 0.7$ or diestrus if $E < 0.3$).

Behavioral Observations. Observations were made in 2.2-m-high rat-proof pens with a floor area of 4.6 × 5.6 m, lit on a 16:8 hr day–night cycle. Abundant food and water were always available. Using random numbers, one male and one female rat were selected to provide interactions between related and nonrelated rats, using estrus and diestrus females. Each animal was used once for each interaction type, using a different partner each time. Ten pairs were used for interactions between males and related estrus females, nine pairs for interactions between males and nonrelated estrus females, 21 pairs for interactions between males and related diestrus females and 22 pairs for interactions between nonrelated diestrus females. The female's reproductive status was assessed after each observation period.

Behavioral observations were made during 10-min sessions. A single male was introduced into the test pen 5 min before a single female, and observations began as soon as the female was released into the pen. Six behavior categories were recorded: sniffing, locomotion, mounting, genital grooming, self-grooming, and other. We inferred that a male had ejaculated when a mount was followed by genital grooming and a refractory period (Dewsbury, 1967).

A sniff was recorded when the male's nose touched the female's body. Each sniff was recorded as directed to one of eight zones depicted in Figure 1; rats were divided into these zones by eye. Zones were chosen because they were sniffed by rats during pilot studies, contained scent glands according to published evidence, or were observationally convenient. When two consecutive sniffs occurred within 5 sec without any apparent interruption, they were deemed to be part of a sequence. Transition probabilities for consecutive sniffs to various body zones were calculated using contingency tables (Bakeman, 1986).

To discover whether sniffs deployed to each zone differed between females on the basis of relatedness, reproductive cycle, or both, we analyzed the data using a three-level nested model II analysis of variance (Sokal and Rohlf, 1981). This test was applied to both the absolute number and the proportions of sniffs per zone during a 10-min trial. Multi-pairwise comparisons of means were made using the GT2 test (Sokal and Rohlf, 1981).

Histology. Corpses of wild rats trapped at the Oxford University Farm,

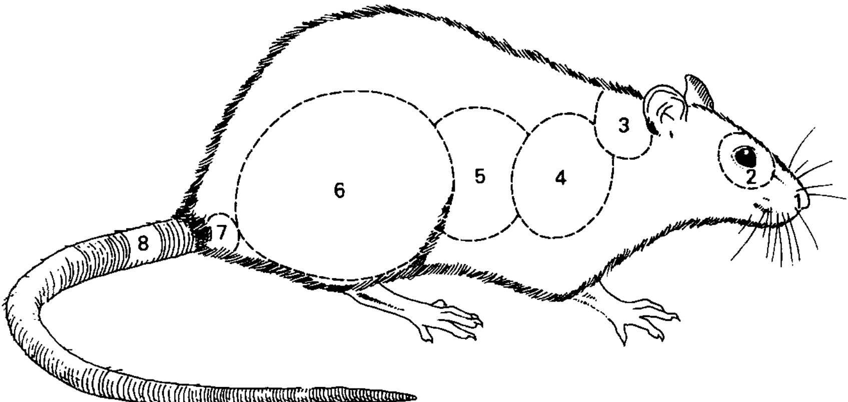

FIG. 1. Approximate locations of body zones to which sniffs were directed; 1, nose; 2, face; 3, neck; 4, shoulder; 5, flank; 6, haunch; 7, genitals; 8, tail.

Wytham, were stored at −20°C until postmortem examination. A skin sample of about 1cm^2 was taken from shoulder and haunch regions of six estrus females and five nonestrus females, then fixed, sectioned, and stained in hematoxylin and eosin. Following Macdonald et al. (1984), secretory activity in each skin sample was measured as the surface area-to-volume ratio for sebaceous alveoli. Stereological techniques (Clarke and Frearson, 1972; Elias et al., 1971; Weibel et al., 1966) were used to measure surface to volume ratios of skin sebaceous glands.

RESULTS

Male Sniffing Behavior. As a proportion of all behaviors shown towards females, males sniffed diestrus females more than estrus females ($\chi^2 = 54$, $df = 1$, $P < 0.001$) and related females more than nonrelated females ($\chi^2 = 8.3$, $df = 1$, $P < 0.01$) (Table 1). However, there were no significant differences between categories of female in the absolute number of sniffs per 10-min trial: in 62 trials, each lasting 10 min, males sniffed females an average of 23.38 times (SD = 1.3, $N = 62$) (Table 1).

Males directed different numbers of sniffs to the different zones of the females' bodies ($F = 38.21_{[32,28]}$, $P < 0.005$, two-tailed; Table 2). In total, the haunches of females of all four classes received more sniffs than at any other zone (genitals were invariably second in this ranking). There was a high rank-order correlation between classes of females for the number of sniffs directed

TABLE 1. NUMBER (AND PERCENTAGE) OF OCCURRENCES OF MALE BEHAVIOR OBSERVED DURING 10-MIN TRIALS TOWARDS FEMALES OF DIFFERENT RELATEDNESS AND REPRODUCTIVE CONDITION[a]

	Female class			
Behavior	RE	RD	NRE	NRD
Mount	37 (8.2%)	28 (3.6%)	58 (13%)	44 (7.6%)
Genital groom	13 (3%)	4 (0.5%)	43 (9.4%)	7 (1.2%)
Sniff	251 (56.7%)	568 (72.8%)	246 (53.8%)	385 (66.9%)
Other	142 (32%)	180 (23%)	110 (24%)	139 (24.2%)
Total	443	780	457	575
N	10	21	9	22

[a] *N* = number of pairs in each experimental group; RE = related estrus female; RD = related, diestrus; NRE = nonrelated estrus; NRD = nonrelated diestrus.

TABLE 2. TOTAL NUMBER (AND PERCENTAGE) OF SNIFFS DIRECTED BY MALES DURING 10-MIN TRIALS TO EACH OF SEVEN BODY ZONES FOR FOUR CLASSES OF FEMALE[a]

	Female class			
Body zone	RE	RD	NRE	NRD
Eye	12 (4.9%)	19 (3.5%)	11 (5.8%)	17 (4.5%)
Neck	22 (9%)	45 (8.2%)	14 (7.6%)	37 (9.9%)
Shoulder	30 (12.3%)	78 (14.3%)	20 (9.6%)	43 (11.5%)
Side	32 (13.1%)	73 (13.3%)	14 (6.7%)	45 (12%)
Haunch	87 (35.7%)	188 (34.4%)	75 (36.1%)	129 (34.5%)
Genitals	49 (20.1%)	120 (21.9%)	62 (29.8%)	83 (22.2%)
Tail	12 (4.9%)	24 (4.4%)	12 (5.8%)	20 (5.3%)
Total	244	547	208	374
N	10	21	9	22

[a] Legend as for Table 1.

to each zone ($r_s = 0.800$). Nonetheless, there was a significant difference between estrus and nonestrus females in the number of sniffs to each zone during 10-min trials ($F_{[2,28]} = 158.34$, $P < 0.001$, two-tailed). However, there were no significant differences based on relatedness, or body zone (using a three-level ANOVA where: group = relatedness, subgroup = reproductive condition, sub-subgroup = zone sniffed). Using a Kruskal-Wallis test, the only body zone that differed between categories of female in the absolute number of sniffs received

was the shoulder ($F = 3.54$, $P < 0.02$): the shoulders of related diestrus females were sniffed, on average, 3.7 times in 10 min, whereas those of nonrelated diestrus females were sniffed an average of 1.9 times in 10 min.

Using a three-level nested ANOVA, there was no significant difference between related an nonrelated females or between estrous and diestrus females in the proportional distribution of male sniffs among different body zones (Table 2). However, the proportion of sniffs directed to the genitals rather than to the remainder of the body was significantly greater for nonrelated estrous females than for any other class of female ($F = 38.21_{[32,28]}$, $P < 0.005$, two-tailed). There were no differences in the proportion of sniffs to the genitals of the remaining three classes of females.

There were no significant differences between body zones in the number of sniffs per unit area of each zone (Table 3), except that the genitals (a very small area) were sniffed at approximately fivefold the frequency per unit area of all other zones (Kolmogorov-Smirnov two-sample test $KS_a = 1.87$, $P = 0.002$). Although the number of sniffs that male rats directed at females' haunches, and to all other zones except genitals, was directly proportional to surface area, the fact remains that a major contributor to a male's experience of a female was the odor of its haunch.

Considering the amount of time spent sniffing each zone during a 10-min trial relative to the size of that zone, males devoted more of their sniffing time per square centimeter to females' genitals than to any other region (GT2 test, $\alpha = 0.05$; for nonrelated nonestrus females $F = 7.8$, $df = 1$, $P < 0.01$; for nonrelated estrus females $F = 18.2$, $df = 1$, $P = 0.0005$; for related nonestrus

TABLE 3. NUMBER OF SNIFFS DIRECTED BY MALE RAT, PER AREA, TO EACH OF SEVEN BODY ZONES FOR FOUR CLASSES OF FEMALE[a]

		Female class			
Body zone	Area (cm^2)	RE	RD	NRE	NRD
Eye	3.6	3.3	5.3	3.1	4.7
Neck	4.12	5.3	10.9	3.4	9
Shoulder	6.21	4.8	12.6	3.2	6.9
Side	10.17	3.1	7.2	1.7	4.4
Haunch	14.86	5.9	12.7	5	8.7
Genitals	1.77	27.7	67.8	35	46.9
Tail	15.42	0.8	1.6	0.8	1.3
Total		50.9	118.1	52.2	81.9
N		10	21	9	22

[a]Legend as for Table 1.

females $F = 29.7$, $df = 1$, $P = 0.0001$), except in the case of related estrus females ($F = 1.96$, $df = 1$, $P > 0.1$; based on arcsine square root transformed fractions of total sniffing time for each rat in each class). In terms of both the absolute amount of time spent sniffing and percentage of time spent sniffing (Table 4), males sniffed the genital and haunch zones of females more than any other zones. Shoulders were also sniffed for long periods of time, particularly shoulders of nonrelated diestrus females. These results arise because of differences in the number of sniffs to each zone according to reproductive condition; the mean duration of individual sniffs (mean = 2.0 sec, SD = 4.8, $N = 1435$) did not vary significantly between the body zones or classes of female to which they were directed.

Sniffing Sequences. Overall, 24.98% of sniffs were followed by another sniff. Our impression, from observation, was that there appeared to be a rearward progression of sniffs between zones from front to back. However, there was no significant difference in the frequency of transitions from forequarters to haunch versus haunch to forequarters. Furthermore, the mean frequency of such transition between the two regions averaged approximately 1 per session for nonrelated females and related diestrus females, but was more than twice as frequent for related estrus females (Fisher's exact test, two-tailed, $P > 0.001$; Table 5). However, overall and irrespective of whether followed by another sniff or not, the ratio of forequarter sniffs to haunch sniffs did not differ between classes of female ($\chi^2 = 5.49$, $df = 3$, $P = 0.139$; Table 5)

Males tended to resniff the genitals of only related estrus females (transition probability = 0.273), but frequently resniffed the haunches of both estrus and

TABLE 4. TIME (MINUTES) (AND PERCENTAGE) OF TIME SPENT BY MALE RATS SNIFFING EACH OF SEVEN BODY ZONES FOR FOUR CLASSES OF FEMALE RAT[a]

	Female class			
Body zone	RE	RD	NRE	NRD
Eye	1.5 (7.7%)	2.1 (4.9%)	1.7 (8.2%)	2.6 (6%)
Neck	2.2 (11.3%)	3.7 (8.7%)	1.4 (6.8%)	5.3 (12.3%)
Shoulder	2.4 (12.3%)	6.1 (14.3%)	3.5 (16.9%)	8.3 (19.3%)
Side	3.6 (18.6%)	5.3 (12.4%)	2.8 (13.5%)	4.5 (10.4%)
Haunch	4.6 (23.7%)	11.2 (26.2%)	4.5 (21.7%)	10.3 (23.9%)
Genitals	4 (20.6%)	11.8 (27.6%)	6.1 (29.5%)	9.9 (23%)
Tail	1.1 (5.7%)	2.5 (5.8%)	0.7 (3.4%)	2.1 (4.9%)
Total	19.4	42.7	20.7	43
N	10	21	9	22

[a] Legend as for Table 1.

TABLE 5. MALES' SNIFFS DIRECTED AT FEMALES' FOREQUARTERS (F) AND HAUNCH (H) IN QUICK SUCCESSION[a]

Female	Sniffs F to H	n	Sniffs H to F	n	Σ	N
RE	1.2500 (0.8292)	11	1.2500 (1.1990)	7	18	8
RD	0.5789 (0.9775)	9	0.5789 (0.8154)	11	20	19
NRE	0.8571 (0.9897)	4	0.5000 (1.3229)	6	10	8
NRD	0.5000 (0.8919)	10	0.4545 (0.7820)	11	21	22

[a]Mean (standard deviation) and number of sniffs observed in quick succession (n) are given for each class of female. N = number of rats in each class; RE = related estrus female; RD = related, diestrus; NRE = nonrelated estrus; NRD = nonrelated diestrus.

diestrus nonrelated females (transition probabilities = 0.429 and 0.28, respectively). Only the following between-zone reverse sequences had transitional probabilities larger than 0.25 (Figure 2): genital region to haunch (transitional probabilities = 0.545 for related estrus; 0.412 for nonrelated estrus; 0.423 for related diestrus; 0.5 for nonrelated diestrus females); tail to genital region (transition probability = 0.667 for nonrelated estrus females); tail to haunch (transition probability = 0.5 for nonrelated diestrus females).

Male Sexual Response. Males mounted estrus females more than diestrus females ($\chi^2 = 21.67$, $df = 1$, $P < 0.001$), and females not related to males were mounted more often than related females ($\chi^2 = 17$, $df = 1$, $P < 0.001$). Thus, levels of mounting were similar in related estrus females and unrelated diestrus females and were lowest in related diestrus females (Table 1).

Not all mounts led to successful intromission or ejaculation as inferred from genital grooming. There were significant differences in genital grooming by males after mounting different categories of female. Males groomed their genitals more with nonrelated females than with related females ($\chi^2 = 23$, $df = 1$, $P < 0.001$), and more with estrus than diestrus females ($\chi^2 = 55$, $df = 1$, $P < 0.001$).

Glandular History. In five of six estrous females the mean surface area-to-volume ratios of sebaceous alveoli were significantly smaller in the shoulder region than in the haunch region (Table 6). In contrast, in four of five diestrus females the surface area-to-volume ratios did not differ significantly between shoulder and haunch. Surface area-to-volume ratios of sebaceous glands in the haunch region varied more than the ratios in the shoulder, suggesting a fluctuating production of sebum in the haunch only, in synchrony with the estrous cycle.

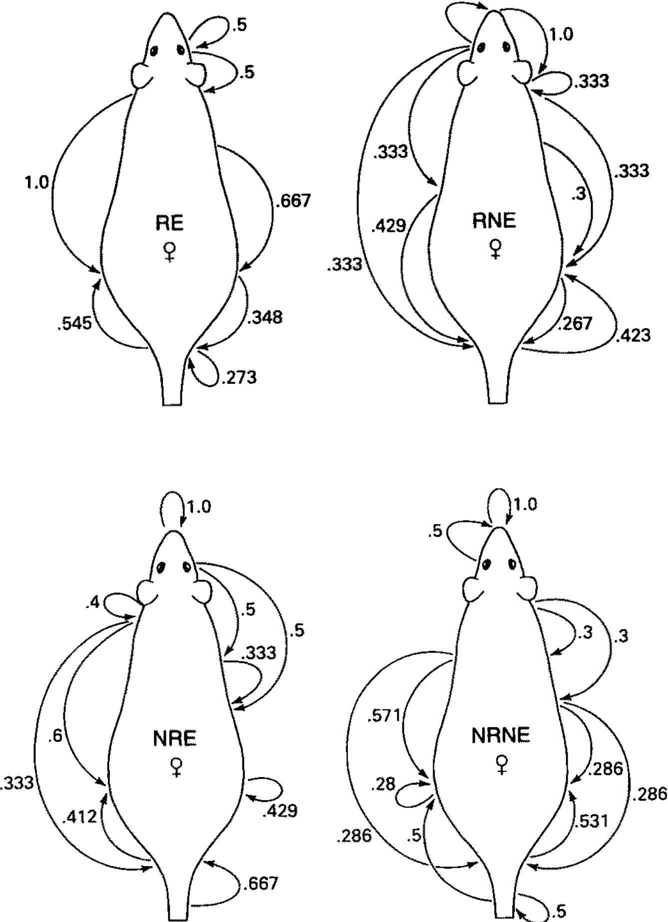

FIG. 2. For those interactions when male rats sniffed female rats of four categories twice in succession, the transition frequencies are shown for a sniff at one zone being followed by a sniff to another (depicting only those cases where the probabilities exceeded 0.25).

DISCUSSION

Social interactions between male and female rats involve sniffing many parts of the body, and Brown (1985a) has shown that whole-body odors of female rats are more attractive to male rats than clitoral odors alone. Our results demonstrate that male rats sniffed females differently depending on their estrus

TABLE 6. DESCRIPTIVE STATISTICS OF SURFACE AREA-TO-VOLUME RATIOS FOR SEBACEOUS ALVEOLI OF ESTRUS AND DIESTRUS FEMALE RATS[a]

	Body zone	Mean ± SD	N	F	P
Estrus					
♀1	haunch	0.681 ± 0.502	152	2.32	<0.0001
	shoulder	0.523 ± 0.330	175		
♀2	haunch	0.581 ± 0.415	132	3.57	<0.0001
	shoulder	0.409 ± 0.219	83		
♀3	haunch	0.642 + 0.437	86	2.20	0.0009
	shoulder	0.464 ± 0.295	67		
♀4	haunch	1.043 ± 0.839	45	4.37	<0.0001
	shoulder	0.533 ± 0.401	122		
♀5	haunch	0.588 ± 0.376	194	1.21	0.2932
	shoulder	0.549 ± 0.345	98		
♀6	haunch	0.429 ± 0.237	32	3.34	0.0003
	shoulder	0.541 ± 0.433	99		
Diestrus					
♀1	haunch	0.808 ± 0.512	94	1.25	0.2498
	shoulder	0.663 ± 0.572	145		
♀2	haunch	0.566 ± 0.391	249	1.19	0.1790
	shoulder	0.543 ± 0.358	219		
♀3	haunch	0.616 + 0.382	53	2.07	0.0343
	shoulder	0.402 ± 0.266	31		
♀4	haunch	0.551 ± 0.367	62	1.03	0.9256
	shoulder	0.533 ± 0.362	52		
♀5	haunch	0.572 ± 0.366	107	1.34	0.1622
	shoulder	0.368 ± 0.316	85		

	SA : V ratio			
	Shoulder	Haunch	% Signif.	N
Estrus	High	Low	83.3	6
Diestrus	High	High	20.0	5

N = number of sebaceous glands measured.

cycle and relatedness, and the males then showed different sexual responses such that most successful matings were with unrelated estrus females, and fewest successful matings were with related diestrus females. Although the genitals received a disproportionate number of sniffs, in total more sniffs were directed at the haunch than anywhere else, indicating the semiochemical importance of this body zone. Males appeared, generally, to sniff first at the nose or head of a female, and then progress down the body, sniffing most at the haunch. Males

frequently returned to areas of the body that they had already sniffed, raising the question of why they did not simply concentrate on signals from each body area in turn.

Haunch odors emanate from sebum, which could provide semiochemicals, either directly or via microbial modification, with the potential to signal the internal physiological state of the animal (Albone, 1984; Flood, 1985; Stoddart, 1980). The scent of rat haunches has at least 22 volatile components, the proportions of which vary greatly between individuals, but which generally characterize males, estrus females, and diestrus females (Natynczuk and Macdonald, 1994).

Our data, in broad agreement with Ebling's findings (Ebling and Skinner, 1967, 1975; Ebling, 1974), indicated a fluctuating production of sebum in the haunch of wild female rats in synchrony with the estrous cycle, while no such cycle was found in the shoulder region. Furthermore, Ebling and Skinner (1967) showed that treatment with androgens changed not only the rate at which sebaceous cells secreted sebum, but also the ratios in which various components were represented: fatty acid ratios $n\mathrm{C}_{16}/n\mathrm{C}_{18}$ and $n\mathrm{C}_{18:1\Delta9}/n\mathrm{C}_{18}$ increased in active sebaceous tissue. Since the rate of secretion appears not to vary with the estrous cycle at the shoulder, we doubt that the nature of the secretion will vary either, while for the haunch area there is good evidence that both the nature and the rate of secretion change with reproductive condition.

We have demonstrated, therefore, that male rats direct most sniffs to the haunch region and that secretions from this region vary with reproductive condition. Furthermore males tended to sniff the haunch after sniffing the shoulder, which does not vary in secretory activity with reproductive condition, as part of a sequence of sniffing, running along a female from head to tail. Assuming that the higher estrogen titer at estrus causes a change in the production of sebum in the haunch region, but not elsewhere, then a male rat running his nose down the body of an estrus female will smell a discontinuity (or follow a gradient) in scent, but there will be no such discontinuity (or gradient) on a diestrus female.

What reference system does the male rat use to calibrate these cyclical changes in the female's sebum production? Our data tentatively support the proposal that the absolute state of a combination of individual zones is not used in isolation, but that the degree of difference between these zones is also taken into account. Thus, a male can judge the precise state of a female's condition by calibrating an odor that changes during the reproductive cycle (e.g., haunch odor) with an odor that remains constant, at least so far as reproduction is concerned (e.g., shoulder odor).

Self-calibration might explain some male sniffing patterns and, in particular, why males return to areas they have already sniffed. For example, males directed a greater proportion of their sniffs to the shoulders of unattractive (related

diestrus) females than to the shoulders of attractive females (unrelated estrus). We suggest that when unrelated females were in estrus, their receptive state was clearly signaled by a large difference between haunch and forequarters. However, when a female was both diestrus and related, the male needed to assess exactly what stage of her reproductive cycle she was in, and how close her relationship to him was, before deciding on his sexual response. The more careful assessment of related diestrus females required a male to gather more "baseline data" for comparison with her haunch odor. Similarly, males would be expected to take more care in deciding their sexual response to related females, and this might be the reason why they repeated the sniffing sequence from fore- to hind quarters only with related estrus females.

The self-calibration model offers four advantages. First, it obviates the need for learning or inheriting responses to a pharmacopeia of scents. Second, self-calibration minimizes the difficulty posed to the recipient by variation, in quality and quantity, between the odors of different signalers. For example, individual females may vary in the secretory rate characteristic of estrus, so evaluating them on an absolute scale would be difficult; remembering how each smelled previously would also be difficult and, in some mating systems, impossible. These difficulties would be obviated by the self-calibrating mechanism, which requires only comparison, on a relative scale, of two parts of the same body. Third, social odors are inevitably mixed with variable and copious background smells, and even one individual's scent may vary over only a few days (e.g., Albone et al., 1978). Distinguishing signal from noise would seem to be a difficult task, likely to be made easier by the opportunity to calibrate the signal against an odorously stable part of the signaler's body. Fourth, self-calibration could work not only with complex mixes of chemicals, but also with single compounds. For example, diestrus could be signaled when the haunch emits more butyric acid than the shoulder, while estrus could be signaled when the haunch emits less butyric acid than the shoulder.

Self-calibration relies on assessment of the degree of difference between odors for the transmission of a signal and might apply to a variety of phenomena detailed in the literature. For example, self-calibration might explain scent signaling of dominance: if there were a dominance pheromone, a dominant male might smell himself into submission; however, he can instead assess relative rank by comparing his own odor with that of a rival. Similarly, the Bruce effect (Bruce, 1959), where pregnancy blockage is caused by exposure to the odor of an unfamiliar dominant male, but not a subordinate male (Hafer, 1990), might rely on a female comparing the odor of her last mate with that of the unfamiliar male.

Self-calibration might also be a mechanism whereby one animal can assess its genetic relationship with another. Individual odors in mice and rats have been linked with genotypic individuality of the major histocompatibility complex

(Beauchamp et al., 1990; Brown et al., 1990). Thus, the degree of difference between two animals' odors might be an expression of genetic distance; an animal that wanted to assess its relationship with another would simply have to compare its own smell with that of the other animal.

The self-calibration model encompassess and extends the matching scent hypothesis (Gosling, 1990). Self-calibration differs from scent matching because it allows animals a means of assesssing subtle, sliding-scale differences both within and between individuals rather than making a simple match between two odors. Self-calibration allows one odor to transmit a message that is dependent on the status of the recipient, and offers a means of accommodating the substantial variation in repeatedly sampled odors from a single individual. There will, of course, be many situations in which self-calibration cannot apply, most obviously in the use of pheromones as primers.

Detailed bioassay, integrated with chemical and endocrinological studies and experimental manipulation, are needed to test whether animals use self-calibration. In particular, the shoulder and haunch of female rats should be sampled through their estrus cycle using the dynamic solvent effect (Apps, 1990; Apps et al., 1987). Dynamic solvent effect sampling now allows rapid and sensitive headspace sampling of scent in a way that reflects the odor profile more closely than previous studies (Natynczuk et al., 1989). One of the wider implications of the self-calibration model for the study of odor communication is that we should test whether animals can assesss sliding-scale differences, rather than presence/absence of certain semiochemicals.

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ANALYSIS OF RAT HAUNCH ODOR USING DYNAMIC SOLVENT EFFECT AND PRINCIPAL COMPONENT ANALYSIS

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Abstract—We used the dynamic solvent effect to sample rat haunch odor, which we then analyzed using principal component analysis. PCA, based on 22 volatile components, indicated that one axis clearly separated rat haunch odor samples according to sex and female reproductive condition (estrus and diestrus), explaining 79.5% of the variation in proportional peak area. We have therefore been able to separate odors along biologically meaningful lines.

Key Words—dynamic solvent effect, haunch odor, principal component analysis, rat.

INTRODUCTION

Odors are extremely important for rats, both directly (Brown 1985a) and as primers (Brown 1985b). The skin sebaceous glands are likely to be a significant source of rat chemical signals (Mykytowycz and Goodrich, 1974; Stoddart, 1980; Albone, 1984; Maderson, 1985; Flood, 1985; Rivard and Klemm, 1989), and the rat haunch is well endowed with sebaceous tissue (Ebling, 1963, 1974; Ebling and Skinner, 1967, 1975; Thody and Shuster, 1975). The size and productivity of sebaceous glands of females' haunches change through the estrus cycle, suggesting a role in signaling reproductive state (Ebling, 1977). Further-

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more, Natynczuk and Macdonald (1994) have shown that, during 10-min encounters, male rats directed a large number of sniffs to a female's haunch. There is evidence, therefore, that haunch odor is a candidate for communication of reproductive status and, perhaps, sex.

It has been common practice in studies of mammalian social odors to analyze the solvent-extracted lipid fraction of scent gland material by gas chromatography. Qualitative and quantitative variations in the lipid fraction have then been examined in the hopes of finding signals communicating, for example, sex, social status, and reproductive condition. Because it samples chemicals irrespective of their volatility, solvent extraction is very different from sniffing, which is the sampling technique generally used by the mammal receiving the message. However, a new technique, based on the dynamic solvent effect (DSE) (Apps, 1990; Apps et al., 1987) allows rapid and sensitive headspace sampling of scent in a way that reflects the odor profile more closely than previous studies (Natynczuk et al., 1989).

We have sampled haunch odor using the dynamic solvent effect and have then used principal component analysis to analyze our results. We demonstrate a successful attempt to use both chemistry and statistics to separate odors along biologically meaningful lines.

METHODS AND MATERIALS

F_1 crosses of wild-trapped male *Rattus norvegicus* and female Wistar and D.A. rats were used. Females' phase of estrus was determined by vaginal epithelial cell counts (Zarrow et al., 1964). Scores (1–5) for the abundance of leukocytes (L), and nucleated (N) and cornified (C) cells were used to determine an index of estrous (E) calculated using the formula $E = C/(C + N + L)$. Females were categorized as estrous if $E > 0.7$ or diestrus if $E < 0.3$).

Semiochemicals of female rats with regular estrous cycles were sampled in diestrus and three days later, at their following estrus. Males' semiochemicals were sampled at the same time intervals. A sample of fur was trimmed from the haunch, weighed, then wetted with 3 ml of distilled water that had been boiled for 10 min to remove any contaminant volatiles. After 5 min, headspace volatiles were purged and analyzed using the dynamic solvent effect (Apps, 1990). Each sample was purged with activated-carbon-filtered hydrogen at 15 ml/min for 15 min. The GC conditions employed were: gas chromatograph: Pye 104 modified for a capillary column; integrator: Hewlett Packard 3090A; sample purge gas: H_2 at 15 ml/min via an activated-charcoal filter; sample time: 15 min; carrier gas: N_2 at 3 ml/min; FID detector: air at 1000 ml/min, H_2 at 30 ml/min; FID temperature: 250°C; column: 25 m × 0.23 mm ID × 3 μm

bonded OV-1; inlet heater on after 6 min; ballistic heating to 200°C in 90 sec; temperature program: 60°C then 4°C/min to 200°C. A blank was run with each batch of specimens analyzed. Freshly distilled *n*-hexane was used for each day's headspace analyses.

Chromatographic profiles of volatiles from the rats' haunch fur were obtained and peaks were matched (by relative retention time) by eye from each chromatogram. Peak areas were corrected for the dry weight of hair used in each analysis, and those that occurred in the headspace analysis of water blanks were excluded. The abundance of compounds in each chromatographic trace was subjected to principal components analysis (PCA) (Chatfield, 1988; Digby and Kempton, 1987; Gilbert, 1989) with the aim of detecting patterns common to rats of a particular sex or reproductive status. Principal component analysis is a multivariate technique for examining relationships among several quantitative variables in the search for structure, both between variables and between individuals (Chatfield, 1988; Digby and Kempton, 1987; Gilbert, 1989). Eigenvalues (the variances of the principal components) provided sample statistics for proportional peak areas (Flury and Riedwyl, 1988).

RESULTS AND DISCUSSION

Chromatographic profiles of volatiles from the rats' haunch fur were obtained, and peaks were matched (by relative retention time) by eye from each chromatogram. Peak areas were corrected for the dry weight of hair used in each analysis, and those that occurred in the headspace analysis of water blanks were excluded. Each peak was scrutinized as a potential indicator of sex or reproductive status according to the assumption that we had detected all possible pheromone candidates.

There was enormous individual variation in the size and ratio of peak areas; no one peak was a reliable indicator of either sex or reproductive condition (Figures 1 and 2). However, post hoc superimposition of the headspace chromatograms suggested that each male's scent profile was more constant over time in the composition and size of its peak than were those of the females (Figures 1 and 2).

Twenty-two peaks common to all chromatograms were located and their proportional peak areas subjected to PCA. The first principal component appeared to define the sexual status of the rats and accounted for 79.5% of the variance in proportional peak areas of three classes (estrous vs diestrous females vs males). Scent profiles from each class were closely grouped (Figure 3), suggesting that the general body odor of the rat's haunch provides a passive signal of its hormone profile.

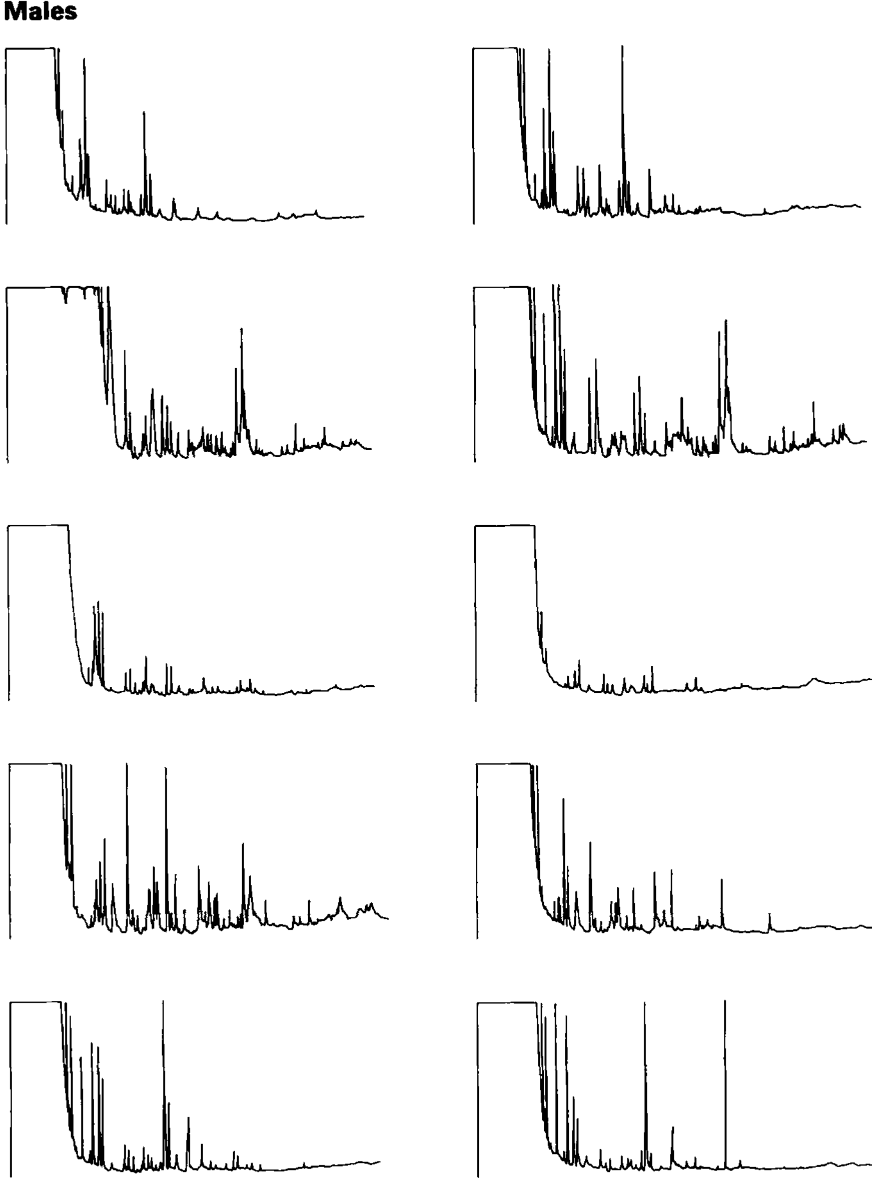

FIG. 1. Male rats' scent profiles obtained using the dynamic solvent effect. Individuals were sampled at three-day intervals to mirror the sampling interval between each female's estrous and diestrous phases. The successive samples from each rate are pictured as pairs.

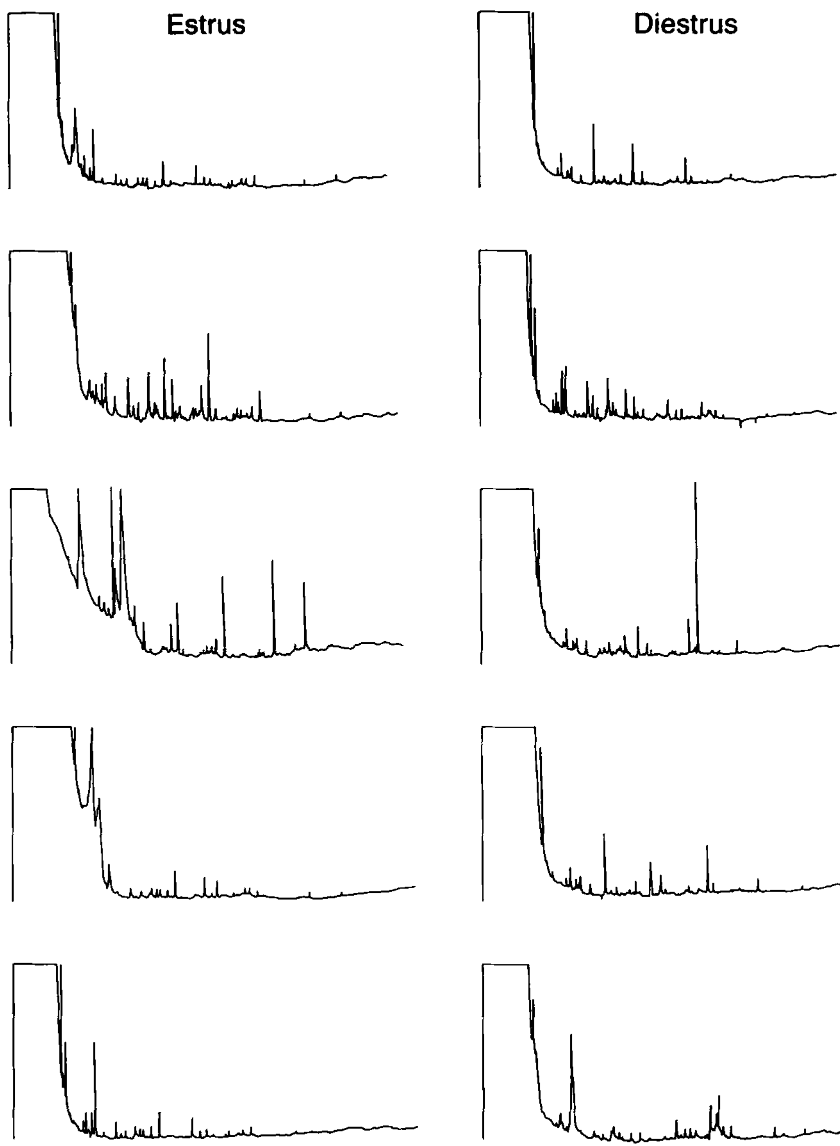

FIG. 2. Female rats' scent profiles obtained using the dynamic solvent effect. Individuals were sampled at three-day intervals to reveal profiles during both estrus and diestrus.

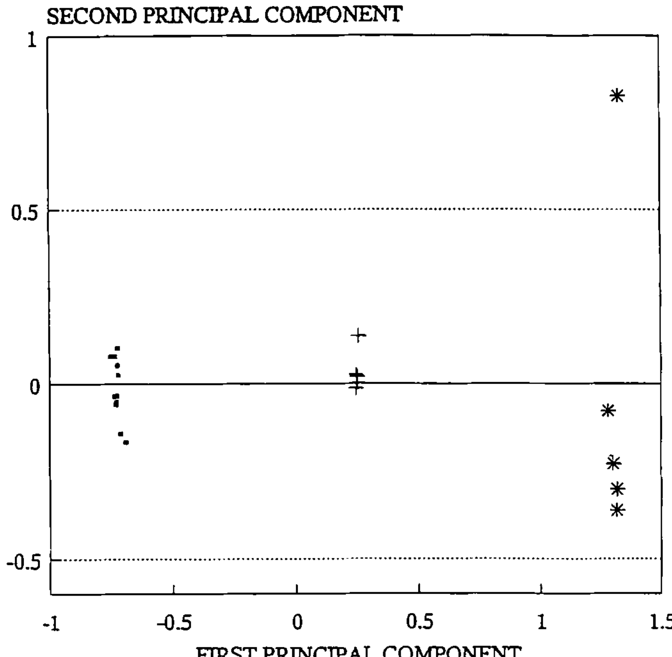

FIG. 3. Principal component analysis of the scent profiles of rat haunches, plotting components 1 and 2 and showing the separation of sex and reproductive condition. (■, males; +, estrous females; *, diestrous females).

The eigenvalues for peaks 1–14 vary in their sign (Table 1) and would appear to depend on two factors (estrous/nonestrous). These contrast with the eigenvalues for peaks 15–22 (excluding peak 21), which are of approximately equal magnitude and would appear to reflect a third, much less variable (male) factor. The other principal components exhibit little variance between samples, so these PCs are candidates for signals of individual identity.

In conclusion, we have shown that the scent of rat haunches has at least 22 volatile components, the proportions of which vary greatly among individuals. Although no single compound emerged as diagnostic of each class, PCA revealed exclusive categories in the odor profiles of rats of each sex and reproductive status. The patterns of abundance of these constituents clearly identified sex, reproductive status, and individuals. While it seems very likely that the rats use this information, proof that the rats make a discrimination analogous to the PCA of the 22 peaks will require a bioassay to compare the rats' response to the natural odor with that to a synthetic mixture of the same composition (Gawienowski, 1977; Abbott et al., 1990; Hall, 1990; Novotny et al., 1990).

TABLE 1. EIGENVECTORS OF FIRST TWO PRINCIPAL COMPONENTS (PC) OF HEADSPACE ANALYSIS OF HAUNCH SCENT OF MALES AND ESTROUS AND NONESTROUS FEMALES

	Eigenvectors:	
Peak number	PC 1	PC 2
1	0.036	−1.322
2	−0.045	−0.047
3	0.109	−0.576
4	−0.030	−0.027
5	0.016	−0.045
6	−0.016	0.053
7	−0.018	0.013
8	−0.022	0.010
9	0.003	−0.006
10	−0.017	0.034
11	−0.014	−0.171
12	−0.051	0.060
13	0.011	−0.032
14	−0.022	−0.009
15	−0.002	0.022
16	0.000	0.002
17	−0.002	0.008
18	−0.002	0.029
19	−0.002	0.029
20	−0.015	−0.001
21	0.003	0.028
22	−0.005	0.010
% variance	79.5	5.8

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TO PENETRATE OR NOT TO PENETRATE? A BEHAVIORAL CHOICE BY BEAN BEETLE FIRST-INSTAR LARVAE IN RESPONSE TO *Phaseolus vulgaris* SEED SURFACE QUALITY

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Abstract—Survival of *Acanthoscelides obtectus* larvae depends on the ability of the first instar to pierce the seed coat of *Phaseolus vulgaris*, which represents a critical sequence because of physical characteristics and toxicity. We have investigated the influence of seed surface quality on larval boring behavior by the usual method of surface washing with different solvents, or by removing the testa, or by coating the testa with a polymer spray. Observations were made on isolated larvae in no-choice and dual-choice bioassays. In the no-choice situation, larval penetration was reduced after seed coats were soaked with chloroform, whereas water, diethyl ether, or methanol had no significant effect. The ratio of boring attempts to successful penetrations was increased on seeds washed with chloroform. In the dual-choice situation, larvae avoided artificially coated seeds and preferred untreated seeds over those washed with chloroform or methanol. These results indicate that boring stimulants exist on the seed coat and that they are removed by chloroform and methanol or made inaccessible by artificial coating. The high mortality of first instars on seeds washed with these two solvents is attributed to a lack of chemicals necessary to initiate and sustain boring behavior, causing larval stress, possibly due to prolonged locomotory activity and starvation. Thus, seed coat quality may influence the population dynamics of *A. obtectus*.

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Key Words—Coleoptera, Bruchidae, *Acanthoscelides obtectus*, Fabacea, seed coat, *Phaseolus vulgaris*, taste, insect-plant relationships, behavior.

INTRODUCTION

The bean beetle *Acanthoscelides obtectus* (Coleoptera, Bruchidae) is regarded as a monophagous species *sensu stricto*; female ovipositing and larval development naturally occur on common beans (*Phaseolus vulgaris*), *Phaseolus coccineus*, and cultivated *Vigna unguiculata* (Leroi and Jarry, 1981). Despite this rather limited range of host plants, *A. obtectus* larvae can develop, under artificial conditions, on a broader spectrum of seed species: 18 species have been reported as suitable for larval development (Johnson, 1989).

As in several other bruchids, *A. obtectus* can complete its development both in maturating seeds and in stored ones. The females deposit batches of eggs either in the cavity of mature pods or on stored beans. Newly hatched larvae display locomotory activity, resulting in dispersion, before selecting a suitable boring site on the seed coat. First-instar larvae are very small (ca. 500–600 μm length), and live only a short time if unfed. In certain conditions, this may represent a critical stage (Simmonds et al., 1989). Both locomotory activity and boring behavior are energetically costly, and high rates of mortality may occur under certain seed storage conditions. Successful establishment may occur for 90% in freshly matured seeds compared to 20% in older ones, and even less than 10% in artificially dried ones (Stamopoulos, 1980; Thiéry, 1981, 1982a). This has been attributed by some authors to the toxicity of the seed coat (Stamopoulos and Huignard, 1980; Stamopoulos and Desroches, 1981). This toxicity has not been borne out in the recent work of Cardona et al. (1989). In all probability, first-instar larvae escape toxicity of the seed coat by avoiding ingestion of powder during the penetration (Thiéry, 1984). Similar behavior has also been proposed for *Callosobruchus maculatus* (Janzen, 1977). Besides a possible toxic barrier, which has not been clearly demonstrated, a mechanical barrier (hardness as a consequence of dryness) very effectively prevents larval penetration (Thiéry, 1981a, 1984).

In the field, observation of infested pods shows up to 95% of the larvae established in the seeds (Jarry, 1984). Almost all larvae pierce the seed coat rather than the inside wall of the pod. This suggests the occurrence of chemical factors that elicit behavioral sequences that lead to piercing of the seed coat. Until now only the mechanical characteristics of the seed coat and its toxicity have received attention, and the influence of possible chemical factors on seed acceptance or rejection has not been investigated. We hypothesized that "biting stimulants" for *A. obtectus* larvae occur on the *P. vulgaris* seed surface and that they influence larval penetration. This work reports on the larval ability to

bore through integuments, the chemical characteristics of which were modified by coating or by washing with different solvents. It shows that biting stimulants to *A. obtectus* larvae do occur on the seed surface of *P. vulgaris* and that they influence boring site selection as well as subsequent feeding by larvae.

METHODS AND MATERIALS

Bioassay. Two different bioassays were used: (A) a no-choice bioassay where whole seeds were placed separately in small glass tubes with one egg, about to hatch; and (B) a dual-choice bioassay with two half seeds in contact with each other (one treated, one nontreated) (Figure 1). Larvae collected within 2 hr of hatching were gently deposited with a brush on the lower half seed surface. The dual-choice bioassay was designed to offer the thigmotactic requirements of *A. obtectus* larvae when trying to penetrate the teguments [most larvae from our strain need an interval of about 0.25 mm to meet a suitable dorsal support for boring, between the seed and dorsal contact (Thiéry, 1981)]. The behavioral response is described by the following parameters: (1) number of larvae that did not penetrate into the seed (2) number of larvae dead while boring, (3) number of unsuccessful boring attempts, and (4) number of developing larvae. Mortality during development was always very low, less than 2%.

Insects and Seeds. Insects were reared from a laboratory strain called the normal strain (Labeyrie, 1961; Pouzat, 1970), the females of which need *P. vulgaris* pods or seeds on which to ovipost. The insects were maintained under constant conditions (complete darkness, 24 ± 1°C, 80 ± 5% relative humidity).

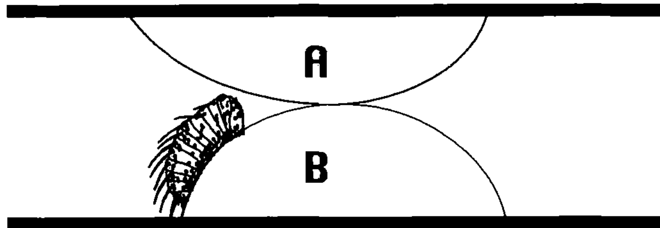

FIG. 1. Scheme of the dual-choice bioassay designed to determine *Acanthoscelides obtectus* first-instar larval preferences between different seed surface qualities. A & B, half seeds previously subjected to different treatments, microscope glass slides (thick black) and *A. obtectus* larva (not to scale). The device is positioned horizontally in order to prevent larvae from falling down. The half seed that is supposed to be less favorable is always placed in the lower position (in a position more favorable for penetration), and one larva per device is deposited with a brush on the lower half seed surface.

P. vulgaris seeds (cv. Soisson Nain) were harvested six months before experiments and stored at 5°C with a saturation deficit (Δpv) of 1–2-mm Hg to limit seed drying. Seeds given to larvae were subjected to different treatments. For both bioassay A and B, entire seeds were soaked in diethyl ether (24 hr) or chloroform (1 min), or methanol (2 hr). Organic solvents were of GC or HPLC analysis grade > 99.95% pure). For bioassay A, a batch of seeds was kept 6 hr in distilled water. Bernays et al. (1976) found that a short soaking in chloroform was sufficient to remove surface waxes from leaves. We therefore dipped the seeds only briefly in chloroform. Soaking times in ether and water (several hours) were adapted from Pouzat (1976), who extracted oviposition stimulants from the seed coat. Seeds were then stored for eight days at room temperature under humid conditions described above. This period allowed complete evaporation of solvents. For bioassay B, seeds were first soaked, stored eight days, cut lengthwise, and stuck on the glass slides. In one group of seeds, teguments were manually removed (naked seeds), and in another one, intact seeds were coated with a commercial polymer spray (coated seeds).

Data were compared by G test or by G subjected to Sidak correction for multiple comparisons (Sokal and Rohlf, 1981; Scherrer, 1984). In the dual-choice situation, distributions between two different half seeds were compared to the distribution observed in the control (first line of Table 2 below).

RESULTS

No-Choice Situation. In the control, 57% of larvae successfully penetrated the seeds, achieved full development, and matured to adults. This percentage decreased with all the treatments, but only significantly after treatment with chloroform (21%). Methanol also reduced successful penetration (39%), although it was not statistically different from diethyl ether, water, or control (Table 1). The ratios of the boring attempts to completed holes were similar in the different treatments (ca. 0.5), except after washing with chloroform, where it was twice as high, but the multiple comparison showed a significant difference only at the level risk $\alpha = 0.07$ ($G = 8.79$, 4 df) (Table 1).

Dual-Choice Situation. Boring behavior was influenced by the half seed position: in the control (two nontreated half seeds), most of the holes and most of the boring attempts were found in the half seed stuck to the floor. The distribution was about 70% in the lower half seed and 30% in the upper one. It differs statistically from a 50–50% distribution ($G = 8.64$, $P < 0.003$, 1 df). This indicates that larvae prefer the lower half seeds and that in the dual-choice situation, gravity is important for boring activity. In the other treatments, the distributions between the upper and lower seeds were compared to the distribution observed in the control treatment taken as reference (Table 2, line 1).

Seed Surface Preferences. On naked seeds, a very low number of unsuc-

TABLE 1. ACCEPTANCE OF SEED COATS BY *Acanthoscelides obtectus* LARVAE AFTER WASHING IN DIFFERENT SOLVENTS[a]

Treatment	Larvae (*N*)	Larvae found dead after piercing (%)	Unsuccessful penetrations (boring attempts) (%)	Successful penetrations (%)	Ratio attempts/ penetrations
Control	186	2	29	57(a)	0.50
Diethyl ether	85	0	34	54(a)	0.63
Chloroform	213	2	23	21(b)	1.11
Methanol	92	2	24	39(a)	0.59
water	100	0	28	51(a)	0.53

[a] Solvents are ranked by increasing polarity. Values with same letters are not significantly different at risk level $\alpha = 0.05$ (multiple G comparison).

cessful boring attempts was observed. This indicates that when the seed coat is removed, almost all boring attempts are followed by larval penetration into the seed. Polymer coating made the seeds less suitable for larval penetration: numbers of both boring attempts and of completed holes were very low (no attempts and only two holes), which suggests that few larvae initiate normal boring activity (Table 2).

The number of boring attempts on the upper and the lower half seeds was not affected by washing with the different organic solvents. The ratios between unsuccessful boring attempts and successful penetrations were also not modified ($G = 3.54$, 5 *df*, NS). The only difference was found when larvae had a choice between naked and normal half seeds ($G = 41.15$, 1 *df*, $P < 0.0001$). This ratio was always lower in the dual-choice as compared to the no-choice situation, indicating that the dual-choice arrangement facilitated more successful penetration.

The half seed selection was affected only by washing with methanol (48–52%; $G = 4.94$, 1 *df*, $P < 0.03$) and chloroform (37–63%; $G = 6.49$, 1 *df*, $P < 0.01$). In the chloroform treatment, only 61% of the total number of larvae develop, whereas 83% survived in untreated seeds. This may be a consequence of a sustained locomotory activity resulting in larval weakness. This clearly demonstrates that seed coat quality indirectly influences further larval development.

DISCUSSION

A. obtectus first-instar larvae exhibit a rather important locomotory activity before selecting an appropriate site to initiate boring behavior (Thiéry, 1981). Therefore, in the dual choice experiments, the testa quality on which larvae are

TABLE 2. PREFERENCES OF *Acanthoscelides obtectus* FIRST-INSTAR LARVAE AMONG DIFFERENT SEED QUALITIES[a]

	Replicates	Larvae found dead after piercing (1)	Unsuccessful penetrations (boring attempts) (2)	Successful penetrations (3)	Seed coats pierced (1 + 3)		Ratio attempts/pierced [2/(1 + 3)]	
Control	56	5	4	12	17	a	0, 24	NS
Control		4	13	34	38		0, 33	
Seed coat removed	40	0	1	38	38	b	0, 03	***
Control		0	14	2	2		7, 00	
Control	42	1	13	39	40	b	0, 33	NS
Coated with plastic		0	0	2	2		0, 00	
Control	40	2	9	17	19	a	0, 47	NS
Diethyl ether		1	12	19	20		0, 57	
Control	41	7	11	18	25	c	0, 42	NS
Chloroform		9	9	6	15		0, 60	
Control	46	7	6	17	24	c	0, 25	NS
Methanol		6	9	16	22		0, 41	

[a]Boring attempts are expressed as a number (several attempts per larva being sometimes observed). Solvents are ranked according to increasing polarity. Same letters within a column indicate a distribution between upper and lower half seeds identical to that found the control (first line) at risk level α = 0.05. NS = no statistical difference in the ratio of attempts to piercing between upper and lower half seeds, $***P < 0.001$. In three lines, the number of replicates differs from the number of seed coats pierced because of escaped larvae.

deposited should have little effect on their choice. In the dual-choice experiment, gravity was found to influence piercing behavior. In order to offset this effect, the expected less favored seed (washed or coated) was always in the lower position. Seed coat hardness, measured by means of a hardness meter (Thiéry, 1984), was not different between the different treatments, but when the seed coat was removed larval establishment was favored. In this situation, few unsuccessful boring attempts were observed. This suggests that larvae may directly start feeding and avoid piercing behavior when offered such an opportunity.

Observed differences between different types of washing may be attributed to chemical modifications. Washing seeds with chloroform, and to a lesser extent with methanol, reduced larval piercing of *P. vulgaris* testa in both bioassays. It had the same effect as coating the seeds with a thin plastic film. This effect is similar to that observed on *Phaseolus lunatus* seeds, where larvae are unable to clear the teguments (Leroi and Jarry, 1981) even if physical characteristics would have allowed it (Thiéry, 1984). A lack of biting stimulant to *A. obtectus* larvae on *P. lunatus* seed surfaces was hypothesized in these cases. We interpret the results of the present experiments as demonstrating removal of biting stimulants from *P. vulgaris* seed surface after washing with chloroform and methanol. The term biting stimulant is used instead of phagostimulant, because previous results showed that first-instar larvae do not ingest testa powder during boring of teguments (Thiéry, 1981).

The removal of semiochemicals from the seed surface by washing with chloroform or methanol caused larvae to reject treated seeds in dual-choice tests; the effect of methanol is, however, not so clear in the no-choice bioassay. We can therefore conclude that biting stimulants are likely to be removed in sufficient amounts by these two solvents. Making stimulants inaccessible to the larvae by coating the seed should cause the same effect, which was the case. In the present experiments, artificially coating seeds may be interpreted as a modification of the seed surface quality and as masking of boring stimulants. However, the fact that coating contains deterrents has not been verified. The results obtained with diethyl ether and water are less clear. One hypothesis is that a prolonged stay in these two solvents may have elicited passage of chemicals from the inner part of the seed (cotyledon) to the surface. Occurrence of phagostimulants to *A. obtectus* is logically suspected in the cotyledon of several legumes (Stamopoulos and Huignard, 1980; Thiéry, 1981); however, no chemical structure is available. A long soaking duration in water or diethyl ether could possibly have balanced the removal of biting stimulant by entailing a migration of phagostimulants from cotyledons to the seed coat surface. The shorter stays in chloroform and methanol would not have allowed such a phenomenon. It is difficult to determine the polarity of molecules that have been removed since no fully apolar solvents have been used (e.g., pentane or hexane). In the present experiments the best extraction was obtained with a solvent of intermediate polarity: chloroform. Soaking

seed coats with this solvent increased the number of unsuccessful boring attempts in the no-choice situation. This is interpreted as a consequence of unsuitable seed coat quality for sustained boring behavior. It has already been shown that prolonged locomotory activity and subsequent stress reduce the larval ability to bore through *P. vulgaris* seed coats (Thiéry, 1982b). In the present experiments the absence of stimulants may impede the piercing behavior and/or increase the number of unsustained attempts. This may explain the low rate of successful penetration when seeds present unsuitable surface characteristics. This is observed in no-choice and dual-choice situations.

First-instar larvae of *A. obtectus* have evolved boring behavior without tegument ingestion (Thiéry, 1981, 1984) and perhaps the boring stimulants also elicit this rejection behavior. First-instar larvae of *A. obtectus* face a balance between physical characteristics (mainly hardness) that impede penetration and chemical ones that stimulate boring activity. Compounds present on the surface of different species of Phaseolinae may control larval penetration behavior. Even if the female's oviposition behavior represents an important step in host-plant selection in this species, larval biting stimulants can also play a role in the recognition and acceptance of *P. vulgaris* by *A. obtectus*. Molecules with similar behavioral effects may also be expected to exist on other legumes on which *A. obtectus* larvae can establish themselves (Johnson, 1989). Comparisons with species whose larvae cannot select seeds (species that cement eggs onto the surface of seeds, e.g., *C. maculatus* or *Zabrotes subfaciatus*) would yield additional information to interpret the role of first-instar larval behavior in the Bruchid–Fabacae relationships.

Our study is a first step before the characterization of chemical structures. The characterization of such chemicals may lead to agronomic applications, which would be useful because bean beetles are still problematic, especially in developing countries. Progress in the knowledge of behavior-modifying chemicals and attempts to manipulate behavior may lead to a future strategy for controlling Bruchids.

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PRIOR ANESTHESIA IMPAIRS A CHEMICALLY MEDIATED FRIGHT RESPONSE IN A GOBIID FISH

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Abstract—Quinaldine and phenoxyethanol, two commonly used fish anesthetics, reduced the intensity of a chemically induced fright response in a marine goby. MS-222, an anesthetic that has been indicated as harmful to fish olfaction, had no significant effect. Understanding these effects must await further study, but it is clear that caution should be exercised. One should definitely not use quinaldine or phenoxyethanol instead of MS-222 in an effort to avoid harmful effects.

Key Words—Chemoreception, olfaction, alarm, pheromone, fright-response, MS-222, quinaldine, phenoxyethanol, *Asterropteryx semipunctatus*.

INTRODUCTION

Rumors of negative effects of various anesthetics on the chemical senses of fishes have long existed. Evidence of any such effect is sparse and contradictory. Lewis et al. (1985) found an adverse effect of MS-222 on the olfactory organ of channel catfish, *Ictalurus punctatus*. They reported that the drug destroyed cilia on the olfactory sensory epithelia. They cautioned that "Although the effects may be subtle, they should be considered when anesthetized fish are to be used in behavioral or chemosensory system studies." In response to this warning, however, Quinn et al. (1988) found no evidence for an adverse effect

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of MS-222 or 2-phenoxyethanol on the chemosensory ability of Pacific salmon, *Oncorhynchus tshawytscha* and *O. kisutch*. The drug did not seem to interfere with either field tests of homing ability, which presumably depended on chemoreception of home stream odor, or laboratory tests of their avoidance response to L-serine. Chivers and Smith (1993) found that fathead minnows, *Pimephales promelus*, that had been anesthetized with MS-222 still evidenced a chemically released fright reaction. In both cases, qualitative chemical responsiveness was not destroyed; odors were detected. We have no knowledge, however, of more quantitative effects: Was chemical responsiveness diminished? Might more subtle response to chemical cues or the chemical senses themselves have been impaired?

Many researchers have been forced to use drugs in the collection of cryptic species with the hope that the negative effects of these chemicals are minimal. Other workers avoid the use of chemicals and must often invest considerable time and effort in collecting evasive species. Some laboratory investigators have avoided the use of anesthetics because they might cause a problem in chemosensory studies. Depending on the procedures involved, this practice could lead to unnecessary suffering that could have been avoided if some harmless anesthetic were used on the subjects.

Asterropteryx semipunctatus is a common Indo-Pacific marine fish of the family Gobiidae. It shows an alarm response to visual contact with predators and to chemical stimuli from injured conspecifics. When frightened, it reduces activity and sometimes shows head-bobbing behavior (Smith, 1989). This defensive response can be visually transmitted to adjacent conspecifics that cannot see or smell the threat stimulus (Smith and Smith, 1989). The alarm pheromone system of this species is similar to the fright reaction (Schreckstoff alarm signal) found in fishes of the superorder Ostariophysi and the North American darters (see Smith, 1992 for review) and likely depends on olfaction.

Here we report the results of a pilot study to examine possible behavioral effects of strong anesthesia by MS-222, quinaldine, and phenoxyethanol on this fright response.

METHODS AND MATERIALS

Gobies, *Asterropteryx semipunctatus* (2.1–3.9 cm standard length) were captured with hand nets in Kaneohe Bay, Oahu, Hawaii, and held in running seawater aquaria. Randomly chosen individuals were placed in a treatment bath for 60 sec and then into seawater for recovery. Treatments were: control and sham (seawater), MS-222 (Finquel, tricaine methanesulfonate, Argent Chemical Laboratories) at 2.5 mg/ml of seawater; quinaldine (Eastman Kodak Co.), at 1 ml of stock solution (1 : 20 quinaldine–methanol) per 500 ml of seawater; and

2-phenoxyethanol at 1 ml/500 ml of seawater. Anesthetic concentrations were chosen to represent a strong "field effective" dose that might be encountered when anesthetics are used to collect fish. They typically produced "stage III immobilization" (Bell, 1987) and complete cessation of movement within 30 sec (Table 1).

Three fish each, of each treatment type, were placed in 30-liter aquaria supplied with a sand bottom, shelter tubes, and running seawater at about 200–300 ml/min. After five days of recovery from anesthesia, fish were tested by first obtaining a 15-min pretest videotape. Test substance was then injected into their seawater supply line: plain seawater for sham-treatment fish, chemical extract from an injured conspecific obtained as per Smith et al. (1991) for control, and anesthetic treatment fish. Videotaping continued for the subsequent 15-min test period.

Behavior (counts and durations) was event-recorded, using the BEAST (University of Hawaii, 1993) system, by an observer who was blind to the treatment of the fish. Behavior recorded was: move, the duration of one or more gobies showing any movement other than bob; Bob, a count of the occurrences of a slow raising and more sudden lowering of the head and anterior trunk without forward locomotion; and perch, the duration of resting on the shelter tubes with the head projecting above the highest tube. BEAST was programmed to sum behavioral durations and counts in three ways: the entire pretest period and the test period; six 5-min bins, three pretest and three test; and 100 bins from the beginning of the pretest to the end of the test to allow visual scanning for changes in behavior. Repeated-measures MANOVA of log-transformed data used the SAS (SAS Institute, 1991) procedure GLM with type III sum of squares, least-squares means (LSM) comparisons, and Duncan's multiple-range (DMR) test.

TABLE 1. RESPONSES TO ANESTHESIA[a]

Response	Anesthesia		
	MS-222	Phenoxyethanol	Quinaldine
Gills still	35(8)	22(6)	20(5)
Gills move	192(124)	167(112)	136(68)
Orienting	302(126)	352(128)	344(107)

[a] Gills still, time lag from administration of anesthesia until total cessation of gill movement; gills move, lag from placement in fresh seawater to resumption of gill movement; orienting, time from placement in fresh seawater to resumption of normal upright posture. Data are means and, in parentheses, standard deviations, in seconds.

RESULTS

We rejected 10 runs in which the activity during the 15-min pretest period was less than 20 sec, or if more than one head bob was seen during the pretest. Trials with little activity during the control period lack power to reveal a reduction in activity during the test period. Trials with head-bobbing during the pretest period indicate some uncontrolled source of frightening stimuli. Eleven groups remained for each treatment.

The effective dosage for each anesthetic produced similar results (Table 1). The time required for recovery from anesthesia did not depend on the anesthetic used.

When we treated the data as per Smith and Smith (1989), we found no effect of anesthesia. There was no difference among treatment groups in the reduction of the number or duration of moves in the 15-min test period as contrasted with the 15-min pretest period (MANOVA, period × treatment, $P > 0.1$). As expected, the sham treatment, with no test substance in the seawater, showed more movement than any other group during the test period (LSM, $P = 0.03$). Only the fish exposed to the test, fright-inducing substance reduced movement during the test period.

Inspection of the results suggested that response on a finer time scale could be masked by summing over the entire 15-min test period. Fish that had been exposed to anesthesia may have shown a fright response for a shorter period of time than the control fish. To explore variability over a finer time scale, we divided the pretest period into three, 5-min subperiods. There was no difference in move duration among the three pretest subperiods (MANOVA, period × treatment, $P > 0.1$). A trend was indicated for MS-222 groups to have less activity than quinaldine or sham groups during the first 5-min subperiod (LSM, $P = 0.05$ and 0.02; DMR, $P < 0.05$). However, this is the reverse of the test results presented below.

The test period was then divided into three, 5-min subperiods. The mean of the three pretest 5-min subperiods was taken as a control observation for repeated-measures contrasts with the three, 5-min test subperiods (Figure 1). Contrast of all groups indicates a significant effect of treatment (MANOVA, period × treatment, $P < 0.025$). For the first and last 5-min test subperiods, sham and quinaldine fish were more active than controls (LSM, $P < 0.05$). Phenoxyethanol-treated fish were more active than the controls only in the last 5-min subperiod (LSM, $P < 0.04$). Removal of the sham group has little effect on the results (MANOVA, period × treatment, $P = 0.05$).

Head-bob was seen in only about half of the test periods for the control group. Only two control groups showed more than one head-bob. Head-bob lacked power for detecting the fright reaction and was not further analyzed.

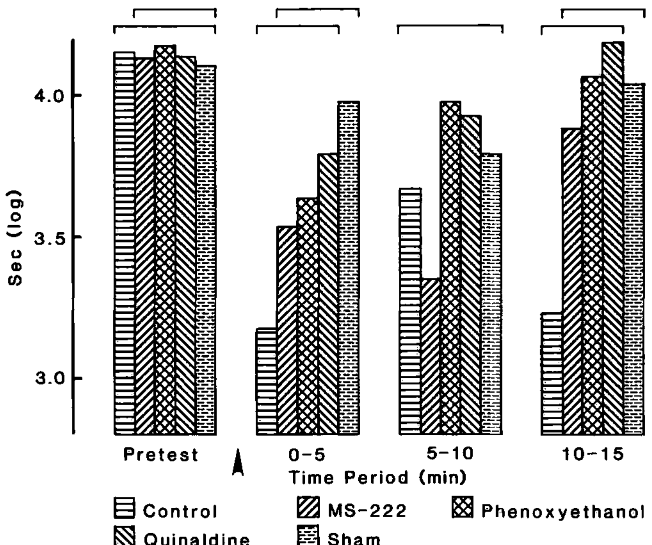

FIG. 1. The number of seconds of activity per 5-min sample period. The pretest is the mean of three, 5-min samples of each group. 0–5, 5–10, and 10–15 are the three consecutive 5-min subperiods of the 15-min test. Each bar is the mean of 11 groups for each treatment. Horizontal lines over the bars indicate the DMR 95% confidence groupings. Experimental treatment groups are: control (no anesthesia, with alarm pheromone), MS-222, phenoxyethanol and quinaldine anesthesia (with anesthesia, with alarm pheromone) and sham (no anesthesia, no alarm pheromone). Alarm pheromone was added at the arrow between the pretest and the 0–5 min time periods.

Other behavior, such as perching on top of the shelters, was evaluated and also found lacking in power.

A few anomalous trials had a strong effect on the control values for the second test subperiod (see Figure 1). Throughout this pilot study, we refined our methods to reduce the variability between runs. Future tests should enjoy greatly reduced variability within treatment groups. We were surprised at the extreme importance of providing live zooplankton as food on the morning of the test day. With live food, the fish were reasonably active with feeding, exploring, and social behavior. Without live food, many fish did almost nothing for the entire 15-min pretest period, thus offering little opportunity to show a reduction in activity due to a fright reaction. Even the lag between feeding and testing was critical, with 90 min resulting in a predictably moderate level of activity that could be inhibited by a fright response.

DISCUSSION

There is ample cause to suspect an impairment of the chemical senses after anesthesia by quinaldine and phenoxyethanol. The effects, however, were quantitative, not all-or-nothing. Several impaired groups did show evidence of a fright reaction, but the response was reduced in duration or intensity. It is likely that the chemical stimulus was perceived as a low-intensity indicator of predatory threat. One might easily obtain positive electrophysiological evidence of signal detection in such fish and not suspect impairment. In their original report of structural effects on the sensory epithelia, Lewis et al. (1985) cautioned that the behavioral effects might be subtle. Affected fish might show the normal olfactory choices even though all chemical stimuli were perceived as less intense. On the other hand, affected fishes could be totally deprived of the perception of more subtle, unritualized olfactory cues and metabolic by-products of the sort that have been suggested by Liley (1982).

The dosages in our study were greater than those of Quinn et al. (1988) and induced full immobilization very quickly. These dosages were intended to serve as an upper limit that might be achieved if the drugs were used to produce rapid and deep anesthesia or to aid in capture under field conditions with highly unpredictable dosage. Recovery from anesthesia was fast and complete. Of more than 200 fish anesthetized, only one died of an apparent overdose. It would be easy to conclude that these were safe and clinically effective dosages.

Despite a dosage 25 time higher than theirs, our results for MS-222 agree with Quinn et al. (1988). With further refinement of our methods, we might be able to show a quantitative effect, but it would be far less than that seen for the other anesthetics. Our phenoxyethanol dosage was about six times that used by Quinn et al. (1988), and our results disagreed with theirs. The question remains as to whether this is an effect of dosage or real differences between methods, criteria, and species. The conservative position is to suspect that our assay was more sensitive to quantitative impairment of the chemical senses. Quinn et al. tested for comparison of water that did and did not contain a chemical. We have no doubt that many of our impaired fish did detect the chemical but probably perceived the concentration to be lower than did unimpaired fish. Structural studies of the olfactory epithelium are necessary before we can fully interpret these findings. We must also confirm that the behavioral deficits are specific to chemical stimuli and not some more central process.

Our results fail to confirm any negative effects for MS-222. They clearly, however, suggest strong caution in the use of anesthetics and that the strategy to avoid MS-222 and instead use quinaldine or phenoxyethanol is most inadvisable.

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SEED GERMINATION AND GROWTH INHIBITORY CADINENES FROM *Eupatorium adenophorum* Spreng

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Abstract—Chloroform extract of the aerial parts of *Eupatorium adenophorum* Spreng was fractionated and examined for growth inhibition. Bioassay-directed fractions of the plant materials afforded three known cadinenes and β-sitosterol. The effects of different fractions as well as isolated cadinenes were determined using *Allium cepa*, *Raphanus sativus*, and *Cucumis sativus* seeds. Three-day exposure to these cadinenes significantly inhibited germination and seedling growth of all three assay seeds. The degree of inhibition was dependent upon seed species and the concentrations of the compounds tested. Cadinene (**1**) was found to be more inhibitory to the seeds tested and the activity of the cadinene (**3**) was less than that of (**1**) and (**4**).

Key Words—*Eupatorium adenophorum*, Asteraceae, cadinene, allelopathy, germination inhibitors, *Allium cepa*, *Raphanus sativus*, *Cucumis sativus*.

INTRODUCTION

E. adenophorum Spreng (Syn. *E. trapezoidieum* Kunth) is a perennial herb of the family Asteraceae. This weed was introduced in India very recently. Since then, it is proliferating extensively and has become a serious dominant weed of

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northeast India, and growing abundantly in the State of Meghalaya, particularly at higher altitudes (553–1750 m) as one of the aggressive weeds of ruderal habitats. The plant also grows abundantly in Nepal, where it has been used by the Tamang tribes as a herbal medicine for treating fever and insomnia (Manandhar, 1991).

The behavior of *E. adenophorum* in the field would appear to fall under the classic definition of allelopathy as defined by Rice (1984); i.e., this plant may adversely affect other nearby vegetation by releasing inhibitors. Reports in the literature (Tripathi et al., 1981; Jha et al., 1985) suggest that allelopathy may play an important role in the overall interference of *E. adenophorum* with other species. Proksch et al. (1990) studied the sesquiterpene derivatives from *E. adenophorum*, which exhibited contact toxicity and growth-retarding activity against larvae of a noctuid species. Prior to this study, Shukla et al. (1986) reported the isolation of four cadinenes from *E. adenophorum*. They also established their absolute stereochemistry by x-ray crystallography to prove that these cadinenes belong to the amorphene subgroup of cadinenes. Sesquiterpenoids containing the basic skeleton of cadinene are produced by a wide variety of plant species. Biological activity associated with cadinenes, e.g., cytotoxicity (Duke et al., 1987), phytotoxicity (Duke et al., 1987; Chen and Leather, 1990; Cutler, 1988), insect antifeedant (Proksch et al., 1990) and growth-regulating activity (Kalsi et al., 1979; Talwar et al., 1992), suggest the possibility that these compounds may play a role in the allelopathy of *E. adenophorum*. The above findings of Tripathi et al. (1981) and those of Jha et al. (1985) prompted our interest to study *E. adenophorum* in an effort to isolate the probable chemical source of the reported allelopathic effects.

The overall objective of the present study was to isolate and identify growth inhibitors from the aerial parts of *E. adenophorum* and to determine the inhibitory effects of specific compounds that may contribute to allelopathic activity.

METHODS AND MATERIALS

Collection of Plant Materials. Plant collections took place in March 1992, from Shillong, State of Meghalaya, India.

Extraction of Plant Material and Column Chromatography of Crude Gum. Aboveground parts (stem and leaves) of *E. adenophorum* (2 kg), were extracted with chloroform and processed in the usual fashion (Shukla et al., 1986). The crude gum, 25 g was chromatographed over silica gel (600 g) packed in petroleum ether (PE), 200-ml fractions being collected in the following order: 1–9, PE; 10–33, PE/EtOAc, 9:1; 34–44, PE/EtOAc, 5:1; 45–50, PE/EtOAc, 4:1; 51–62, PE/EtOAc, 2:1; 63–72, PE/EtOAc, 1:1; 73–78, EtOAc; 79–86, EtOAc/MeOH, 19:1.

Thin-Layer Chromatography (TLC) of Biologically Active Fractions. Fractions 53–62 showed two major spots on TLC and were combined to give 2.5 g of crude. The crude gummy material was subjected to preparative TLC (PE/EtOAc, 5:1; five developments). The more polar compound was obtained as gum and identified as **3** (Figure 1), yield 0.5 g, IR ($CHCl_3$): 1700, 1670, 1600, 1100, and 975; ^1H NMR and MS as reported by Shukla et al. (1986).

The less polar band provided 0.525 g of a colorless solid, **4**, mp 173–176°C (CH_2Cl_2), which was identified by comparing with an authentic product (TLC, IR, ^1H NMR, and MS). Earlier compound **4** had been reported as gum (Shukla et al., 1986).

Fractions 63–72 were combined (2.5 g) and contained one major compound and other minor components. The major component was purified by preparative TLC (PE/EtOAc, 1:4; three developments) to give 1.5 g of gummy material identified as **1** by directly comparing with the authentic sample (TLC, IR, ^1H NMR, and MS) (Shukla et al., 1986).

Chromatography. Column chromatography was carried out through a glass column using a definite amount of silica gel (60–120 mesh, BDH, Bombay, India). TLC and preparative TLC were performed on silica gel (silica gel-G,

(1) (2) (3) (4)

FIG. 1. Structures of cadinene **1**, **2**, **3**, and **4**.

BDH) plate activated at 110°C for 1 hr. Bands of the chromatograms were visualized by exposing the plates to iodine vapor, spraying with sulfuric acid, and heating.

Melting Point and Spectroscopic Study. Melting points (mp) were determined on Kofler block and uncorrected. Infrared spectra (IR) were measured as Nujol mulls for the compounds on a Shimadzu-440 spectrometer. ^{1}H NMR were recorded on a 60-MHz spectrometer with TMS as an internal standard. Low-resolution mass spectra (MS) were recorded on a MS-30 instrument.

Chemicals used in the present investigation were purchased from BDH Chemicals (E. Merck, Bombay, India). Assay seeds were purchased from IARI, New Delhi, India.

Seed Germination and Seedling Growth Bioassay. Test solutions of isolated compounds were prepared using dimethyl-sulfoxide (DMSO, 0.1% v/v) as the initial solvent carrier followed by diluting with distilled water to a final concentration of 1 mM. Other test solutions (i.e., 50, 100, 250, 500 μM) were prepared by dilution with an aqueous solution of DMSO. Each test solution (5 ml) was added separately to a sterile glass Petri dish lined with Whatman No. 1 filter paper. Inhibitory activity of test solutions was assayed using seeds of onion (*Allium Cepa* L. cv. Red Globe), radish (*Raphanus sativus* L. cv. Jap white) and cucumber (*Cucumis sativus* L., cv. Long green). Seeds were surface-sterilized by immersion in 1% NaOCl for a brief period and then rinsed several times with distilled water. Treatments consisted of 25 evenly spaced seeds per dish. Four replicates of each treatment were arranged and were repeated twice. Treated seeds were kept in the dark in a BOD incubator at 25 ± 2°C. Germination (radicle protrusion through the seed coat) and radicle and hypocotyle lengths of all assay seeds were recorded after 72 hr.

Statistical Analysis. The germination and root and shoot length data were subjected to one-way analysis of variance (ANOVA) followed by Duncun's new multiple-range test (DMRT) to determine significant differences among mean values at the 0.05 probability level.

RESULTS AND DISCUSSION

The aerial parts of the plant were extracted with chloroform as described in Methods and Materials. Thc gummy residue (25 g) obtained after evaporation of the solvents in vacuo was fractionated in a silica gel column. Elution was made successively with petroleum ether (PE) and petroleum ether–ethyl acetate (PE/EtOAc), increasing stepwise, and finally eluted with 1% MeOH. Then eluates were bioassayed using onion, radish, and cucumber seeds to determine the fractions possessing significant inhibitory activity (Table 1). These results showed that the fractions 45–50 (PE/EtOAc, 4 : 1) and 63–72 (PE/EtOAc, 1 : 1)

TABLE 1. GERMINATION OF ONION, RADISH, AND CUCUMBER SEEDS EXPOSED FOR 3 DAYS TO VARIOUS FRACTIONS OF EXTRACTS OF *E. adenophorum* SPRENG[a]

		Germination[c]		
Fraction[b]	Eluent	Onion	Radish	Cucumber
1-9	PE	62a	85a	83a
10-33	PE/EtOAc (9:1)	60a	81a	80a
34-44	PE/EtOAc (5:1)	57a	78a	77a
45-50	PE/EtOAc (4:1)	45b	58b	65b
51-62	PE/EtOAc (2:1)	58a	80a	78a
63-72	PE/EtOAc (1:1)	36c	62b	50c
73-78	EtOAc	58a	81a	78a
79-86	EtOAc/MeOH (19:1)	60a	84a	81a

[a] Values in the same vertical column followed by the same letters do not differ ($P < 0.05$) according to Duncan's new multiple range test (Duncan, 1955).
[b] Extracts were applied to filter paper disks (15 cm) and solvents were completely evaporated before testing. Solutions of the crude were prepared using 0.1% DMSO with distilled water to a final strength of 100 ppm.
[c] Values are the mean of four observations.

showed significant inhibition of germination of the three assay seeds. Much lower reduction of germination of all the test species was observed for the other fractions and were insignificant. On the basis of the bioassay results, both lots of the fractions were chosen as the most probable source of inhibitors in *E. adenophorum*.

Fractions 63-73, eluted with PE/EtOAc (1:1), have the most potent activity which, on further purification by preparative TLC, furnished compound **1** as gum from the less polar band of the chromagogram. The most polar TLC bands contained traces of minor components that were not further investigated due to the lack of material.

Fractions 45-50, PE/EtOAc (4:1), the second most potent lot, on further purification by preparative TLC provided two compounds. The less polar band of the chromatogram contained compound **4** as a colorless solid, mp 173-176°C (CH_2Cl_2) while the more polar band furnished its isomer **(3)**.

In addition to the cadinenes, fraction 34-44 (PE/EtOAc, 5:1) was found to contain β-sitosterol **(2)**, which was identified by TLC, IR, ^{1}H NMR, and MS spectral comparison with an authentic sample. These fractions showed lower and insignificant reduction of germination of all three types of assay seeds. Moreover, sterols are common products in all higher plants, and they have not been reported to have effects on seed germination per se (Bradow, 1985). Therefore, in the present study, the effect of β-sitosterol on seed germination and growth was not examined.

Table 2 shows the inhibitory effect of different concentrations of all three compounds on three different test species. All concentrations of the compounds were inhibitory to seed germination of all three assay seeds. Increasing concentration of the compounds led to increasing inhibition of seed germination. However, the three cadinenes were not significantly inhibitory to seed germination at 50 μM, but cadinene (**1**) inhibited the shoot length of onion at that concentration, which was statistically significant (Table 2). The difference between patterns of inhibitions of seed germination and growth by cadinenes suggested that the mechanism of the growth inhibition by them might be different for growth and germination. Our results also are in accord with Duke et al. (1987) concerning bioassays of artemisinin, a cadinene derivative, where seedling growth appears to be more sensitive to the effect of inhibitors than does germination. Furthermore, the shoot and root of the test species exhibited different responses of the three assay seeds to three different chemicals, confirming the contention of Whittaker (1971) and Akhtar et al. (1978) that allelopathic activity depends on the nature of the test species and the concentration of the allelochemicals. Insofar as structures of the compounds are concerned, the important groups are at the C-3 and C-8 positions. Cadinene **1**, which is the most active of the triplet, has an OH group in C-8 and an α,β-unsaturated ketone at C-3, and it inhibited seed germination and seedling growth of all three assay seeds from 50 to 1000 μM. Cadinene **4** has two keto functions at C-3 and C-8; one of them, an α,β-unsaturated ketone, inhibited germination and seedling growth above 100 μM. Cadinene **3**, an epimer of compound **4** having the epimeric center at C-7, was slightly less active than **4**. With respect to the inhibitory effect on germination and seedling growth of three test species compared to control, cadinene **1** exhibited the most potent activity, followed by **4**, and least by **3**.

From the foregoing discussion, it becomes evident that the effect of cadinenes as inhibitors of germination and seedling growth is highly significant. Growth of both radicle and hypocotyl were reduced, but the relative effect on the hypocotyl growth was greater than that of the radicle growth. This is in agreement with Tripathi et al. (1981), who reported that inhibitors present in the aqueous extract of *E. adenophorum* caused more inhibition of hypocotyl growth than radicle growth.

From these results, it may be seen that the cadinene components of *E. adenophorum* showed inhibitory activity in seed germination and seedling growth of all three assay seeds. These compounds probably have a role in other cases of allelopathy besides the observed inhibition of onion, radish, and cucumber seeds. The presence of these compounds in this plant suggests that they may be active in the regulation of seed germination and growth of other associated species, as reported by Tripathi et al. (1981). This result suggested that the released compounds from the plant contribute at least partly to the inhibition

TABLE 2. GERMINATION AND SEEDLING GROWTH OF ONION, RADISH, AND CUCUMBER EXPOSED FOR 72 HR IN DIFFERENT CONCENTRATIONS OF COMPOUNDS **1**, **3**, AND **4**[a]

		Onion			Radish			Cucumber		
Compound	Concn. (μM)	Germination (%)[b]	Root length (mm)[c]	Shoot length (mm)[c]	Germination (%)	Root length (mm)[c]	Shoot length (mm)[c]	Germination (%)[b]	Root length (mm)[c]	Shoot length (mm)[c]
1	0	63a	15.8a	6.5a	85a	23.6a	9.4a	83a	16.6a	6.3a
	50	58ab	14.9a	5.7b	80ab	23.1a	9.3a	79ab	16.4a	5.9a
	100	54ab	12.7b	4.9c	74b	22.2b	6.1b	72b	15.3b	4.6b
	250	48b	9.8c	3.0d	62c	17.9c	4.8c	63c	11.6c	3.3c
	500	31c	5.8d	1.6e	49d	13.4d	3.2d	43d	8.8d	2.0d
	1000	18d	2.7e	0.7f	36e	8.9e	2.4e	25e	5.8e	1.0e
3	0	65a	14.9a	7.4a	86a	25.5a	9.2a	82a	23.7a	8.8a
	50	62ab	14.8a	6.9a	82ab	25.2ab	8.8a	78ab	23.4a	8.3a
	100	56bc	13.9b	5.8b	76bc	24.7bc	8.1b	73b	21.7b	7.9b
	250	53c	12.6c	4.8c	73c	24.3c	8.0b	70c	21.6c	7.3c
	500	34d	10.4d	2.9d	65d	21.3d	6.4c	61d	18.6d	6.1d
	1000	20e	5.2e	1.7e	44e	12.0e	3.8d	47e	15.4e	4.4e
4	0	67a	13.5a	7.8a	88a	25.0a	11.0a	81a	15.0a	7.0a
	50	64a	13.0ab	7.4a	85ab	24.5a	10.1a	79a	14.8ab	6.7a
	100	55b	12.3b	6.7b	80b	23.5b	9.3b	72ab	13.7b	6.1b
	250	47c	10.0c	4.8c	71c	21.4c	7.3c	65b	12.2c	5.2c
	500	34d	6.3d	3.1d	51d	16.2d	3.9d	35c	10.7d	3.3d
	1000	14e	3.1e	1.8e	33e	12.3e	1.7e	30d	5.1e	1.8e

[a]For each compound, means within a column sharing the same letter are not significantly different ($P < 0.05$) according to new DMRT.
[b]Values are means of four observations.
[c]Mean of 20 observations.

of growth of neighboring plants. These activities are possibly related to the ecological interaction of *E. adenophorum* with its surrounding environment.

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COMPARISON OF LARVAL AND ADULT P-450 ACTIVITY LEVELS FOR ALKALOID METABOLISM IN DESERT *Drosophila*

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Abstract—The cytochrome P-450 monooxygenase system has been implicated in plant utilization by at least three species of *Drosophila* (*D. nigrospiracula*, *D. mettleri*, and *D. mojavensis*) that are endemic to the Sonoran Desert of the southwestern United States and northwestern Mexico. Basal and induced levels of total cytochrome P-450 were determined for third-instar and decapitated 2- to 5-day post eclosion adults of the three desert species. Total P-450 levels, both basal and induced for all species assayed, were significantly higher for adults than for larvae by up to 20-fold. On a per organism basis, the levels of in vitro metabolism of the cactus alkaloid, carnegine, and patterns of response to induction by cactus tissue for adult desert *Drosophila* approximated those of larvae. Induction by phenobarbital, however, resulted in levels of in vitro carnegine metabolism that were up to 5.6-fold higher in adults than in larvae.

Key Words—*Drosophila*, Diptera, Drosophilidae, cytochrome P-450, polysubstrate monooxygenase, cactus, alkaloids, resistance.

INTRODUCTION

The ability of many pest organisms to rapidly acquire resistance to high levels of widely used synthetic organic compounds as well as naturally occurring plant chemical defenses has been an area of intense research for several decades

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(Heidelberger, 1975; Brattsten et al., 1977; Brattsten, 1979). Examples of behavioral adaptations and modified physiological processes are not uncommon as a means of defense against toxic xenobiotics, but biochemical resistance mechanisms, especially those involving detoxication by polysubstrate monooxygenases, have long been considered the primary mode of acquired resistance to xenobiotics (Plapp, 1976; Guthrie, 1980; Georghiou and Saito, 1983). As early as 1971, Krieger et al. (1971) suggested that tolerance of synthetic insecticides might be the result of the preadaptation of some insects to feed on plants which contain toxic allelochemicals.

The cactus-*Drosophila*-microorganism model system of the Sonoran Desert of the southwestern United States and northwestern Mexico provides an excellent vehicle for research into interactions between insects and toxin-containing host plants (Barker and Starmer, 1982). The system features four species of *Drosophila* that are endemic to the Sonoran Desert (*D. nigrospiracula*, *D. mojavensis*, *D. mettleri*, and *D. pachea*) and five species of columnar cacti, the necrotic tissues of which are utilized as feeding and breeding substrates. The cacti are saguaro (*Carnegiea gigantea*), cardón (*Pachycereus pringlei*), senita (*Lophocereus schottii*), agria (*Stenocereus gummosus*), and organ pipe (*Stenocereus thurberi*). The patterns of host plant utilization by the desert drosophilids involve interactions that are behavioral (Fogleman et al., 1981) and/or chemical (Kircher et al., 1967; Fogleman and Heed, 1989). Utilization of the cactus tissue depends ultimately on the ability of the resident fly species to tolerate the presence of allelochemicals that have shown to be toxic to nonresident species (Fogleman and Abril, 1990). For *D. nigrospiracula* (which lives on saguaro and cardón), *D. mettleri* (which lives primarily in the rot-exudate-soaked soils of saguaro and cardón), and *D. pachea* (which lives exclusively on senita), this means the ability to tolerate varying concentrations of the simple isoquinoline alkaloids, gigantine and carnegine, in saguaro (1–2% dry weight) and the monomers and trimers of lophocereine in senita (5–20% dry weight). Recent studies by Frank and Fogleman (1992) that have investigated in vivo tolerance to and in vitro metabolism of saguaro alkaloids have established a central role for the cytochrome P-450 monooxygenase enzyme system in the detoxication of these plant allelochemicals. This initial work, which focused on the larvae of the cactophilic *Drosophila*, demonstrated the likely involvement of P-450 enzymes on the basis of several independent lines of evidence. First, larval survival through eclosion on cactus substrate was significantly reduced by addition of the P-450 inhibitor, piperonyl butoxide (PBO). Second, the in vitro metabolism of saguaro cactus alkaloids by isolated microsomes was significantly induced by exposure of larvae to alkaloid-containing cactus tissue and to the general P-450 inducer, phenobarbitol. Third, alkaloid metabolism was inhibited by the addition of piperonyl butoxide. Finally, exposure to alkaloid-containing cactus

tissue resulted in significantly increased total P-450 content in two of the three desert *Drosophila* species studied.

Polysubstrate mixed-function monooxygenases, which are more commonly referred to as cytochrome P-450s, comprise a superfamily of mostly microsomal hemoproteins that were initially described on the basis of their unique spectral absorbance peak at 450 nm (Omura and Sato, 1964). Over 200 members of 36 families have been described to date (Nebert and Gonzalez, 1987; Gonzalez, 1989; Nelson et al., 1993). In addition to their involvement in the processing of a number of endogenous compounds, insect cytochrome P-450s have been identified as the primary means of metabolizing xenobiotics, including terpenes, sterols, phenolics, quinones, and alkaloids, all of which have been implicated as natural plant defense mechanisms against herbivores.

It has been shown in *D. melanogaster* that the response by larvae to mutagenic compounds differs from that of adult flies (Vogel, 1977; Vogel et al., 1980). Hälström et al. (1983) investigated and identified significant differences in the activities of several drug-metabolizing P-450s between larval and adult *D. melanogaster*. The current study represents an extension of the initial work by Frank and Fogleman (1992) by investigating total P-450 content and in vitro alkaloid metabolism by enzymes from adult *Drosophila*. When analyzed in combination with the larval data, these provide a basis for additional insight into the ability of and the mechanisms by which P-450 enzymes respond to and metabolize the plant secondary compounds that the drosophilids encounter in their natural breeding substrates. Furthermore, since P-450 enzymes have been found to be responsible for the activation of a number of carcinogenic compounds (Wolf, 1986; Guengerich, 1987), knowledge of P-450 activity and substrate specificity at different stages of the *Drosophila* life cycle is essential to the effective and continued use of *Drosophila* as a test organism for genotoxic compounds as well as for the rational evaluation of potential pesticides.

METHODS AND MATERIALS

Drosophila *Species and Pretreatments*. Larvae and adults of three species of cactophilic *Drosophila* (multifemale lines of *D. nigrospiracula*, *D. mettleri*, and *D. mojavensis*) originally isolated (1988) from a mainland region of the Sonoran Desert were used in the current study. All *Drosophila* stocks had been maintained under ambient laboratory conditions on yeast-supplemented instant *Drosophila* media. Early third-instar larval and 1- to 3-day post eclosion adult-*Drosophila* were induced for 48 hr with 1 g (dry weight) of either rehydrated saguaro tissue or senita tissue or with 20 mg of phenobarbital dissolved in 1 ml of water (2% w/v, pH 9.0). Each inducer was distributed evenly across the surface of the instant *Drosophila* media.

Microsomal Harvest. Prior to homogenization, larvae were collected by dumping medium plus larvae into a 20% sucrose solution, collecting the floating larvae on a 0.8-mm^2 wire-mesh screen, and rinsing them briefly with deionized water. Headless adult flies were prepared by repeated vigorous vortexing in liquid nitrogen followed by separation of frozen heads and other appendages from bodies by rapid vibration on a 0.8-mm^2 wire-mesh screen. The efficiency of separation of heads and bodies approached 100%. This was necessary to avoid the putative inhibitory effect of the eye pigment xanthomattin on P-450 content and in vitro metabolism assays (see Discussion). The total microsomal fractions of homogenized, mid-third-instar larval (20 g) and 3 to 5-day post eclosion headless adult (10 g) *Drosophila* were isolated by standard methods (Waters et al., 1983; Lee and Scott, 1989). Briefly, larvae and adults were thoroughly homogenized in a 100 mM sodium phosphate buffer (pH 7.5) containing 100 μM dithiothreitol, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM 1-phenyl-2-thiourea, and 10% glycerol. The crude homogenate was filtered through 10 layers of cheesecloth and then centrifuged twice at 20,000 *g* for 20 min at 4°C to pellet large cellular debris. Wax from the larval cuticle was removed by filtration of the supernatant through six layers of cheesecloth following each centrifuge run. The total microsomal fraction was then pelleted by centrifugation of the supernatant at 145,000 *g* for 1 hr at 4°C. Microsomes were resuspended in 100 mM sodium phosphate buffer (pH 7.5) containing 40% glycerol (0.33 ml buffer/g larvae; 0.8 ml buffer/g adults) and stored at −70°C until needed.

Total P-450 content and in vitro alkaloid metabolism assays were standardized on the basis of microsomal protein concentration, which was determined spectrophotometrically using the Pierce Coomassie Protein Assay Reagent system (Pierce Chemical Company, Rockford, Illinois). Bovine serum albumin fraction V in 100 mM sodium phosphate buffer (pH 7.5) was used to generate a standard curve.

Total P-450 Content. Total P-450 content was determined spectrophotometrically by the method of Omura and Sato (1964) with the following modifications. NADPH was used as the reductant in place of sodium dithionite. Larval microsome suspensions were reduced and assayed at a microsomal protein concentration of 2 mg/ml, while adult microsome suspensions were first reduced at 5 mg/ml and then diluted to 2 mg/ml immediately before reading the background absorbance. This was necessary because the 5 mg/ml suspension was too opaque to read accurately, and reduction at a microsomal protein concentration of 2 mg/ml failed to yield a detectable difference in the absorbance spectra of the unbound versus the CO-bound P-450 species. No differences in the measured P-450 content of larval microsome suspensions were detected between samples reduced at 2 mg/ml and those reduced at 5 mg/ml.

In Vitro Metabolism of Carnegine. Microsomal suspensions (6 mg micro-

somal protein/ml for adults; 2 mg microsomal protein/ml for larvae) were incubated at 37°C for 20 min to oxidize any endogenous NADPH. Reaction mixtures contained 1.6 mg microsomal protein, TKEM reaction buffer (50 mM Tris HCl, pH 7.4, 50 mM KPO_4, 1 mM EDTA, and 1 mM $MgCl_2$), 2 mg NADPH tetrasodium salt, and extracted saguaro alkaloids suspended in 7 ml of ethylene glycol monomethyl ether. Alkaloids were extracted from cactus tissue using standard techniques (Meyer and Fogleman, 1987). The final protein concentrations for the microsomal suspensions were 5 mg/ml for adults and 1.6 mg/ml for larvae. Since P-450 enzymes are NADPH-dependent, reaction mixes without NADPH served as controls. P-450 inhibition was investigated by the addition of 1 mg of the known P-450 inhibitor, piperonyl butoxide (PBO), to microsomal suspensions during the preincubation period. All experiments and controls were set up in triplicate. Following a 2-hr incubation period at 37°C with constant shaking, 25 μl of 72% trichloroacetic acid was added to each reaction mix to precipitate the protein component, which was subsequently pelleted by centrifugation for 15 min in a benchtop centrifuge. The supernatant was transferred to a fresh tube and was made basic by the addition of 200 μl of strong ammonia solution. The unmetabolized alkaloids were extracted from the aqueous phase with ether (two extractions using 2 ml of ether each). The ether from the two extractions was then pooled and dried under N_2. The extracted compounds were resuspended in 100 μl of chloroform, and unmetabolized alkaloids were quantified using a Hewlett-Packard 5890A gas chromatograph with a flame ionization detector and a HP Ultra 2 column. Samples were run isothermally at 220°C with H_2 as the carrier gas (9 psi head pressure). The injector and detector temperatures were 300°C. Under these conditions, carnegine had a retention time of approximately 4.07 min. The identity of the carnegine peak was confirmed by comparison of the retention time of a commercially prepared standard of carnegine (Pfaltz and Bauer Inc., Waterbury, Connecticut). The amount of carnegine metabolized was then determined by comparing the amount of carnegine extracted from the NADPH-containing reaction mixes to that in the control mixes containing no NADPH. Alkaloid quantities were expressed in terms of peak area count units.

Statistical Analysis. One-way analysis of variance (ANOVA) tests were used for larvae versus adults, induced versus uninduced, and PBO-inhibited versus PBO-uninhibited comparisons.

RESULTS

Preliminary Experiments: Whole versus Decapitated Adult D. mojavensis. Total measurable P-450 content was determined spectrophotometrically for the microsomal fraction of adult *D. mojavensis* that were either whole or that had

been decapitated immediately prior to homogenization. The detectable P-450 content expressed as nanomoles P-450 per milligram microsomal protein was found to be 2- to 3.7-fold greater in homogenates prepared from decapitated *D. mojavensis* versus those from whole flies. On the basis of one-way ANOVA tests, the difference was significant at the $P < 0.01$ level, regardless of whether or not the organisms had been induced with cactus tissue (saguaro or senita) or phenobarbital prior to being harvested. No P-450 activity was seen in assays of in vitro saguaro alkaloid metabolism using suspended microsomes harvested from whole *D. mojavensis*, while microsomes from decapitated flies produced significant levels of detectable activity.

Total Microsomal Protein Content: Adults versus Larvae. Using a Coomassie dye-based assay system, total microsomal protein content was determined on the basis of the spectral absorbance at 595 nm. The larval drosophilids in the current study were found to have 2.52 ± 0.28 mg (mean $\pm$SD) total microsomal protein/g of organism while decapitated adult flies had significantly more ($P < 0.001$) at 7.84 ± 0.96 mg microsomal protein/g of organism.

Total P-450 Content. Total microsomal P-450 content was determined for adult decapitated desert *Drosophila* (*D. mojavensis*, *D. mettleri*, and *D. nigrospiracula*). Basal (i.e., uninduced) P-450 levels, as well as levels after induction with saguaro alkaloids, senita alkaloids, and phenobarbital, were determined for all desert *Drosophila* species used in the current study with one exception: the P-450 content of senita-induced *D. nigrospiracula* was not determined because this species cannot tolerate exposure to senita alkaloids. The results are presented in Table 1, and clearly show that adults have significantly greater P-450 content than larvae. The ratio of adult content to larval content (A/L ratio) varies from 5.08 (phenobarbital-treated *D. mojavensis*) to 35.82 (uninduced *D. mettleri*).

The lowest basal level of total P-450 in adults was seen in *D. nigrospiracula*, while *D. mettleri* had the highest. Differences in the basal P-450 levels for larvae of the three species were not significant. Treatment with phenobarbital resulted in a significant increase in total P-450 in both adults and larvae for each of the desert species. Except for *D. nigrospiracula* ($F_s = 243.8$; $df = 1,4$; $P < 0.001$), exposure to cactus alkaloids did not produce a significant detectable increase in overall P-450 levels in adults. Indeed, total P-450 content was slightly reduced for *D. mettleri* and *D. mojavensis* by exposure to saguaro or senita tissue. This is in contrast to the situation in larvae where exposure to cactus alkaloids was associated with significant increases in total P-450 content in both *D. mettleri* and *D. mojavensis* (reported in Frank and Fogleman, 1992).

In Vitro Metabolism of Carnegine. Suspensions of isolated microsomes were incubated with the purified saguaro alkaloid, carnegine, to assay specifically for carnegine-metabolizing P-450 activity. Basal, cactus alkaloid- (saguaro and senita) and phenobarbital-induced activity levels were determined for each of the desert *Drosophila* species. *Drosophila nigrospiracula* was not induced

TABLE 1. TOTAL P-450 CONTENT (NANNOMOLES MOLES/PER GRAM OF ORGANISM) OF ADULTS VS. LARVAE[a]

Species and inducer[b]	Adults	± SD	*N*	Larvae	± SD	*N*	F_S^c	A/L Ratio
D. melanogaster								
None	4.42	0.38	3	0.45	0.10	6	650	9.82
pb	4.75	0.13	3	1.20	0.53	8	122	3.96
D. mojavensis								
None	2.60	0.41	6	0.15	0.04	7	246	17.33
Saguaro	2.43	0.08	3	0.17	0.04	10	4500	14.29
Senita	2.37	0.26	4	0.30	0.05	8	497	7.90
pb	3.30	0.25	3	0.65	0.05	7	837	5.08
D. nigrospiracula								
None	2.31	0.12	3	0.12	0.03	4	1241	19.25
Saguaro	3.84	0.12	3	0.14	0.06	9	5371	27.43
pb	3.80	0.49	3	0.28	0.11	8	438	13.57
D. mettleri								
None	3.94	0.34	3	0.11	0.05	7	1024	35.82
Saguaro	3.68	0.57	3	0.19	0.07	5	206	19.37
Senita	3.59	0.14	3	0.19	0.03	3	1710	18.89
pb	5.01	0.04	3	0.93	0.27	6	643	5.39

[a] Results of one-way ANOVA are also presented.
[b] pb = phenobarbital.
[c] All F_S values were significant at $P < 0.001$.

with senita alkaloids for the reason stated above. An additional induction of *D. mojavensis* with 10× concentration of saguaro alkaloids was also performed to determine if induction of enzyme activity was a function of the specific alkaloids used or a function of the concentration of alkaloid. The results of this work are shown in Table 2 where activity is expressed as area count units per gram of organism. Significant basal levels of in vitro carnegine-metabolizing activity were seen in both adults and larvae for all the cactophilic *Drosophila* species studied. The basal activity of microsomal suspensions prepared from adult *Drosophila* was found to be highest for *D. mettleri* and lowest for *D. mojavensis*. Treatment with phenobarbital resulted in a significant ($P < 0.001$) increase in assayed in vitro metabolism in both *D. mojavensis* and *D. mettleri*. Significant induction of in vitro P-450 activity by cactus alkaloids was observed in *D. mojavensis* treated with senita alkaloids ($P < 0.001$) or 10× concentrated saguaro alkaloids ($P < 0.05$). No induction by saguaro tissue was observed for any of the desert species. Relative to levels of in vitro metabolism reported for larvae (Frank and Fogleman, 1992), P-450 activity in adults was approximately the same or greater. Thus, the correlation in the overall pattern of response by adults versus larvae to induction by cactus alkaloids and phenobarbital was highly significant ($r = 0.947$; $df = 10$; $P \ll 0.001$).

Inhibition of P-450 activity was investigated by addition of the general monooxygenase inhibitor, piperonyl butoxide (PBO), to the microsomal suspension at the start of the in vitro assay. As was the case with larvae, both the cactus alkaloid- and phenobarbital-induced increases in carnegine metabolism were significantly ($P < 0.05$–0.001) reversed by PBO.

DISCUSSION

The primary objective of the current study was to determine whether or not significant differences existed in the total cytochrome P-450 content or the in vitro alkaloid-metabolizing activity of larval versus adult cactophilic species of *Drosophila*. In order to develop a meaningful basis for comparison, however, it was necessary to address some of the qualitative differences that exist between the larval and adult stages of the life cycle. These included the impact of eye pigments, which are only present in the microsomal suspensions prepared from adult organisms and the use of microsomal protein content as a basis for expressing total P-450 content and in vitro metabolic activity.

Xanthomattin is an abundant pigment in the eyes of many insects, including *Drosophila* (Ryall and Howells, 1974). Hodgson (1985) has suggested that as a potent electron acceptor, xanthomattin may act to divert electrons away from cytochrome P-450s, resulting in an overall lowering of the apparent P-450 content and in vitro activity levels. Since P-450 activity in dipterans has been shown

TABLE 2. CARNEGINE METABOLISM (AREA COUNT UNITS × 10^{-3} ADJUSTED FOR DIFFERENCES IN MILLIGRAMS OF MICROSOMAL PROTEIN PER GRAM OF ORGANISM) OF ADULTS VS. LARVAE[a]

Species and inducer[b]	Inhibitor	Adults	± SE	*N*	Larvae	± SE	*N*	F_S^c
D. mojavensis								
None	None	59.4	9.2	3	21.8	5.3	9	12.68**
Saguaro	None	105.0	40.1	3	37.0	5.7	3	2.82
Saguaro	1 mM PBO	31.6	5.7	3	7.9	19.3	3	3.59
10 × Saguaro	None	238.0	40.3	6	100.6	3.9	3	5.41
10 × Saguaro	1 mM PBO	6.8	37.8	3	56.0	18.2	3	1.37
Senita	None	252.1	16.4	3	144.9	9.0	3	32.86**
Senita	1 mM PBO	67.5	43.7	3	99.0	9.0	3	0.50
pb[c]	None	1609.1	22.7	3	286.1	10.9	3	2763.82***
pb	1 mM PBO	1045.8	35.0	3	85.4	3.0	3	745.82***
D. nigrospiracula								
None	None	70.6	18.8	2	35.0	2.6	3	6.05
Saguaro	None	97.5	12.7	3	28.3	12.0	3	15.64*
Saguaro	1 mM PBO	34.9	15.3	3	7.2	19.1	3	1.28
pb	None	194.5	53.6	6	50.7	3.2	3	3.36
pb	1 mM PBO	54.8	29.5	6	33.4	3.5	3	0.24
D. mettleri								
None	None	204.3	20.1	6	59.2	5.5	6	48.46***
Saguaro	None	92.1	14.3	6	57.3	4.1	3	2.72
Saguaro	1 mM PBO	0.0	29.8	6	26.9	12.4	3	0.37
Senita	None	246.5	7.0	3	120.5	6.9	3	163.85***
Senita	1 mM PBO	0.0	22.1	3	48.0	4.1	3	4.58
pb	None	1311.9	11.2	3	292.5	2.3	3	7992.13***
pb	1 mM PBO	1072.2	33.9	3	188.2	5.7	3	659.99***

[a] Results of one-way ANOVA are also presented.
[b] pb = phenobarbital.
[c] $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

to be primarily associated with the abdomen (Tsukamoto and Casida, 1967), removal of the entire head immediately prior to homogenization was considered the most efficient means of producing pigment-free microsomal suspensions without adversely impacting the P-450 or total protein content of the microsomal suspension. Total measurable P-450 content was consistently found to be 2.0- to 3.7-fold greater when assayed using microsomes prepared from decapitated versus intact flies, suggesting that the inhibitory effect of endogenous inhibitors associated with *Drosophila* heads, including xanthomattin, is a significant concern. This is further supported by experiments that measured in vitro alkaloid metabolism in which there was a complete suppression of metabolic activity in microsomal suspensions prepared from intact flies.

With a rare exception from work on rats (Shayiq and Avadhani, 1989), xenobiotic-metabolizing activity has been primarily associated with the microsomal fraction (i.e., the endoplasmic reticulum) of both insect (Hansen and Hodgson, 1971) and mammalian (Nebert et al., 1982; Black and Coon, 1987) tissue homogenates. Total P-450 content and enzyme activity, therefore, have traditionally been expressed on a per milligram of microsomal protein basis. In the current study, expression of P-450 content and enzyme activity on a per milligram microsomal protein basis was considered inappropriate for comparing adults and larvae due to the large difference in total microsomal protein content between the two stages of the organism's life cycle. While a decapitated adult fly and a third-instar larva were found to be equivalent in weight per individual, the adult was found to have just over three times as much microsomal protein as a larva. This 3:1 ratio of adult of larval microsomal protein content was consistent for each of the *Drosophila* species studied. Expression of either P-450 content or in vitro enzyme activity data on a per milligram microsomal protein basis, therefore, appears to have little relevance at the individual level. In effect, adults having only one third the in vitro enzyme activity of larvae, on a per milligram microsomal protein basis, would still have the same total substrate-metabolizing capacity as larvae by virtue of there being three times as much microsomal protein in the adult. This becomes a significant concern when using in vitro enzyme activity data in studies on insecticide resistance in pest organisms at different stages of their life cycle or investigations involving cross-species comparisons (Hällström et al., 1983; Leonova et al., 1987; Yu and Hsu, 1993). Failure to address the issue of total microsomal content per organism, in such cases, therefore, may result in an inaccurate assessment of conditions at the organismic level. This may, in turn, lead to questionable conclusions, especially when evaluating the use of different life cycle stages in mutagenicity assays or selecting optimal conditions for pesticide use. Such concerns are not limited to studies of insects. It has been suggested (Philpot, 1993) that differences in the isolation efficiency of the microsomal fraction from different tissues of mammals

or following administration of some inducers may artificially bias activity and content measurements.

Total cytochrome P-450 content was at least fivefold (phenobarbital induced *D. mojavensis*) and as much as 36-fold (uninduced *D. mettleri*) higher for adult flies than for third-instar larvae. The enormous increase in total cytochrome P-450 levels in the adult stage of the life cycle was not, however, associated with a correspondingly large increase in alkaloid-metabolizing activity except when induced by phenobarbital.

Cytochrome P-450s are produced by a large superfamily of genes (Nelson et al., 1993), many of which are expressed simultaneously (Wilkinson and Brattsten, 1972; Ronis et al., 1988). As such, the relationship between total P-450 content and in vitro enzyme activity can be used as a crude indicator of the degree of isozyme multiplicity responsible for a specific metabolic activity. Two findings of the current study, therefore, are of particular significance: (1) the greater total P-450 content seen in uninduced and cactus tissue-induced adult flies, relative to larvae, was not associated with correspondingly higher in vitro carnegine-metabolizing activity, and (2) the increased in vitro carnegine-metabolizing activity observed following exposure of adult and larval *Drosophila* to high concentrations of naturally occurring cactus alkaloids or phenobarbital was not associated with an equivalent increase in total P-450 content. While it remains a possibility that alkaloid metabolism is the result of the concerted action of numerous P-450 enzymes, these findings, taken together, suggest that only a small number (possibly only one) of alkaloid-inducible P-450 isozymes are responsible for the observed in vitro carnegine-metabolizing activity of microsomal suspensions.

The induction of in vitro enzyme activity by phenobarbital was greater than that by cactus alkaloids for both adult and larval *Drosophila*. Phenobarbital has been shown to induce a large number of P-450s (Perry et al., 1971; Narhi and Fulco, 1982; Whitlock, 1986), including several in family III, from which the xenobiotic metabolism-associated family VI P-450s are thought to have most recently diverged (Feyereisen et al., 1989). This may suggest that the increase in both total P-450 content and in vitro activity, which is most pronounced in phenobarbital-treated flies, is the result of induction of several P-450s that are closely related to the cactus alkaloid-inducible species of P-450 and that have activity on carnegine but that are not cactus alkaloid-inducible.

Alternatively, phenobarbital may simply be a stronger inducer than cactus tissue of the P-450(s) responsible for alkaloid metabolism. If this were the case, the degree of P-450 induction may be regulated in a dose-dependent manner. There is preliminary evidence for this based on the observation that exposure of either larvae or adults to concentrations of cactus alkaloids in excess of those encountered in the rot pockets or exudate-soaked soils in which the organisms normally breed produces levels in vitro enzyme activity that are intermediate

between those obtained with induction by cactus alkaloids at concentrations that the organisms normally encounter and those obtained with induction by phenobarbital.

In conclusion, the pattern of induction of alkaloid-metabolizing P-450 activity in adult cactophilic *Drosophila*, both by cactus tissue and phenobarbital, was significantly correlated with that reported for larvae. Furthermore, when adjusted for differences in total microsomal protein content, the in vitro carnegine-metabolizing activity of microsomal suspensions (non-PBO-inhibited) prepared from adult cactophilic *Drosophila* was at least as great as that in larvae. The observed pattern of induction also appears to suggest that a small number of P-450 isozymes are responsible for the detoxication of the cactus alkaloid carnegine. Further work, including identification of the gene(s) that code for alkaloid-metabolizing P-450(s), will lead to a greater understanding of the roles, regulation, and evolution of P-450 enzymes responsible for xenobiotic metabolism and the observed patterns of insect–host plant interaction.

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SURFACE DISPOSITION AND STABILITY OF PEST-INTERACTIVE, TRICHOME-EXUDED DITERPENES AND SUCROSE ESTERS OF TOBACCO

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Abstract—The precise physical location of trichome-exudate biochemicals on the plant surface is undoubtedly important in plant–pest interactions, perhaps particularly those involving fungal and bacterial pathogens that invade the plant through the epidermal layer. The chemical stability of exuded compounds is also important in this regard. Here we have studied these two aspects of trichome biology using the highly exuded tobacco line, *Nicotiana tabacum*, T.I. 1068. Particularly under high relative humidity growth conditions, sucrose esters (SE) were found to migrate from the exudate droplet around the gland down the trichome stalk to the epidermal cells below. Six days after labeling leaf midveins on plants grown in a high humidity environment, 29 and 71% of label found in SE were recovered with trichome glands and below gland regions, respectively. Corresponding disposition in the moderate humidity environment was 40 and 60%, respectively. Migration of less polar duvatrienediols (DVT) was less marked. Staining of SE with rhodamine B showed the occurrence of more extensive and physically different migration in the high humidity versus moderate humidity case. Both SE and DVT were stable between six and 18 days postlabeling, the period encompassing the time of maximum exudate formation through the beginning of tissue senescence. Our results suggest that even under conditions that avoid mechanical disturbance of tissue, SE and DVT are chemically stable, at least until senescence, and appear to migrate from the gland region to the epidermal surface, apparently according to their relative polarity.

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Key Words—Trichome, trichome exudate, surface deposition, exudate stability, surface chemicals, sucrose esters, duvatrienes, terpenes, pest resistance, humidity, tobacco.

INTRODUCTION

Glandular trichomes occur on the aerial surfaces of 20–30% of plant species, and most of these exude biochemicals (Dell and McComb, 1978; Duffey, 1986; Fahn, 1988; Kelsey et al., 1984; Wagner, 1991). In numerous cases exuded substances are shown to influence plant–pest and -pollinator interactions (Kelsey et al., 1984; Severson et al., 1985, 1991; Tingey, 1991). Exudate biochemicals are often terpenes, phenolics, and resins and are generally thought to be accumulated and contained within a space between gland cells where they are produced (Keene and Wagner, 1985; Kandra and Wagner, 1988; McCaskill et al., 1992) and an outer cuticular barrier. Accumulated or accumulating substances may move from this containment when the cuticle is mechanically disrupted (by insects, airborne particles, rain, etc.) or possibly through pores in the cuticle (Dell and McComb, 1978; Fahn, 1988). In certain high-exudate-accumulating, arid plants, exudates can form a continuous layer on the plant's surface, and this layer is thought to function in light reflectance and thereby leaf temperature control (Dell and McComb, 1978).

The precise location and composition of exudate below the gland and on epidermal cells of the laminar surface is difficult to determine and is little studied. This aspect is, however, potentially important to the effectiveness of exuded biochemicals in fungal, bacterial, and insect resistance.

Another aspect of trichome-exudate biology that is relatively little studied is that of turnover or recycling within the plant. In what is perhaps the best studied case, Croteau and colleagues showed that in *Mentha*, the trichome-exuded monoterpene 1-menthone is in part reduced, then glycosylated and transported from the gland to the roots where it is degraded (Croteau, 1986). A sesquiterpene of *Marrubiium vulgaris* (presumably formed in trichomes) appears to be degraded shortly after synthesis (Breccia and Badiello, 1967), and radiolabeled sclareol appears to be degraded when supplied to *Salvia sclerea*, and plant that produces this labdane diterpene (Nicholas, 1964). Regarding trichome-produced diterpenes, studies of turnover are few and not conclusive.

A third aspect of trichome biology regards the influence of environmental and agronomic factors on exudate accumulation and disposition. The effects of nitrogen supply, water stress, mechanical disturbance by rain, light quality and intensity, and agronomic factors such as soil type and planting date on exudate content and chemical stability have been studied in tobacco (Severson et al., 1985). Effects of relative humidity and temperature have received less attention.

The diterpenes α- and β-duvatrienediol (DVT), sucrose esters (SE), and labdane diterpenes are principal components of *N. tabacum* trichome exudates (Severson et al., 1985). There is considerable evidence that these components are involved in plant–pest interactions (Severson et al., 1991). Much of this evidence is derived from studies in which exudates were collected, fractionated, and applied to various exudateless or exudate-depleted tobaccos as pure compounds or mixtures (Severson et al., 1985; Wagner, 1991). More recently *in vivo* bioassays have been developed to test such interactions (Menetrez et al., 1990; Kennedy et al., 1992; Cutler et al., 1992). These approaches, while extremely valuable, do not allow manipulation of physical disposition on the leaf surface (i.e., on the gland versus the trichome stalk versus below the stalk) and may suffer from uneven deposition (Menetrez et al., 1990).

Here we have attempted to address these little-studied aspects of trichome biology using the high-exudate-producing tobacco line T.I. 1068. We have studied the disposition of SE and DVT on the undisturbed surface and the possibility of their turnover over the developmental period during which exudate is primarily accumulated. The effects of high humidity and temperature on SE and DVT accumulation and surface disposition and accumulation were investigated in the course of the study.

METHODS AND MATERIALS

Plant Materials and Labeling of Leaf Midveins. Nicotiana tabacum L., tobacco introduction 1068 (T.I. 1068), plants were grown in the greenhouse as previously described (Kandra and Wagner, 1988). About 2-month-old plants were transferred to a growth chamber and grown under continuous light (cool white fluorescent plus incandescent) at 24–26°C. The high humidity treatment [relative humidity (RH) of 80 ± 2%] was maintained using two household humidifiers and the moderate humidity treatment (control RH of 60 ± 2%), consisted of growth under the same conditions but without humidifiers. In the high temperature, moderate RH condition, temperature was 35–35°C and RH was 60 ± 2%. For all experiments, fully expanded leaves 14–18 days after unfolding (No. 5 and 6 counting from the youngest expanded leaf) were used for radiolabeling. Leaves were detached about 10 cm from the point of attachment to the stalk and this portion was discarded. Leaf blade tissue was removed from the remaining portion leaving only midveins attached to the plant stalk. A core (0.15 × 4.3 cm) was removed from the cut end of the midvein section by inserting a thin-wall, sharpened (garnet paper) glass capillary (product number 22897, SMI, Berkeley, California) using a twisting motion (Figure 1A). After blotting the well with a twisted tissue, 150 μl of about 20 × 10^6 dpm of [U-^{14}C] sucrose (530 m Ci/mmol, ICN, Irvine, California) in H_2O was intro-

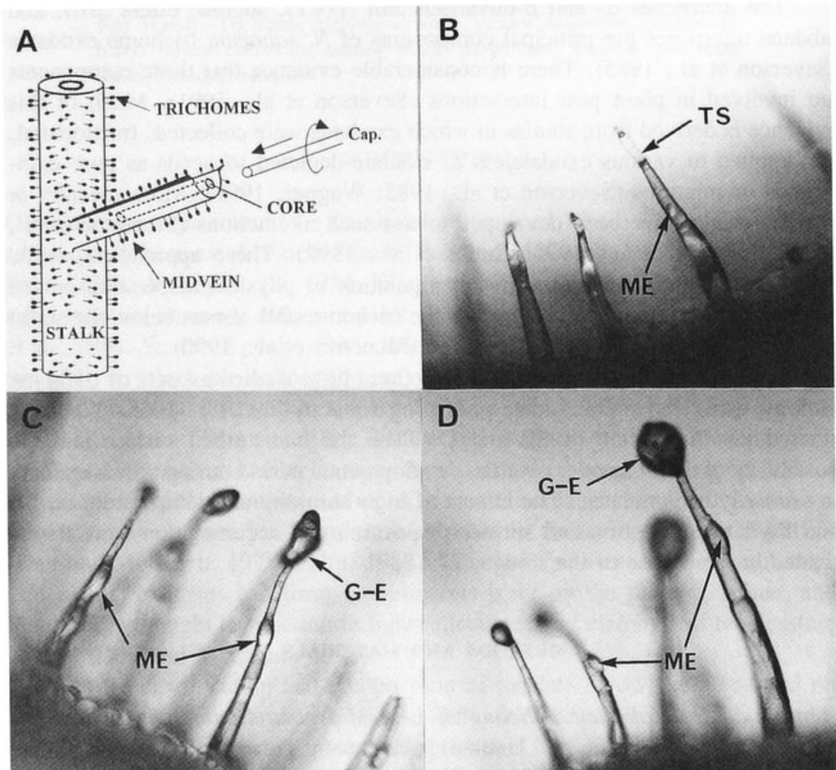

FIG. 1. (A) Diagramatic representation of tissue coring method. (B) Midrib after brushing to remove trichome glands and exudate surrounding them. (C) Undisturbed trichomes from midribs of plants grown in a high RH environment for 28 days. Note the dehydrated appearance of exudate around the glands and substantial exudate droplets migrating down the stalk. (D) Undisturbed trichomes of midribs from plants grown in a moderate RH environment for 28 days. Note the retention of substantial exudate around the gland, but some migration down the trichome stalk. Cap = glass capillary; TS = trichome stalk; G-E = gland with exudate; ME = migrated exudate. Magnification is 146×.

duced. A small piece of a wooden dowel was inserted in the end to prevent tissue dehydration. Six duplications (2 midribs from 3 plants) were used in each treatment.

Collection of Gland Cells and Recovery of Exudate. To follow the synthesis and location of exudate chemicals on the midvein surface with time, 0.6-cm-long pieces of labeled midvein sections were detached using a razor blade at 0- to 50-hr or 2-, 6-, 10-, 14-, 18-day intervals after labeling, unless otherwise

noted. The first terminal segment was discarded. A wooden dowel was reinserted into the core after each section was removed in order to minimize tissue dehydration. During all manipulations, disturbance of surface trichomes was avoided. Glands (head cells of trichomes) were removed by brushing the surface of the section gently using a camel's hair brush (Keene and Wagner, 1985). A watch glass was held under the section during brushing to capture glands that were detached but did not stick to the brush (about 30% of total). The extent of gland removal was monitored at 20× using a dissecting microscope. The brush and the brushed section from which the gland cells were removed were immersed (20 sec) in separate volumes (10 ml) of 100% acetonitrile to dissolve the exudate. The former (plus rinse of watch glass) was regarded as the exudate retained outside the gland cells and the latter as the exudate that had migrated below the trichome gland region of trichomes. These two different locations were verified by light microscopy (see Results) as was completeness of gland removal (Figure 1B). Acetonitrile solutions were evaporated at 35°C and residues were extracted three times with 2 ml $CHCl_3/H_2O$, 2:1 (v/v) to remove H_2O-soluble materials. $CHCl_3$ phases were pooled and evaporated. About 79 ± 10% (N = 124 separate samples) of the radioactivity in acetonitrile extracts was recovered in $CHCl_3$ phases.

Analysis of SEs and DVTs. For HPLC analysis, two different sample types were used: (1) samples collected from one typical midvein section; and (2) pooled samples (pooled $CHCl_3$ phases) from the remaining five duplicate midvein sections. $CHCl_3$-soluble components were resuspended in 200 μl acetonitrile. After addition of unlabeled standard SE and DVT (Kandra and Wagner, 1988), and aliquot (90 μl) was fractionated by HPLC using an Econosil C_{18}, 5-μm column (250 × 4.6 mm Altech Associates, Inc.). Gradient elution was achieved by introducing (at 1.2 ml/min) 100% acetonitrile into 40 ml of stirred 70% acetonitrile in H_2O at room temperature. The column flow (from the 40 ml solvent container) was 1.2 ml/min and detection was at A_{214}. Fractions containing SE and DVT were evaporated and radioactivity was determined. Typically, >90% (92 ± 10%, N = 29) of injected ^{14}C was recovered in collected fractions.

Histochemical Staining of SE. Tissue pieces were submerged in 0.2% rhodamine B in H_2O for 60 min, then submerged in four separate vessels containing distilled H_2O (5 sec in each) to remove unbound stain. Samples were photographed using Kodak TMAX-100 film.

RESULTS AND DISCUSSION

The use of a cored, leaf-midvein radiolabeling system allowed us to monitor synthesis, stability, and surface location of tobacco trichome exudate chemicals over an 18-day interval. Typically, 18 days after labeling, ^{14}C in acetonitrile-

solubilized, chloroform-extractable surface (exudate) components of cored midveins (all sections combined) was about 0.4–0.7% of label introduced into the midrib core (or 0.07–0.12% per midvein section) on day 0 (14-day-old leaves) (Table 1). DVT (combined α and β epimers) and SE accounted for about 50% of exudate, $CHCl_3$-extractable counts. Similar total conversion was observed in moderate and high RH and moderate RH high temperature treatments. An advantage of the cored midvein system is that it allows analysis over the period of maximum exudate synthesis (under growth conditions used here about 14–28 days after leaf unfolding) to leaf senescence (about 34 days after leaf unfolding). Another advantage of this system, as compared to labeling of detached midvein epidermal peels or detached glands (Kandra and Wagner, 1988; Keene and Wagner, 1985), is that the tissue remains attached to the plant for the entire period of the experiment. Cored and undisturbed midveins senesced (yellowed) at the same rate (observation). Conversion of [^{14}C]sucrose to DVT and SE was, however, less efficient in cored midveins (0.63–0.67% for high and moderate RH, respectively) as compared with about the same surface area of detached epidermal peels (Kandra and Wagner, 1988; Keene and Wagner, 1985) by a factor of 4–17. This difference is presumably due to the utilization of more label by nontrichome tissue in the cored midvein system. In the case of high temperature and moderate RH, percent incorporation was similar to that of other treatments, and the DVT/SE ratio was higher (1.4; Table 1). Epidermal peels, when radiolabeled at high temperature (29°C), also produce exudate having a high DVT/SE ratio (2.4) (Kandra and Wagner, 1988).

TABLE 1. EFFECTS OF RELATIVE HUMIDITY (RH) AND TEMPERATURE ON CONVERSION OF [^{14}C] SUCROSE TO $CHCl_3$-SOLUBLE DVT AND SE COMPONENTS OF TRICHOME EXUDATE.

	Incorporation (%)			
Treatment	$CHCl_3$-soluble	DVT	SE	DVT/SE
High RH				
Single petiole[a]	0.51	0.11	0.12	0.92
Pooled data (5 petioles)[b]	0.63	0.16	0.18	0.89
Moderate RH				
Single petiole[a]	0.43	0.11	0.12	0.92
Pooled data (5 petioles)[b]	0.67	0.15	0.18	0.83
High temperature				
Single petiole[c]	0.46	0.14	0.10	1.40

[a]Data represent means from four pieces (6-, 10-, 14-, 18-day samples) from a single petiole.
[b]Data represent means from 20 pieces (6-, 10-, 14-, 18-day samples) from five petioles.
[c]Means from two pieces (six-day samples) from two petioles.

The time course of labeling midveins of plants growing in a moderate RH environment was examined first over a period of 50 hr. Labeling of total $CHCl_3$-phase exudate components, and DVT and SE fractionated from it was linear over 50 hr. This indicates reliability in comparing different pieces of midvein sections detached at various times (Figure 2). Results are for a typical experiment. While the magnitude of labeling varied in separate experiments (midveins), trends in labeling of $CHCl_3$-soluble, DVT and SE products versus time were similar. DVT and SE together accounted for about 50% of $CHCl_3$-extractable label after about 20 hr, and the ratio of DVT/SE label was about 1. Of the chloroform-soluble label not separated as SE or DVT by HPLC, about 23% eluted with several components between DVT and *cis*-abienol (not shown). This region is expected to contain at least labdenoid diterpenes, which are known to occur in the exudate of T.I. 1068 (Severson et al., 1985). No attempt was made to further characterize these labeled components. While *cis*-abienol is a major diterpene component of T.I. 1068 exudate (Severson et al., 1985), it was not

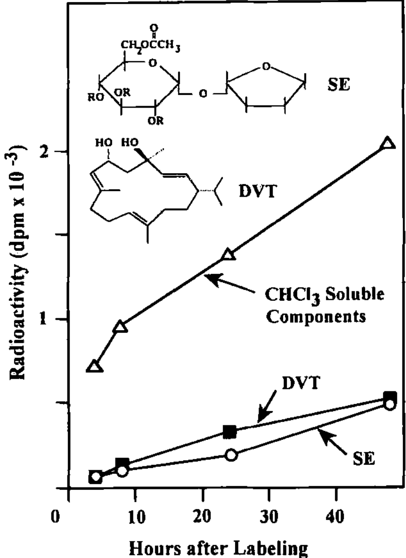

FIG. 2. Time course of labeling trichome exudate components of midveins (moderate RH environment). Principal biochemicals of *N. tabacum* cv. T.I. 1068 are: sucrose esters (6-*O*-acetyl 2,3,4-tri-*O*-acyl-α-D-glucopyranosyl-β-D-fructofuranosides), acyl or R groups are C_3–C_8 branched or unbranched acids; and α- and β-duvatriene diols (DVT) (1*S*,2*E*,4*R*,6*R*,7*E*,11*E*)- and (1*S*,2*E*,4*S*,6*R*,7*E*,11*E*)-2,7,11-cembratriene-4,6-diols, respectively. The α or 4*S* epimer is shown.

significantly labeled from sucrose in these experiments. Rates of DVT and SE synthesis over 50 hr were similar.

To determine if biosynthesized DVT and SE were turned over after synthesis, midveins were monitored for 18 days after pulse labeling with [^{14}C]sucrose (Figure 3 represents a typical experiment). This experiment was performed under conditions of moderate and high RH and moderate RH–high temperature. Results obtained with the last condition are not shown because they were similar at six days to the moderate RH case and plants grew very rapidly at high temperature. In all treatments, labeling of DVT and SE reached a maximum after six days and was stable thereafter, indicating that little or no significant net turnover of accumulated exudate components had occurred, at least until onset of senescence. Label in total $CHCl_3$-phase fractions was also stable after six days (not shown). We expect that DVT triols (oxidation products of DVT; Severson et al., 1985) would have been detected by HPLC if present. Minor modification of SE (loss of certain acyl or acetyl constituents) might have escaped detection by HPLC, but SE appear to be stable even during leaf senescence and curing (Severson et al., 1985). Thus, DVT and SE formed in T.I. 1068 trichome glands

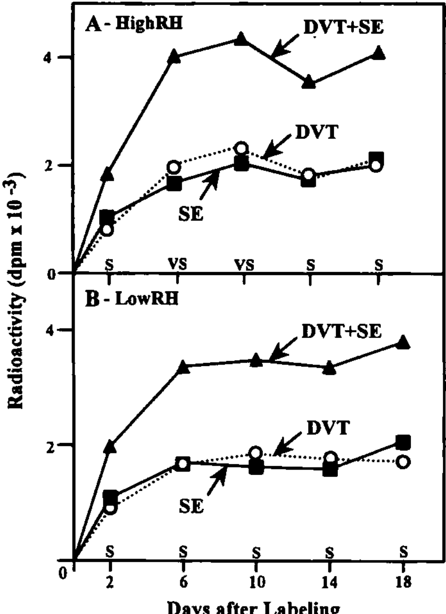

FIG. 3. Time course of labeling midveins in high and moderate RH environments with [^{14}C]sucrose. Leaves were 14 days old when labeled. (A) High RH; (B) Moderate RH. The labels (S = sticky, VS = very sticky) relate to tackiness of the midrib surface.

appear to be stable until senescence. We observed a small decrease in DVT and SE levels in the moderate RH experiments between 18 and 22 days after labeling (data not shown). However, 22-day sample data were somewhat variable. Further study is needed to determine the stability of DVT and SE after the onset of senescence in the system used here. Severson et al. (1985) reported an 8–24% reduction in DVTs from leaves of ripe (senescing) versus young field-grown tobacco. This may reflect changes after the onset of senescence or effects due to mechanical disruption of trichome accumulations that occur in the field. Flower development is known to result in decreased plant DVT levels and topping prevents this effect.

Leaves of plants grown in the high RH condition were found to be considerably more sticky to the touch (due to accumulated trichome exudate) than those in the moderate RH treatment, suggesting accumulation of more exudate in the former treatment. Therefore, total accumulated exudate was quantitatively assessed gravimetrically from leaf pieces (18 days after labeling equivalent) and was found to be 0.70 ± 0.12 $\mu g/cm^2$ and 0.37 ± 0.08 mg/cm^2 on high and moderate RH plants, respectively. Although substantially more exudate was produced in the high RH environment, both the time course and DVT/SE ratios were unaffected by RH (Table 1, Figure 3). We conclude that this is because maximal labeling in these pulse experiments was achieved six days after labeling and the bulk of exudate was observed to be produced thereafter. This was evidenced by microscopic observation and increased stickiness, the requirement for larger volumes of $CHCl_3$ in partitioning, and increased radiolabeling in older petioles (date not shown).

The physical location of exudate is very relevant to the question of the role of these compounds in resistance to microbial and insect damage. In previous work, we established that the trichome gland of T.I. 1068 is the only site of exudate DVT, SE, and *cis*-abienol synthesis in tobacco (Kandra and Wagner, 1988; Keene and Wagner, 1985; Guo et al., 1994). Here we assessed the physical location of exudate compounds over the 18-day period of maximal exudate accumulation under high RH, moderate RH, and moderate RH–high temperature conditions. Our hypothesis was that high RH would enhance movement of exudate (particularly more polar constituents such as SE) past the cuticular boundary to outside the trichome gland, down the stalk, and down to leaf epidermal cells adjacent to trichomes where microbes are most likely to invade the leaf. Observations of midvein pieces (light microscope) during collection of glands (with exudate) and exudate below glands indicated that exudate had migrated from the gland region, particularly in the high RH case six days after coring and labeling (Figure 1C and D). After 14 days, migrated exudate appeared to be gradually "dehydrated" (Figure 1C) on high RH plants. In the moderate RH and moderate RH–high temperature treatments, the exudate droplet appeared to be, in greater part, retained around the gland throughout the time course.

Some small drops of exudate were observed below the gland region (Figure 1D) and evidence of "dehydration" was found only at about 18 days. Presumably in this environment, exudate was mostly retained by the cuticle surrounding the gland. It was also observed that exudate was stickiest between six and 10 days in the high RH treatment (noted as VS for very sticky in Figure 3). Reduction in stickiness coincided with the "dehydrated" appearance at 14 and 18 days.

We observed that significantly more [^{14}C]SE occurred with the isolated gland fraction (removed by brushing) in the moderate RH treatment (62 ± 10%, mean of 2–18 days) than in the high RH treatment (37 ± 5%) (Figure 4). This suggests movement (or greater movement) of SE away from the gland region in the high RH environment. Less movement of [^{14}C]DVT versus [^{14}C]SE was apparent in both treatments. Results obtained for high temperature–moderate RH treatments were similar to those of the moderate RH experiment shown in Figure 4B and D (see legend of Figure 4). We anticipate that the brushing method for removing glands with exudate overestimate the amount of DVT and SE associated with glands because brushing may also remove some exudate that had migrated to the stalk. This is suggested by the appearance of migrated exudate on stalks of undisturbed tissue (Figure 1C) versus that on stalks after removal of glands (Figure 1B). It is also suggested by the large amount of SE

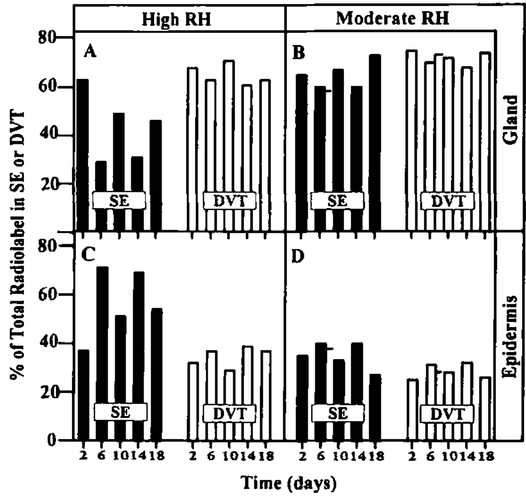

FIG. 4. Physical location of [^{14}C]SE and [^{14}C]DVT on the midvein surface versus time after radiolabeling. (A and B) Constituents recovered with glands; (C and D) those remaining on the epidermis after removal of glands; (A and C) high RH, and (B and D) moderate RH experiments. Horizontal bars on six-day data, in B and D represent results from the high temperature–moderate RH experiments.

found on the epidermal surface below the stalk by histochemical staining (see below). Nevertheless, the brushing procedure indicates migration of exudate, increased migration in a high humidity environment, and greater migration of SE than DVT.

A histochemical staining procedure for SE was devised to verify results described above and to determine if migrated exudate reached the epidermal surface below the trichome stalk. This procedure was specific for SE (DVT and labdanoids not stained) and can be used as a quantitative measure of SE. Details regarding this method are presented elsewhere (Lin and Wagner, in press). On leaves, particularly in the high RH case, stained SE were found to migrate onto the epidermal surface radially from the base of the stalk (Figure 5A). Migration clearly occurred along cell–cell junctures. Less SE appeared to be retained around glands (Figure 5B). The later observation was consistent with that seen with unstained tissue (Figure 1C). In the moderate RH case, migration was less extensive (Figure 5C) and SE was observed to be retained to a greater extent around the gland (Figure 5D). Results are again consistent with that seen for exudate with unstained tissue (Figure 1D). Similarly, on leaf midveins, SE migration onto the epidermis was more extensive in the high RH treatment (not shown).

Staining with 0.2% rhodamine B for 10 min gave similar results in terms of the migration to the epidermis, but staining intensity was lower. Figure 6 shows a red aphid (*Myzus nicotinae*) contaminated with SE. Aphids were placed on rhodamine B-stained leaf segments (high RH) on glass slides and allowed to walk on the surface for 15 min. Slides were then placed in a chloroform atmosphere to anesthetize insects, then mounted for photography. It was observed that antennae and legs were extensively contaminated with SE while the abdomen and stylus generally were not.

Results from histochemical staining, together with direct measurements presented above and light microscope observations of unstained tissue, suggest that RH—particularly, but not exclusively—influences the downward movement of (particularly) more polar SE, perhaps through pores in the cutile, down the trichome stalk to the epidermal surface. Physical disturbance of the gland cuticle by insects, dust, etc., in the natural environment (absent in our experiments) would undoubtedly increase such movement. Heavy rainfall has been shown to substantially decrease exudate levels in the field (Severson et al., 1985). Loss of components was not found to be related to their polarity. The effects of humidity and light rainfall on exudate movement and loss in the field have apparently not been investigated.

Our observations regarding the apparent lack of DVT or SE turnover and their physical disposition on the leaf surface are relevant to plant–pest interactions in several ways. First, the amount and composition of trichome exudate reaching the leaf surface below the gland is undoubtedly important to exudate

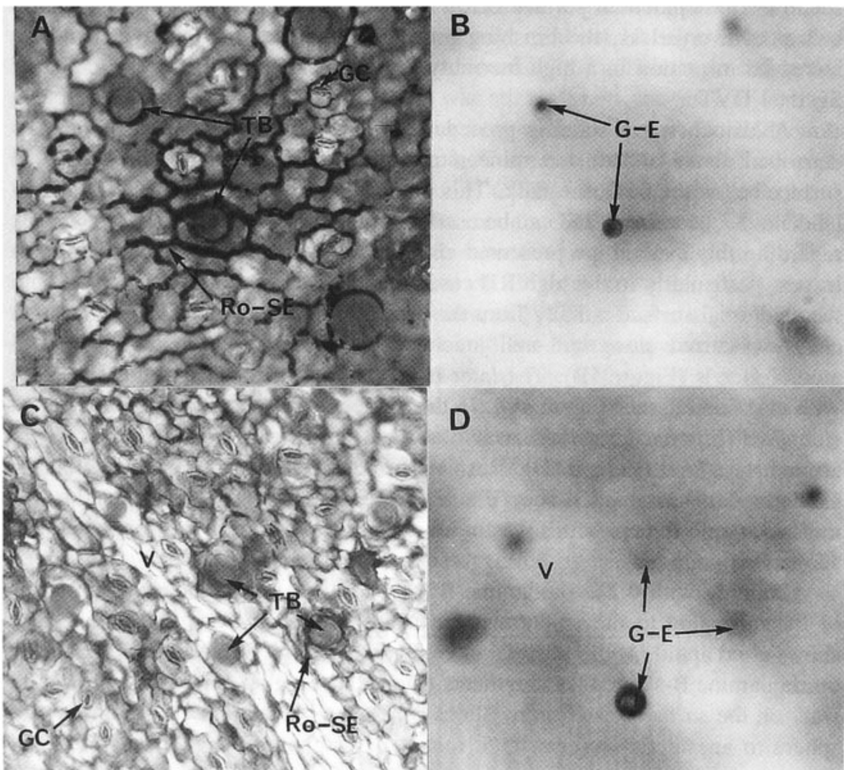

FIG. 5. Migration of SE to the leaf epidermal surface as observed by histochemical staining. (A) Epidermal surface–high RH treatment. (B) Focal plane of trichome gland tips above epidermis in A. (C) Epidermal surface—moderate RH treatment. (D) Plane of trichome gland tips above epidermis in C. TB = trichome base; Ro-SE = rhodamine SE; G-E = gland with exudate; GC = guard cell; V = vein. Magnification is 146×. Alignment of TB with G-E for trichomes in A–B and C–D pairs are imperfect in some cases because trichomes are not always perpendicular to the surface.

chemical effectiveness as natural antimicrobial and antifungal agents. Pathogenic bacteria and fungi generally invade plant tissue by penetration into leaf epidermal cells or through stomates. Second, the matrix (Severson, personal communication; Kennedy et al., 1992) in which antipest chemicals occur at various locations on the surface and their hydration state may be important to their ability to penetrate into and deter chewing and nonchewing insects and microbes. Insects, as they walk on tissue surfaces, may acquire less exudate if it is less sticky once outside the cuticular boundary of the gland. If different chemicals

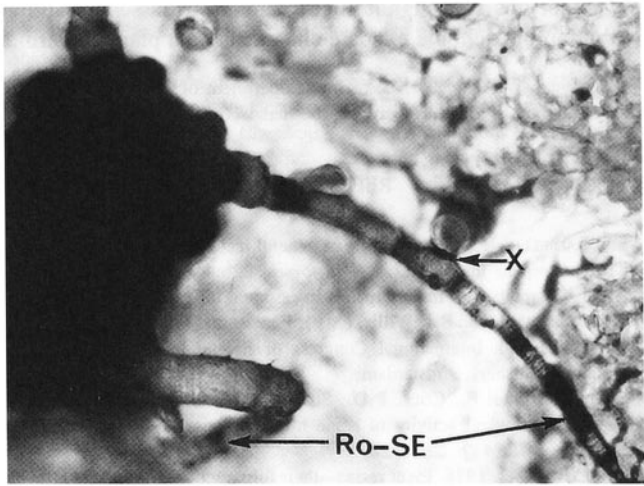

FIG. 6. Red aphid contaminated with SE. Ro-SE = rhodamine-SE. X = antenna stuck to exudated trichome gland. Magnification is 36×.

are differently disposed on the surface (as observed here for SE), they may be more or less abiotic because they are in a different matrix than when contained in the gland droplet. Certain exudate chemicals (i.e., *cis*-abienol), when placed on aphids in pure form, crystallize upon evaporation of solvent and remain outside the insect body, but this is not thought to occur when they are within a matrix with other exudate components (R. Severson, personal communication). The point at which chemicals contact insects (i.e., legs, abdomen) also may affect their toxicity.

Numerous studies have monitored plant-pest interactive properties of surface chemicals by spraying isolated, purified chemicals or mixtures on leaves from which endogenous chemicals had been removed and then applying them to insects (Severson et al., 1991; see Wagner, 1991). Such studies have been very valuable in assessing the pest-interactive properties of a number of natural, antibiotic chemicals. It is, however, difficult to compare quantitatively the effects observed with this method to the in situ case because of the matrix, surface disposition, etc., aspects discussed above. Uneven distribution after spraying may impact the results of such experiments (see Menetrez et al., 1990).

The three different approaches (direct separation of gland exudate from stalk-epidermis-associated exudate, microscopic observation of undisturbed tissue, and histochemical staining) used in this study lead to the same conclusions regarding the surface disposition of SE, a major trichome exudate components.

To our knowledge, this study is the first to attempt to assess quantitatively the precise distribution of trichome exudate chemicals on a plant epidermal surface.

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PRESENCE OF A HYDROXAMIC ACID GLUCOSIDE IN WHEAT PHLOEM SAP, AND ITS CONSEQUENCES FOR PERFORMANCE OF *Rhopalosiphum padi* (L.) (HOMOPTERA: APHIDIDAE)

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Abstract—Phloem sap of wheat seedlings differing in whole leaf hydroxamic acid (Hx) concentrations was collected by cutting stylets of feeding aphids. DIMBOA-glucoside was the only Hx-related product found. Concentration of DIMBOA-glucoside in phloem sap showed a tendency to be negatively correlated with aphid performance.

Key Words—Hydroxamic acids, phloem sap analysis, aphid resistance, cereals, wheat, *Rhopalosiphum padi*, Homoptera, Aphididae.

INTRODUCTION

Several investigations have pointed out the importance of hydroxamic acids (Hx) in the resistance of wheat and other cereals against aphids (Niemeyer, 1988). These compounds are present in the intact plant as glucosides, which are hydrolyzed by *endo*-β-glucosidases when the tissue is injured (Hofman and Hofmanova, 1969). The main aglucone in wheat extracts is DIMBOA (Figure 1).

Hx affect aphid feeding behavior and performance through their antibiotic and antixenotic properties (Givovich and Niemeyer, 1991; Niemeyer, 1991).

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DIMBOA-glucoside —enzyme→ DIMBOA —ΔT→ MBOA

FIG. 1. Biochemical and chemical transformations of hydroxamic acid glucosides present in wheat extracts.

The exact role of Hx in host plant discrimination by aphids is still unknown. Important issues are the localization of Hx in the plant and the stages in the assessment of the plant during which an aphid encounters them. Hx have been found both in the mesophyll and in the vascular bundles of wheat (Argandoña et al., 1987). Moreover, honeydew from aphids feeding on wheat contained Hx-glucosides (Leszczynski and Dixon, 1990; Givovich et al., 1992), suggesting the presence of these compounds in the plant sieve elements.

Here, we present the results of collection and analysis of wheat phloem sap of seedlings of wheat cultivars known to differ in total Hx concentrations and data on the performance of the aphid *Rhopalosiphum padi* (L.) reared on seedlings of these cultivars.

METHODS AND MATERIALS

Insect Material. Individuals of *R. padi* were collected in wheat fields near Santiago and reared on oat seedlings in a growth chamber at 19–25°C, and 16L:8D photoperiod.

Plant Material. Wheat seeds were obtained from Instituto de Investigaciones Agropecuarias (INIA). Three wheat cultivars (*Triticum aestivum* L. cvs. Millaleu, Nobo, and Maitén) were chosen on the basis of their Hx concentrations at the seedling stage (Givovich and Niemeyer, 1991). Experiments were carried out with seedlings in the one-leaf stage (decimal growth stage 10) (Zadoks et al., 1974), which contain the highest concentrations of Hx (Argandoña et al. 1980). Oat seedlings (*Avena sativa L.*, cv. Nahuen) lack Hx and were used as the control. Hx concentrations in plants was determined by HPLC according to Niemeyer et al. (1989a).

Phloem Analysis. Phloem sap collection was carried out by aphid stylet microcautery (Unwin, 1978). Six-day-old wheat seedlings grown under similar conditions in a plant growth chamber at 25°C were each infested with 5–10 adult aphids of similar weights. Aphids were allowed to remain on the plants for *ca.* 12 hr. The aphid whose stylet was to be cut was chosen among those feeding in a given position on the abaxial leaf surface (2 cm from the top of

the leaf, and within 2 mm of the central vein). Honeydew production was taken as an indication that the aphid chosen was indeed feeding. Phloem sap was collected for 3 hr following stylet excision. Only one sap collection was made per day, starting almost at the same time of the day. Sap was collected with a 0.5-μl micropipet in an environment of 20–25°C and 95% relative humidity. After collection, the micropipet was rinsed with 30 μl of distilled water. Hx concentrations in these solutions were determined by injecting them directly into a high-performance liquid chromatograph and analyzing them under conditions similar to those described by Niemeyer et al. (1989a).

The presence of DIMBOA-glucoside in the phloem sap of wheat cultivars was determined by comparison of its chromatogram with that of an authentic sample obtained from maize (*Zea mays* L. cv. Tracy T129) (Lyons et al., 1988). Confirmation of the identity of the compound was obtained by: (1) subjecting the sample to enzymatic glucoside hydrolysis by cell-free wheat extracts and detecting DIMBOA in the product, and (2) subjecting the product of enzymatic hydrolysis to heating under basic conditions (pH 9, 80°C, 1 hr) (Bravo and Niemeyer, 1986) and detecting MBOA—the main decomposition product of DIMBOA—in the mixture (Figure 1). Retention times for DIMBOA-glucoside, DIMBOA, and MBOA were 2.43, 3.47, and 6.17 min, respectively (Figure 2). Cell-free extracts of wheat seedlings were prepared by macerating leaf tissue in

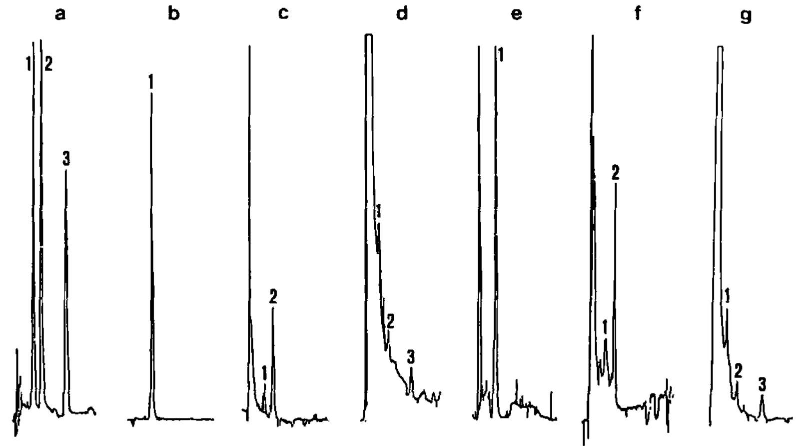

FIG. 2. Confirmation of the presence of DIMBOA-glucoside in the phloem sap of a wheat seedling: (a) standards of DIMBOA-glucoside (**1**), DIMBOA (**2**), and MBOA (**3**); (b) DIMBOA-glucoside standard; (c) product of the enzymatic transformation of (b); (d) product of the decomposition of (c); (e) phloem sap sample; (f) enzymatic transformation of (e); (g) decomposition of (f).

aqueous buffered solution, centrifuging at 20,000*g*, and filtering the resulting suspension through Sephadex G-25 M (Cuevas et al., 1992).

Determination of Aphid Performance. Mean relative growth rate (MRGR), a parameter highly correlated with the intrinsic rate of increase (r_m; Leather and Dixon, 1984), was determined (Adams and van Emdem, 1972) in order to evaluate aphid performance on the seedlings studied. First- or second-instar nymphs were weighed, and those with very similar weights were caged individually onto the abaxial leaf surface of a test plant and removed for weighing 96 hr later. Twenty replicated measurements were made for each wheat cultivar. MRGRs determined in different wheat cultivars were compared using ANOVA/ Duncan's test.

RESULTS AND DISCUSSION

If a plant secondary metabolite is to be important for plant resistance to aphids through antibiosis, it must be present in the sieve elements, since that is the tissue fed on by aphids. Hence, the presence of Hx in the sieve elements is of importance for establishing Hx as the causative factor of the negative correlations described between aphid performance and Hx content of cereals (Niemeyer, 1991). DIMBOA-glucoside had previously been found in the honeydew of aphids feeding on Hx-containing wheat seedlings (Leszczynski and Dixon, 1990; Givovich et al., 1992), suggesting its ingestion from sieve elements. The present study is the first unambiguous demonstration that Hx are present in the phloem sap of wheat seedlings. Our results show the presence of DIMBOA-glucoside as the only hydroxamic acid or related compound detected in the phloem sap of wheat seedlings (Figure 2). Other compounds derived from DIMBOA-glucoside were found earlier in whole aphids (Niemeyer et al., 1989b) and in aphid honeydew (Leszczynski and Dixon, 1990). It is likely that the presence of these compounds was the result of metabolization within the aphid (Leszczynski and Dixon, 1992; Leszczynski et al., 1992) or of chemical transformation of the honeydew samples (Bravo and Niemeyer, 1986).

Hx have been found in high concentrations in meristematic tissue (Epstein et al., 1986). Their translocation from undifferentiated tissues to differentiated ones has been proposed to occur via the apoplastic fluid (Zúñiga and Massardo, 1991). The present results suggest the phloem as an alternative translocation route.

Individual determinations of the concentration of DIMBOA-glucoside in the phloem were not correlated with Hx concentrations in the whole leaf extracts (Figure 3). However, mean Hx content of whole leaf extracts showed very large and clear differences between wheat cultivars (Figure 4), and DIMBOA-glucoside concentrations in the phloem sap showed no significant differences among

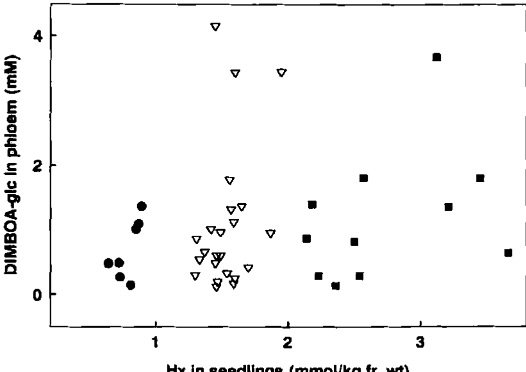

FIG. 3. Concentration of hydroxamic acids in whole leaf and of DIMBOA-glucoside in the phloem sap of wheat seedlings of equivalent developmental stages. Phloem sap was collected through excised aphid stylets. Wheat cultivars used were: Millaleu (●), Nobo (∇) and Maitén (■).

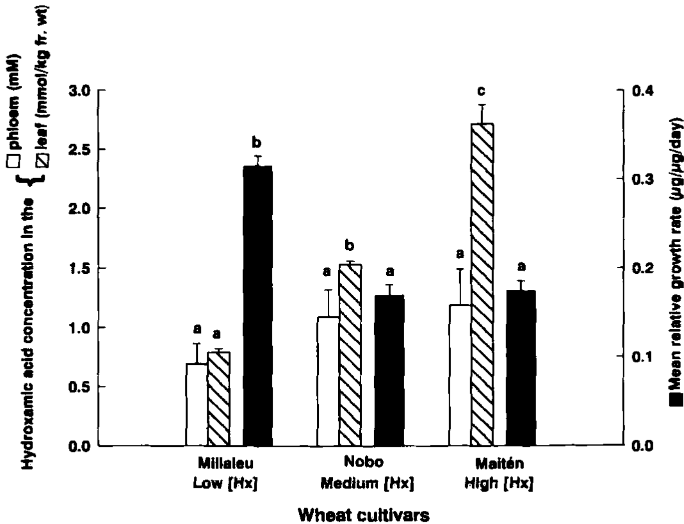

FIG. 4. Mean relative growth rates of first instars of the aphid *Rhopalosiphum padi* reared in seedlings of three cultivars of wheat (■), and concentration of DIMBOA-glucoside in the phloem sap (□) and Hx concentration in the aerial part (▨) of the seedlings.

cultivars (Figure 4). This could indicate that the turnover of Hx outside the phloem differs between wheat cultivars. It is noteworthy that in each of the three cultivars studied, the variability of the glucoside concentration in the phloem sap is considerably larger than in the whole leaf extracts. High variations in concentrations of other compounds, such as amino acids and sugars, have been reported in phloem collected from severed aphid stylets (Girousee et al., 1991). The variation may be explained by the fact that aphids feed from a single sieve tube and concentrations may differ between sieve tubes, possibly reflecting the biosynthetic activity of the area where the sieve tube is loaded. This variation could also be a result of unknown differences in the physiological condition of the plants or the presence of gradients of Hx along the phloem vessels. Our results thus indicate that aphids feeding on wheat seedlings are exposed to considerable differences in the concentration of DIMBOA glucoside in the phloem sap.

Aphids feeding on the wheat cultivars studied showed significant differences in mean relative growth rates, which tended to be negatively correlated to the mean concentration of DIMBOA glucoside in the phloem sap and also to the mean Hx concentration in plant extracts (Figure 4). Interestingly, however, the duration of committed phloem ingestion from sieve elements in the wheat cultivars chosen is independent of the Hx concentration in the aerial parts of a seedling (Givovich and Niemeyer, 1991). This may be interpreted as a combination of several factors: (1) nutrients, as well as other secondary metabolites in the sieve elements, may be masking the known feeding deterrent effect of Hx; (2) the concentrations of Hx in the sap samples may be below the threshold necessary to elicit feeding deterrency in an aphid; or, in view of the present results, (3) aphids may select sieve elements with low concentrations of Hx in plants with relatively high whole leaf Hx concentrations. Figure 4 shows that in spite of an almost 100% increase in whole leaf Hx concentration in going from the cultivar Nobo to Maitén, mean phloem Hx level remains unchanged and, interestingly, mean relative growth rate does not change either. This may be interpreted as the aphid needing to make a selection of sieve tube when it encounters high concentrations of Hx in the plant.

Hx fulfill a double role in host plant discrimination by aphids. At the mesophyll, they act as antixenotics (feeding deterrents), which lead to the selection of plants with lower content of Hx by wingless aphids (Givovich and Niemeyer, 1991) and also by winged ones (Nicol et al., 1992). At the sieve elements, they may act as antixenotics as suggested above and also as antibiotics, which decrease aphid performance, measured either as mean relative growth rate (Thackray et al., 1990a), survival (Argandoña et al., 1980), reproductive rate (Corcuera et al., 1982), or intrinsic rate of increase (Bohidar et al., 1986; Thackray et al., 1990b). Hence, Hx constitute a double barrier towards aphids. They display, within the same molecule, the characteristics of a behavioral pest

control agent and a physiological pest control agent, thus providing for the possibility of efficient and stable resistance (Rice, 1993). Breeding wheat cultivars with high Hx concentrations appears to be a desirable component of integrated pest management of aphids (Escobar and Niemeyer, 1993).

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MOLECULAR BASIS OF *Morinda citrifolia* (L.): Toxicity on Drosophila

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Abstract—The ripe fruit of *Morinda citrifolia*, host plant for *Drosophila sechellia* is highly toxic for three closely related species (*D. melanogaster*, *D. simulans*, and *D. mauritiana*). Green and rotten fruits are not toxic for all species tested. Short fatty acids were found to be present in large quantities in the extract of the ripe fruit. The most abundant (octanoic acid) was tested pure for its toxicity in a dose-dependent manner; *D. sechellia* is five to six times more resistant than *D. melanogaster* to octanoic acid. Octanoic acid alone seems to be sufficient to explain the toxic effect of the pulp. It is less abundant in the rotten fruit and absent in the green fruit.

Key Words—Toxicity, *Morinda citrifolia*, fruit, octanoic acid, *Drosophila melanogaster*, *Drosophila sechellia*, Diptera, Drosophilidae.

INTRODUCTION

Classically, five chemical classes of insecticides are distinguished; some are inhibitors of acetylcholine esterase (organophosphorus, carbamates), and others act on sodium channels and disturb nervous transmission (organochlorides, cyclodienes and pyrethroids) (Narahashi, 1976). Many species of invertebrates have developed resistance to different insecticides, especially DDT (Georghiou, 1986; Poirié and Pasteur, 1991). Thus many investigations were performed recently to look for new types of insecticides, especially from plants.

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Morinda citrifolia (Rubiaceae) is a shrub distributed all around the Indian Ocean, including the Seychelles archipelago where it is common near shores. These plants were introduced in islands such as Australia, Hawaii, Tahiti, Moorea, and even in the West Indies probably by Asian people who used components of this plant for traditional medicine and for dyeing (Balakrishna et al., 1961; Caiment Leblond, 1957; Levand and Larson, 1976).

Fruits of *Morinda citrifolia* seem to be the sole feeding resource of *Drosophila sechellia* larvae (Tsacas and Bächli, 1981), but this species gathers the only flies that can live on this fruit when it is ripe, which is year round in the Seychelles, while other species of the same subgroup, such as *D. melanogaster* or *D. simulans*, also now present in the Seychelles, are killed by the ripe fruit but may survive on the rotten fruit (Louis and David, 1986).

Using the pulp of this fruit, Legal et al. (1992) designed a simple bioassay for quantitative measurements of toxicity. Flies first show a strong excitation, reduce their locomotor activity, then start losing equilibrium while wings strongly vibrate, and finally die. This sequence of events is different from that described for pyrethroids (Beeman, 1982) and organochloride insecticides (Chang and Plapp, 1983).

Preliminary tests with *D. melanogaster* using a physical barrier allowing only the passage of volatile components, showed that mortality occurred after a much longer time (24 hr) with equal amounts of *Morinda* fruit pulp than if physical contact was possible (40 min); this strongly suggested that toxic products might have a moderate volatility (Legal, 1988).

This study deals with the characterization of the molecular basis of *Morinda* fruit toxicity. As only the ripe fruit is toxic, extraction and fractionation of toxic components were performed with this physiological state of the fruit in parallel with green and rotten fruits, which are not toxic. Fractions were then bioassayed and submitted to chemical analysis.

METHODS AND MATERIALS

Flies. Outbred strains of two different *Drosophila* species were used for this study: an old laboratory strain, Canton S. of *D. melanogaster* (meCS) and a *D. sechellia* strain derived from a number of females collected in 1981 in the Seychelles archipelago (se). meCS flies were bred on standard cornmeal medium and se on the same medium plus a small amount of axenic plus alcohol to increase viability, at 25°C, with a 12L:12D photoperiod.

Fruits. Morinda fruits were collected in Guadaloupe (French Caraibs); they were not different from fruits collected in other locations and were tested for toxicity. Three stages of this fruit were distinguished according to the following criteria: (1) green fruit—marked green color, hard consistency, thick peel, (2)

ripe fruit—yellow color, soft consistency, thin peel, and (3) rotten fruit—dark brown color, still harder consistency, thick peel.

Toxicity Bioassay. According to the method described by Legal et al. (1992), *Drosophila* were tested in groups of 10 of either sex, and each test was repeated at least five times. Flies were introduced into Petri dishes (air volume of 8 ml) on the bottom of which a given amount of either pulp, dried extracts, dried fractions, or synthetic products were placed. Mortality was determined when fly wings were vertical. Values of either LD_{50} [dose at which 50% of flies died during a given time (0–120 min)] and T_{50} [time at which 50% of flies died for a given dose (0–500 μg)] were calculated and submitted to statistical analysis (ANOVA with Statview software).

All commercial chemicals were from Merck and solvents from Prolabo.

Extraction. Fifty grams of fruit of each stage were dipped into 150 ml of acetone for 18 hr; the extract was then filtered, dried, and weighed. Such extracts were tested after being resuspended in acetone [1]. After an hour of decantation in a mixture of 1/3 acetone + 1/3 water + 1/3 hexane, the two phases obtained were separated, dried, and weighed. For further uses, they were redissolved in acetone [2] or hexane [3] to get a concentration of 1 mg/ml.

Gas Chromatography (GC) Analysis. A Carlo Erba GC6000 Vega (ICU 600) equipped with a column injector and a polar column Carbowax CP58 (25 m, 0.25 mm diameter) was used, with a temperature gradient of 15°C/min from 40°C to 230°C and isothermal at 230°C for 30 min. GC chromatograms were analyzed on a PC computer using CHROMA 3.0 (S.P.I.R.A.L. software).

Identification of Products. A similar gas chromatograph with the same column was coupled with a mass spectrometer (Kratos MS 80) using either electronic impact or chemical ionization (methane or ammonia). Experimental conditions were identical. To confirm identifications, commercial products were injected into the GC-MS and retention times and fragmentation patterns were compared.

Quantification of Products. An internal reference, dodecanoic acid, was used. A quantity of this product corresponding to 20% of the dry weight of the extract was added to get a final solution of 1 mg/ml concentration. Areas of chromatographic peaks were measured and compared.

RESULTS

Determination of Extraction Procedure. The first step was to determine which solvent led to the best extraction of toxic materials for *D. melanogaster*. Dichloromethane, hexane, methanol, water, and acetone were tested in parallel using the bioassay described by Legal et al. (1992). Acetone was the most efficient, and an extract in a mixture of acetone and water from the fruit was obtained. Methanol and hexane were much less efficient.

Although several methods of extraction, purification, and fragmentation were tried, only one produced an extract containing most of the fruit toxicity with a relatively simple gas chromatogram.

After bathing a fruit in acetone for 18 hr, most of the toxicity was recovered in the extract, while no toxicity was found in the remaining parts of the fruit. At this stage, the chromatographic analysis still suggested a complex mixture. A second step of purification was added: the dry residue from the first step was mixed with a solvent mixture consisting of 1/3 acetone, 1/3 water, and 1/3 hexane; after a 1 hr decantation, two phases could be separated, and the toxicity of each phase was tested. The hexane phase—and only this phase—retained most of the toxicity of the fruit. After each step the different extracts and/or fractions were tested using the toxicity bioassay. After the first extraction, an average of 6.6 g extract/100 g of fresh fruit was obtained; after the second step with three solvents, on average 114 mg residue/100 g of fresh fruit was recovered in the hexane fraction. As toxicity tests with pulp had been performed with 1.5 g, toxicity comparisons were performed with 99 mg of the first type of extract and 1.7 mg of the second type of extract.

Mortality kinetics were compared for the various steps of fractionation and 50 *D. melanogaster* meCS males (Figure 1). Various fractions resulting from the successive extraction and purification were enriched in toxic compound: 100% of flies died in 70–80 min, with lower and lower doses (15 times less in

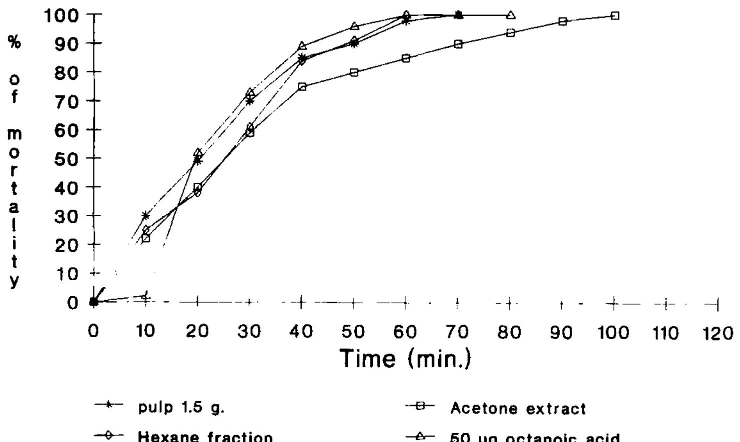


FIG. 1. Mortality kinetic curves for *D. melanogaster* meCS males with various toxic *Morinda* materials: 50 flies were exposed to either (1) (*) 1.5 g of fresh pulp of *Morinda citrifolia* ripe fruit; (2) (□) 99 mg of acetone extract (first step of extraction); (3) (◇) 1.7 mg of the hexane fraction (second step of extraction); (4) (△) 50 μg of commercial octanoic acid. The bioassay used is described by Legal et al. (1992).

the first step, almost 900 times less in the second step). So the simple fractionation method described here allowed an isolation of most of the toxic molecules of the fruit.

Determination of Composition of Most Active Fraction. Similar extraction and fragmentation procedures were applied to *Morinda* fruits of either physiological stage—ripe fruit, which is toxic, and green and rotten fruits, which are not—and gas chromatograms of the hexane fractions were compared. Only a small number of compounds appeared regardless of the stage (Figure 2).

Peaks 1 and 2 were the only large ones apparent in extracts of ripe fruit and were much larger than those present or commigrated in extracts of other stages. The products corresponding to either peak were good candidates to play a role in toxicity of ripe *Morinda* if the toxic components were not minor products not visible in GC.

GC peaks that appeared in either type of extract of different physiological states of the fruit—1–7 in Figure 2—were submitted to a structural analysis by coupled mass spectrometry–gas chromatography (GC-MS) with electron impact and a spectral library. For each product, chemical ionization GC-MS with either methane or ammonia was used to confirm the molecular weights (Figure 3). Finally, comparisons of the retention times and fragmentation patterns of natural and commercial compounds were performed.

Five of the seven visible products on GC chromatograms (Figure 2) where linear carboxylic acids: hexanoic acid, 1; octanoic acid, 2; decanoic acid, 3; palmitic acid, 4; and one 1-octadecenoic acid, 5. The remaining peaks were the ethyl esters of the first two acids; hexanoic ethyl ester, 6 and octanoic ethyl ester, 7.

Thus, octanoic acid was the major product of the purified extract of the ripe fruit. As this compound was inexpensive and available commercially, it was possible to study its toxicity in a dose-dependent fashion.

Bioassay with Commercial Octanoic Acid. Figure 4 compares the mortality dose dependencies for both sexes of both species. LD_{50} values were then calculated: 37.5 ± 7.5 μg for *D. melanogaster* meCS, and 211.5 ± 23 μg for *D. sechellia* (Table 1). *D. melanogaster* meCS males were five times more sensitive than *D. sechellia* males, while the species difference was sixfold among females.

Other mortality indices (T_{50}) were calculated from mortality kinetic curves similar to those shown in Figure 1. One may note the sigmoidicity of the curve obtained with pure octanoic acid.

T_{50} values showed a stronger intraspecific difference between sexes than LD_{50} values (Table 2). For *D. sechellia* females, at the dose of 200 μg, it was not possible to reach 50% mortality. All *D. melanogaster* flies were dead before the first *D. sechellia* began to die.

All these results clearly suggested that octanoic acid might play a major

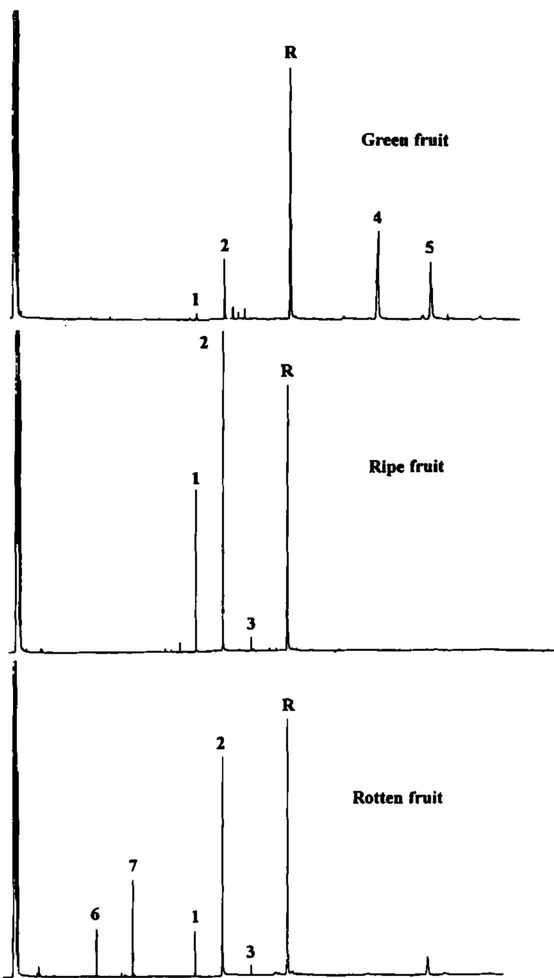
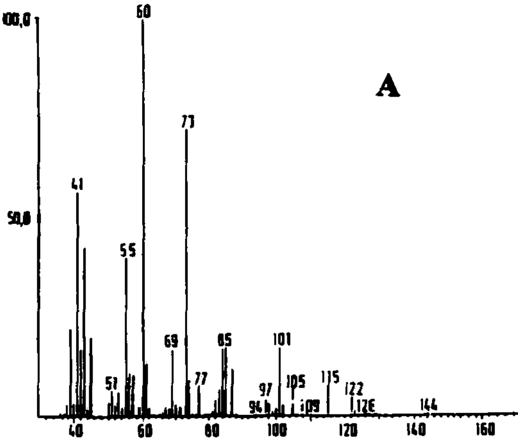


FIG. 2. Gas chromatograms of fractions obtained from green, ripe, and rotten fruit of *Morinda citrifolia*. The following products were identified by GC-MS: R, internal reference, dodecanoic acid; 1, hexanoic acid; 2, octanoic acid; 3, decanoic acid; 4, palmitic acid; 5, octadecenoic acid; 6, hexanoic ethyl ester; 7, octanoic ethyl ester (see legend of Figure 3).



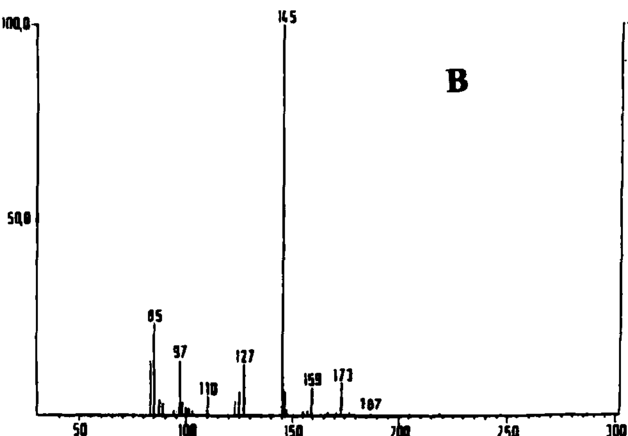


FIG. 3. GC-MS analysis after extraction and fractionation of ripe *Morinda* fruit (see text): (A) Electronic impact mass spectrum of peak 2 (octanoic acid) (70 eV) (MW = 144 g); (B) chemical ionization mass spectrum of peak 2 (octanoic) acid with methane (145 = MW+1; 173 = MW+29; 187 = MW+43).

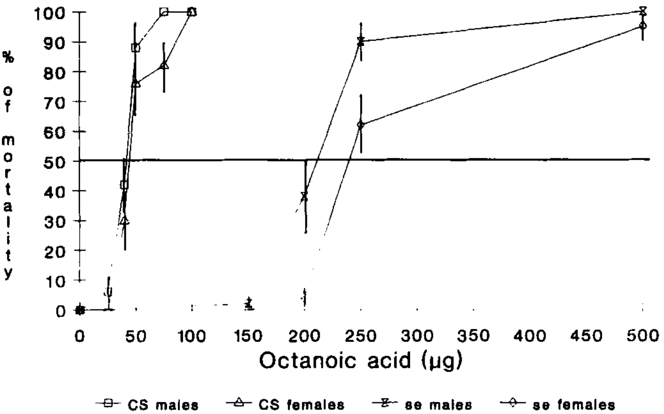


FIG. 4. Dose-mortality response curves of *D. melanogaster* and *D. sechellia* with commercial octanoic acid; 50 flies were tested for each species and each sex for 40 min.

TABLE 1. MEAN (± SD) LD_{50} OF MORTALITY FOR HEXANOIC ACID (C_6) AND OCTANOIC ACID (C_8) ON *D. melanogaster* CS AND *D. sechellia*[a]

	CS males (µg)	CS females (µg)	se males (µg)	se females (µg)	CS/se males	CS/se females
C_6	102 ± 12	127 ± 19	92 ± 13	155 ± 20	t = 2.4 p = 0.021	t = 3.27 p = .003
C_8	36 ± 07	39 ± 08	185 ± 24	238 ± 24	t = 22.38 p < .001	t = 34.11 p < .001

[a]One hundred fifty flies tested for each species and sex during 40 min. ANOVA (Student's *t*-test, SPSS/PC+ software) for interspecific comparison, *df* = 28.

role in the toxicity of the ripe *Morinda* fruit. However, the dose range in which all *D. melanogaster* are dead and all *D. sechellia* are alive is rather narrow.

Thus, it was important to quantify the amount of octanoic acid and other products present in *Morinda* fruits. Dodecanoic acid was used as a standard, as it seemed absent or in very low concentration in the fruit whatever its stage. Contents of eight fruits were compared after purification (Table 3).

For green fruits, palmitic and octadecenoic acids were the most abundant compounds. For ripe fruits, these products could not be detected, but there were large quantities of hexanoic and octanoic acids, 72 and 47 times more, respectively, than in the green fruit, and some decanoic acid. However the content was very variable for these acids; the variation coefficient was as high as 60%

TABLE 2. T_{50} VALUES FOR *D. melanogaster* MECS AND *D. sechellia*[a]

Dose (T_{50})	CS males (min)	CS females (min)	se males (min)	se females (min)
40 μg	48 ± 13	105 ± 22		
50 μg	18 ± 6	19 ± 9		
100 μg	7 ± 3	8 ± 5		
150 μg	6 ± 5			
200 μg			47 ± 8	
250 μg			22 ± 7	30 ± 10
500 μg			7 ± 5	14 ± 8

[a] Five hundred flies tested for each species and sex with several doses of commercial octanoic acid.

TABLE 3. MEAN QUANTITIES (μg/g FRUIT) OF MAIN PRODUCTS OF HEXANE FRACTIONS OBTAINED WITH FRUITS OF DIFFERENT STAGES[a]

Name (μg/g)	Green fruit (μg/g)	Ripe fruit (μg/g)	Rotten fruit (μg/g)
1. Hexanoic acid	0.9 ± 1	65 ± 80	19 ± 12
2. Octanoic acid	7.5 ± 10	352 ± 210	147 ± 85
3. Decanoic acid	0	19 ± 10	15 ± 9
4. Palmitic acid	31 ± 18	0	0
5. Octadecenoic acid	35 ± 25	0	0
6. Ethyl hexanoate	0	0	4 ± 3
7. Ethyl octanoate	0	0	60 ± 59

[a] See text and legend of Figure 2.

for the main compound, octanoic acid, and it was still higher for hexanoic acid. For the rotten fruits, these three last products were still present but in lower quantities, 29%, 42%, and 79% on average, respectively, and ethyl esters of the former two acids became abundant (Table 3).

Variability. Finally, the strong variability of toxicity between fruits was examined, and a possible correlation between the toxicity levels and the octanoic acid contents (and/or contents of other compounds) was searched for. The same eight ripe fruits were characterized for toxic effects using T_{50} values. There was a clear increase of T_{50} values with lower and lower quantities of octanoic acid present in every fruit (Figure 5). A linear correlation between both parameters were observed with a coefficient of 0.91. These data strongly support the earlier suggestion that octanoic acid might play a major role for the toxicity of these fruits for *D. melanogaster* Canton S flies.

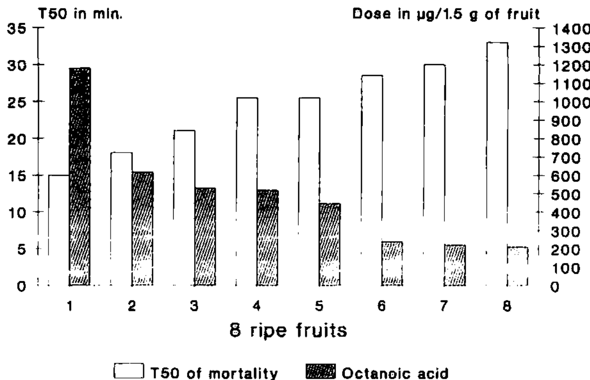


FIG. 5. Correlation between toxicity of different fruits and their intrinsic amounts of octanoic acid; eight ripe fruits were compared. T_{50}: mean time of 50% of mortality for 50 *D. melanogaster* males. Doses in micrograms per 1.5 g of ripe fruit.

Bioassay with Minor Compounds The toxicity of other short-chain fatty acids present in the hexane fraction was also investigated; LD_{50} values of hexanoic acid were not very different for *D. melanogaster* and *D. sechellia* flies (115 μg and 124 μg, respectively, in Table 1). These values correspond to about twice the quantity of hexanoic acid present in *Morinda* ripe fruit (on average 65 μg/g). LD_{50} values for decanoic acid were higher than 250 μg for the two species, while the average quantity measured in ripe fruit was 10 times smaller.

DISCUSSION

The ripe fruit of *Morinda citrifolia* is toxic for most species of the genus *Drosophila*, although *D. sechellia* can develop on it at larval stages. Isolation and characterization of toxic components were performed in parallel with green and rotten fruits, which are not toxic.

Chemical compositions of ripe and rotten fruits may raise a few problems. It cannot be excluded that the extraction and fragmentation procedures might have distorted the actual quantitative compositions, for example, leading to underestimation of more volatile products such as hexanoic acid. There is also a problem concerning the determination of the different physiological states of the fruit and the discrimination criteria used were more or less arbitrary. However, ethyl esters of hexanoic and octanoic acids were detected only in the late stage.

The presence of ethyl esters in rotten fruit might be linked to ethanolic fermentation. Then hexanoic acid and octanoic acid would be esterified by

ethanol. Such a hypothesis is supported by the fact that in the rotten fruit there is much more ester of octanoic acid than of hexanoic acid, which was less abundant in the ripe fruit. Moreover, the amounts of both free fatty acids decrease markedly during rotting. As far as the biosynthesis of those fatty acids is concerned, ripening of the fruit might involve the classical mechanism of β-oxidation steps affecting medium-size fatty acids such as palmitic or oleic acid, which are seen in the green fruit, although in small quantities. These fatty acids result from the action of fatty acid synthetases, which have been characterized in many animal and plant species.

The pulp of the ripe *Morinda* fruit contains large quantities of octanoic acid, which seems to be a major toxicity factor for *Drosophila*. Indeed, the toxic effect of *Morinda* pulp with all its premortem symptoms can be mimicked by commercial octanoic acid. A dose of 50 μg of this product yields mortality kinetics very similar to that obtained with 1.5 g of *Morinda* pulp. This dose of pure octanoic acid leads to about 75% mortality for either meCS males or females (Figure 4). In 1.5 g of whole ripe fruit, on average 530 μg octanoic acid may be chemically extracted, which is 10 times more. However, the content variability from one fruit to another is very important (213–843 μg). One also has to consider the possibility that octanoic acid might be sequestered in internal tissues of the fruit—possibly in the vacuoles—which would be disrupted by acetone. Then octanoic acid would be released in larger amounts after chemical extraction than those naturally encountered by flies in the presence of the fruit.

Is octanoic acid the only chemical responsible for toxicity? Based on its LD_{50} value—when it was the only compound—and the average amount present in ripe fruit, it cannot be excluded that hexanoic acid might play a role in toxicity: its level is highly variable but in some fruits may approach 145 μg/g; it is more volatile than octanoic acid, and thus may have a larger effect at a distance; synergies between both products are not to be excluded. However, as the average LD_{50} value for hexanoic acid is about three times lower than that of octanoic acid, it is probable that the contribution of the former compound is less important than that of octanoic acid. Using the same type of argument, the role of decanoic acid is probably negligible.

Can the reported data explain the resistance of *D. sechellia* at the adult age for the fruit that is toxic for sibling species? There is a marked five- to sixfold times difference in LD_{50} levels of *D. sechellia* and *D. melanogaster* flies for octanoic acid (for pooled sexes: 212 and 38 μg, respectively). The amount of octanoic acid chemically extracted from ripe fruit was on average higher than the LD_{50} values for *D. sechellia* (lower limit: 163 μg), and experiments using adsorbent traps have evaluated among volatiles of ripe fruits, a level of octanoic acid six times lower than the LD_{50} for *D. sechellia* flies (Farine and Le Quéré, personal communication). This difference might explain why *D. sechellia* flies were not affected by vapors of ripe fruit.

Species related to *D. melanogaster* and *D. sechellia*, such as *D. simulans* and *D. mauritiana* are as sensitive as *D. melanogaster* to *Morinda* "ripe" fruit (Legal et al., 1992) and to octanoic acid with close dose dependence (unpublished results). It is also the case for insects of most orders (cockroaches, bees, and ants). No insect studied was found to be as resistant to *D. sechellia* (unpublished results). Thus, octanoic acid might be used as an alternative insecticide, even if its specific activity is less than that of pyrethroids. It is easily available, cheap, and of low toxicity for vertebrates. Its mixed hydrophobic/hydrophilic character makes its permeation through insect cuticle very easy.

Linear organic compounds containing six to nine carbons, including alkanoic acids, are very common in many plants and may be emitted by both aerial and root tissues (Bradow, 1991). Moreover some of them with seven and eight carbons, although less frequently with a carboxylic function, were shown to inhibit germination of seeds of several plants (Bradow, 1991).

A simple molecular basis for the ecological isolation of *D. sechellia* and its sibling species of the *melanogaster* subgroup such as *D. melanogaster* has been documented in this paper. While flies of the sibling species were killed by the ripe fruit and its abundant octanoic acid, *D. sechellia* flies have adapted by developing a resistance mechanism and even choosing the same plant for egg-laying. Moreover, Legal et al. (1992) have shown a good correlation between sensitivity/resistance on one hand and chemorepulsion/chemoattraction on the other hand. Such an example of ecological isolation is not unique in this *melanogaster* subgroup of Drosophilidae. In the *repleta* subgroup, species of the Nannoptera complex including *D. pachea* were linked to cacti. One cactus, *Lophocerus*, contains unusual alkaloids and sterols. The alkaloid, lophocereine, is highly toxic to most *Drosophila* species except *D. pachea*. Moreover one *Lophocerus* sterol, 7-stigmastenol, favors the growth and reproductive physiology of *D. pachea* (Heed and Mangan, 1986; Heed et al., 1990). The *Morinda* toxic principle is more simple and may provide a good model system, together with *Drosophila*, to study the mechanisms of resistance at both enzyme and gene levels.

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BIOLOGICALLY ACTIVE SECONDARY METABOLITES OF BARLEY. IV. HORDENINE PRODUCTION BY DIFFERENT BARLEY LINES

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Abstract—Forty-three lines of barley, including ancestral (wild), landraces, Middle Eastern lines, and modern cultivars, were grown under two different sets of environmental conditions. Hordenine production in barley roots was determined at the one-leaf stage by HPLC analysis and, in two lines only, over a period of 35 days. Forty-two of the 43 lines produced significant amounts of hordenine, although there was no variation among groups. Middle Eastern lines had the highest production with 327 μg/g on a dry weight basis. Production was, however, determined more by environmental conditions during growth than by genetic factors. Hordenine production was up to seven times higher in plants grown under lower light intensities.

Key Words—Hordenine, gramine, barley, *Hordeum* spp. genetics, stress.

INTRODUCTION

Alkaloids are known to have wide biocidal activity. Hordenine and gramine, both produced by the barley plant (*Hordeum* spp.), are typical of this group of secondary metabolites. Although a certain amount has been published about the production and activity of gramine in barley and other species, little is known about the production and activity of hordenine.

Apart from being phytotoxic (Overland, 1966), gramine has been implicated in the self-defense of cultivated barley (*Hordeum vulgare*) against bacteria (Sepulveda and Corcuera, 1990) and aphid infestation (Zuniga et al., 1988;

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Kanehisa et al., 1990). Gramine also inhibits the growth of the fungus, *Drechslera teres*, and armyworm, *Mythimna convecta*, larvae (Lovett and Hoult, 1993) and is considered to be an antipalatability factor toward grazing animals (Marten et al., 1976). In contrast, all that has been reported about hordenine is that it inhibits seedling growth of white mustard, *Sinapis alba* (Liu and Lovett, 1989), and *Dreschslera teres* and *Mythimna convecta* (Lovett and Hoult, 1993). These findings indicate a potential for self-defense by barley against other organisms through the production of hordenine and gramine. Consequently, the level of production of these compounds and the factors controlling their production are worthy of investigation.

The production of gramine and other alkaloids has been shown to be under genetic and environmental control (Moore et al., 1967; Gentry et al., 1969; Hanson et al., 1981, 1983; Lovett and Hoult, 1992). Hanson et al. (1981) surveyed 23 lines of barley of different origins and found gramine production ranged from 0 mg/g to 8 mg/g dry weight and concluded that high gramine production was typical of lines originating in hot areas of the world. They subsequently showed that gramine production by barley increased with temperature (Hanson et al., 1983). Analogous data for hordenine are not available.

Breeding for low alkaloid content, including low gramine content, has been achieved in reed canary grass (*Phalaris arundinacea*) (Marten et al., 1981), the object being to reduce the adverse effects of this alkaloid on the productivity of grazing animals. Hanson et al. (1981) suggested that this should also be possible in barley forage crops. However, if the self-defense role of allelochemicals in grain crops is accepted, the ability to produce large amounts of these compounds would be of advantage and could ultimately lead to reduced use of synthetic crop protection agents. Lovett (1982) suggested that, in breeding crop plants for improved yield, self-defense capability may have been reduced or lost. To reintroduce this capability, it may be necessary to look to ancestral types to find a source of appropriate genes.

Lovett and Hoult (1992) investigated the gramine content of 43 lines of barley chosen to represent the continuum in exploitation and breeding from ancestral types (*H. spontaneum* and *H. agriocrithon*) through to contemporary cultivars, and confirmed genetic control as found by Hanson et al. (1981). In this paper we report on the hordenine content of the roots of these 43 lines and discuss this in relation to genotypic and environmental control of production and in relation to the gramine content of their leaves.

METHODS AND MATERIALS

Plant Material. Six *Hordeum spontaneum* lines, four *H. agriocrithon* lines, eight landraces, nine lines from the Middle East representing modern lines bred close to the center of diversity, and 16 cultivars grown in Australia past and

present were used. Genetically pure seed of all lines were multiplied in Armidale prior to use.

Chemicals. AR or HPLC grade chemicals were used throughout. Authentic hordenine and gramine hemisulfate were obtained from Sigma Chemicals.

Experiment 1. Seed of cv. O'Connor and *H. spontaneum* var. *spontaneum* were sown in sand, replicated four times and grown in a glasshouse in October 1991. The plants were harvested daily from day 6 to day 9, inclusive, and thereafter at 2, 3, 4, and 5 weeks of age. The roots and shoots were separated and the remains of the grain discarded. The plant material was carefully blotted dry and stored in a freezer until representative subsamples were weighed out for analysis. Alkaloid contents are expressed as micrograms per gram fresh weight, as there was not always sufficient plant material to measure dry weight.

Experiment 2. The barley lines selected were grown under two sets of environmental conditions. One environment was a growth cabinet with a 12-hr day, 430 W/m^2, 8–18°C, and total incident light of 5160 $W/m^2/day$. The growth conditions were outside in March 1991; mean daylength 10.3 hr, mean total incident light 6030 $W/m^2/day$, mean maximum incident light 890 $W/m^2/day$, and mean temperature range 10–23°C. Fifty grains of each line, replicated three times, were sown in sand and harvested as each line reached the stage of one fully expanded leaf (approximately eight to 10 days after sowing) as described by Hoult and Lovett (1993). Plant material was stored as in experiment 1. Grain of all lines was not immediately available in sufficient quantities; therefore, the growth chamber material consisted of 30 lines whereas the outside-grown material consisted of all 43 lines. Dormancy problems were encountered in seven of the eight landraces; all were resown at a later date (July 1991) along with cv. Prior and *H. spontaneum* var. *spontaneum* as controls.

Extraction and HPLC Analysis. The frozen root material was extracted in 0.01% acetic acid at room temperature for 24 hr, the extracts filtered through glass wool, the pH adjusted to 9.15, and then centrifuged at 3000 rpm for 5 min before being purified and concentrated using Sep-pak C_{18} cartridges. Purification consisted of applying 10-ml aliquots of extract to Waters C_{18} Sep-pak cartridges prepared before use with 5 ml ACN (acetonitrile) and 2 ml 0.01 M KH_2PO_4, pH 7. The root samples were rinsed once with 2 ml 0.05 M KH_2PO_4, pH 9.5, isopropanol (85:15) before being eluted with 1.5 ml 0.05 M KH_2PO_4, pH 2.3, isopropanol (70:30). All eluates were evaporated to dryness at 40°C under a stream of nitrogen and taken up in 1 ml mobile phase for analysis by HPLC. Recovery of hordenine from these procedures was monitored by "purifying" two 10-ml aliquots of standard authentic compound with each batch of plant root extracts.

Extracts were analyzed by injecting 10-μl aliquots into a Waters HPLC system comprising a M40 pump, flow rate 2 ml/min, U6K injector, μBondapak phenyl column 10 μm (3.9 mm × 30 cm), and UV-vis spectrophotometer

wavelength 221 nm. Results were recorded as peak areas using a Waters 745 data module. Elution was isocratic, the mobile phase consisting of 0.025 M KH_2PO_4, pH 7.15, + 0.1% triethylamine/ACN (67:33). The presence of hordenine and gramine having been confirmed in barley cv. Triumph (included in this survey) by GC-MS (Liu and Lovett, 1990a), identification was by retention time relative to, and by coelution of, representative samples with authentic compound. Quantification was by peak area relative to standards of authentic compound.

RESULTS AND DISCUSSION

Hordenine production within lines was much more variable than gramine production. Table 1 shows the pattern of gramine and hordenine production by two lines, *H. spontaneum spontaneum* and cv. O'Connor, over the first five weeks of growth. The gradual increase in gramine production in both lines up to day 9 after sowing indicates that sampling and estimation of gramine production was reliable. However, the production of hordenine by the two lines followed different patterns, and the sharp peak and decline of hordenine production by *H. spontaneum* made estimation of peak production difficult. Nevertheless, over three replications, production potential over the first few days of growth was probably estimated reliably. Liu and Lovett (1990b) found cv. Triumph to exude hordenine into hydroponic solution much in the *H. spontaneum* pattern, but without knowing the pattern of production by individual lines

TABLE 1. PRODUCTION OF HORDENINE AND GRAMINE OVER TIME BY TWO DIFFERENT BARLEY LINES[a]

	Hordenine content of roots		Gramine content of shoots (μg/g fresh weight)	
Day	*H. spontaneum*	*H. vulgare* cv. O'Connor	*H. spontaneum*	*H. vulgare* cv. O'Connor
6	13.75 abc	31.77 cd	880 def	364 ab
7	28.75 bcd	31.84 cd	936 efg	539 bc
8	12.19 ab	35.69 d	1059 efg	596 bcd
9	8.13 a	11.77 ab	1121 fg	620 bcd
14	5.81 a	4.13 a	1185 g	457 ab
21	3.07 a	4.22 a	605 bcd	507 abc
29	4.62 a	2.25 a	451 ab	321 ab
35	6.70 a	5.15 a	796 cde	206 a

[a]Entries carrying the same letter do not differ from each other significantly at the 5% level.

it is not possible to estimate with confidence the peak production potential of lines. We consider that the data for hordenine production presented here (Table 1) are an acceptable estimate of the production of hordenine by these lines over the first few days of growth.

All lines, with the exception of *H. spontaneum* var. Borgiyyora, in which trace amounts were found, produced hordenine in measurable quantities. This indicates that hordenine production is a normal function of the barley plant and is consistent with our finding that, contrary to a previous report (Hanson et al., 1981), gramine production is also probably the norm in barley (Lovett and Hoult, 1992).

Regression analysis of hordenine production by the 30 lines grown both in the growth cabinet and outdoors gave an r^2 value of 0.2370, indicating little genetic control of hordenine production. The corresponding r^2 value for gramine production was 0.9079, indicating strong genetic control (Lovett and Hoult, 1992). The difference in degree of genetic control over the production of the two alkaloids was exemplified by the relatively large differences in hordenine production between the growth cabinet and plants grown outdoors. Gramine production was enhanced when the plants were grown outdoors, at most by 100% and generally by much less than this amount. By comparison, hordenine production was found to be as much as seven times greater in the plants grown in the growth chamber (Table 2). The enhanced production of gramine under conditions of greater light intensity and higher temperatures may be explained by an elevated metabolic rate leading to a greater excess of substrate available for the production of secondary metabolites. Application of the model of Johnson et al. (1993) for crop photosynthesis to our data indicated that the greater mean maximum light intensity, rather than the higher temperatures encountered by the plants grown outdoors, was the major factor contributing to this greater production. This is consistent with the data of Hanson et al. (1983), who found little effect of temperature on gramine production below 21°C.

The greater hordenine production at lower light intensity cannot be explained in this way. We suggest that at the establishment phase a plant is most vulnerable to adverse environmental conditions, competition from other species, and invasion by pathogens. Seedling vigor is reduced if less than ideal conditions for growth prevail. This results in a concomitant shift in survival strategy from aggression, evidenced as high competitive ability through vigorous growth, to defense, in which a greater proportion of plant product is diverted to the production of secondary metabolites. It is submitted that a maximum light intensity of one third to one half full daylight would constitute stressful conditions.

Hordenine production by the *H. spontaneum* group, in general, was found to be less than in the other groups (Table 3). However, analysis of variance for groups of unequal replicates performed on the five groups grown outdoors (experiment 2) showed that the difference only reached significance between the

TABLE 2. IDENTITY, ORIGIN, AND GRAMINE AND HORDENINE CONTENT OF 43 BARLEY LINES

Aust. No./ Q Code	Cultivar	Origin	Gramine content of shoots (μg/g fr wt)		Hordenine content of roots (μg/g dry wt)	
			Growth cabinet	Outdoors	Growth cabinet	Outdoors
H. spontaneum						
400154	spontaneum	unknown	1147	1506(1158)[a]	717	135
N/A	Borgiyyora	Israel		1725		1
N/A	Eyzaria	Israel		1312		145
N/A	Tel Hay	Israel	1254	1028	1600	222
N/A	Mt. Meron	Israel	844	1215	921	217
N/A	Akziv	Israel		1679		75
H. agriocrithon						
402775		unknown	229	285	871	169
402776		unknown		866		87
402777		unknown	1100	1522	2275	435
402779		unknown		719		126
H. vulgare						
1LQ89	landrace	Syria		(746)		(238)
2LQ89	landrace	Syria		(773)		(160)
3LQ89	landrace	Syria		(854)		(353)
4LQ89	landrace	Syria		(697)		(235)
5LQ89	landrace	Syria		(787)		(250)
6LQ89	landrace	Syria		(1069)		(83)
7LQ89	landrace	Syria		851(717)		(64)
8LQ89	landrace	Syria		(964)		(272)

400110	N/A	Ethiopia	700	885	1361	130
400117	N/A	Ethiopia	915	1061	1100	151
400120	N/A	Ethiopia	5	4	1042	203
400039	N/A	Syria	18	7	2141	599
490224	N/A	Syria	0	5	1820	322
490314	N/A	Syria	449	679	763	151
490187	N/A	UAR	793	1434	2038	315
490281	N/A	UAR	40	1208	2080	247
490288	N/A	UAR	558	1075	1919	356
401786	Prior	England	640	746(565)	2078	226
401781	Proctor	England	3	5	1236	341
400189	Triumph	England	3	2	1274	83
401834	Resibee	Australia	595	913	2307	178
401259	Lara	Australia	548	741	2325	224
402239	Weeah	Australia	449	717	1680	150
400188	O'Connor	Australia	435	509	2625	265
400190	Clipper	Australia	329	306	1604	172
402996	Yagan	Australia	205	394	2200	378
400180	Forrest	Australia	1	24	1534	274
400187	Schooner	Australia	1	5	1200	153
400182	Galleon	Ausralia	1	5	1585	206
400186	Grimmett	Australia	0	3	2133	256
402997	Windich	Australia	0	8	1314	458
402713	Moondyne	Australia	0	4	1842	357
403001	Skiff	Australia	0	3	634	133

[a] Data in parentheses refer to lines sown in July 1991.

TABLE 3. MEAN GRAMINE CONTENT OF SHOOTS AND HORDENINE CONTENT OF ROOTS OF FIVE GROUPS OF BARLEY LINES GROWN OUTDOORS

Group	Gramine content (μg/g fresh weight)	Hordenine content (μg/g dry weight)
H. spontaneum	1410	133
H. agriocrithon	848	204
H vulgare		
Landraces	830	207
Middle Eastern	706	327
Australian	274	241

H. spontaneum and the Middle Eastern group ($P > 0.05$). Recent reviews indicate that allelochemicals may confer fungal (Rizvi and Rizvi, 1992) and nematode (Hasan, 1992) resistance on a plant. Grodzinsky (1992) reported that barley root exudates inhibited germination and germ tube growth of *Fusarium oxysporum* f. *vasinfectum*. It is possible, therefore, that the greater hordenine production by bred lines of barley may be explained in terms of defensive benefits conferred on the barley plant. We infer, therefore, that hordenine production has been favored by selection for pathogen resistance.

In plants grown outdoors, production of gramine and hordenine were independent of each other with a slight trend towards a negative relationship. The exception to this was the *H. spontaneum* group, where the negative relationship was significant ($r^2 = 0.8848$). A similar independence was found in the growth of cabinet-grown plants, with the only relationship approaching significance being a positive one in the group Australian cultivars ($r^2 = 0.4398$). This evidence of a marked change in the balance of hordenine and gramine production in response to environmental changes was interpreted to be another manifestation of the effects of stress (i.e., low light intensity) on the metabolism of the plant and, hence, on its potential to produce allelochemicals.

CONCLUSION

Hordenine production by barley roots appears to be the norm, and there is a tendency to greater production by modern cultivars. This is in contrast to gramine production by the shoots in which alkaloid production is greatest in the ancestral lines and may be suppressed almost entirely in some modern cultivars. It is possible that hordenine production in modern cultivars has been favored because it confers on the barley plant a degree of resistance to fungal pathogens

or other pests. Furthermore, in contrast to gramine production, hordenine production does not seem to be under strong genetic control but is much more responsive to changes in the environment in which the plant is grown. Hordenine may have a role to play in protecting the plant under stressful conditions.

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PLANT–NATURAL ENEMY ASSOCIATION IN TRITROPHIC SYSTEM, *Cotesia rubecula–Pieris rapae*–BRASSICACEAE (CRUCIFERAE). III: COLLECTION AND IDENTIFICATION OF PLANT AND FRASS VOLATILES

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Abstract—To elucidate the identity of the volatile compounds that could be involved in the searching behavior of the parasitoid *Cotesia rubecula* Marshall (Hymenoptera: Braconidae), the volatiles released by cabbage and frass of Lepidoptera feeding on cabbage were collected and analyzed using a gas chromatograph–mass spectrometer. The volatiles emitted by intact cabbage were α-pinene, β-pinene, myrcene, 1,8-cineole, *n*-hexyl acetate, *cis*-3-hexen-1-yl acetate, and dimethyl trisulfide. Mechanical damage on an intact plant induced the release of two more compounds, *trans*-2-hexenal and 1-methoxy-3-methylene-2-pentanone. Current feeding by larvae of *Pieris rapae* L. (Pieridae) induced the plant to release all the compounds released after mechanical damage and additionally 4-methyl-3-pentenal and allyl isothiocyanate. Current feeding by larvae of *Plutella xylostella* L. (Plutellidae) induced the plant to release all the compounds present after mechanical damage and additionally allyl isothiocyanate. The volatiles emitted after feeding by the lepidopterans had ceased were the same as those emitted by cabbage damaged by mechanical means. The blend of volatiles emitted by frass was comprised of plant chemicals, mainly sulfur compounds. Frass of *P. rapae* emitted allyl isothiocyanate, methyl isothiocyanate, methyl propyl sulfide, dimethyl trisulfide, *S*-methyl methane thiosulfinate, 4-methyl-3-pentenal, *trans*-2-hexenal, and 2,3-dihydro-4-methyl furan. Frass of *P. xylostella* emitted only dimethyl trisulfide and *S*-methyl methane thiosulfinate. The blend of volatiles emitted by frass is herbivore-species specific.

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Key Words—*Pieris rapae*, Lepidoptera, Pieridae, Plutellidae, *Plutella xylostella*, *Cotesia rubecula*, Hymenoptera, Braconidae, *Brassica oleracea capitata*, infochemicals, plant volatiles.

INTRODUCTION

Plants continuously release volatile compounds into the surrounding air. Some herbivores utilize these volatiles to locate their food plants (Visser, 1986), and natural enemies of the herbivores (predators, parasitoids) may utilize these plant infochemicals at some stage of their search for prey or hosts (Vet and Dicke, 1992). Feeding by herbivorous insects induces plants to release a volatile blend of chemicals different from that released during the intact state (Turlings et al., 1990, Dicke et al., 1990a,b) and natural enemies of the herbivores may utilize the plant volatile chemicals related to feeding by herbivores to locate their prey or hosts (Turlings et al., 1990, 1991; Dicke and Takabayashi, 1991; Dicke et al., 1990a,b).

Cotesia rubecula Marshall (Hymenoptera: Braconidae) is an endoparasitoid of *Pieris rapae* L. (Lepidoptera: Pieridae). Despite a distinct preference for *P. rapae* as a host (Wilkinson, 1945; Wiskerke and Vet, 1991), the range of other hosts that geographically separated populations of the species utilize is not well known (Shenefelt, 1972). Females of *C. rubecula* are attracted from a distance to cabbage plants infested by its host *P. rapae* (Nealis, 1986; Keller, 1990; Kaiser and Cardé, 1992). Strong indications of plant involvement in host location are apparent in the work of Kaiser and Cardé (1992) where females of *C. rubecula* were attracted to uninfested leaves held in a vial. A limited attraction to uninfested leaves was also observed by Keller (1990). The involvement of host-plant in leading *C. rubecula* to its host was further investigated by Agelopoulos and Keller (1994a,b). In a single-choice situation, females did not fly to intact cabbage plants (host plant), but they flew to host plants bearing damage. Different means such as mechanical, larvae of the host, larvae of the nonhost lepidopteran *Plutella xylostella* L., and snails (a nonhost, noninsect herbivore) were used to produce different types of damage on the host plant. Attraction was also observed to frass of the host and to frass of the nonhost lepidopteran *P. xylostella*. In a choice situation, *C. rubecula* preferred cabbages damaged by the lepidopterans, *P. rapae* and *P. xylostella*, over those damaged by mechanical means or snails, but it was not able to differentiate between cabbages damaged by the two different lepidopterans.

Here, we sought to identify chemicals that may influence the searching behavior of *C. rubecula*. Our aim was (1) to identify the volatiles released by intact and mechanically damaged cabbage and then to compare them to the volatiles released when feeding by the lepidopterans, *P. rapae* and *P. xylostella*,

occurred, and (2) to identify the volatile chemicals released by frass of the two lepidopterans.

METHODS AND MATERIALS

Plants. Cabbage plants, *Brassica oleracea capitata* cv. Green Coronet were grown under greenhouse conditions. Because nutrient availability affects the chemistry of plants (for an overview see: Waterman and Mole, 1989), a hydroponic system was used to grow the cabbages so that nutrient depletion associated with soil leaching and consumption of nutrients by the developing plant would not be a factor in the experiment. The plants were grown in unfertilized soil and were watered with Hoagland nutrient solution (Silsbury, 1984) at regular intervals depending on the developmental stage of plants. During development, plants were regularly checked for root exposure or other accidental damage. Damaged plants were rejected as damage may affect the physiology and subsequently the emission of volatiles by plants (Dicke et al., 1990b; Turlings et al., 1990, 1993). All the plants used for this experiment were of the same developmental stage (five fully expanded and two small leaves).

Apparatus for Collecting Volatiles from Plants. To avoid damaging the plants, volatiles were collected from potted plants. The apparatus was constructed from Perspex and was divided in two parts: a lower part that contained the pot and an upper part that contained the aerial part of the plant (Figure 1). To exclude volatiles from the soil and pot that could contaminate the plant volatiles, the lower part containing the pot was isolated from the upper part by using a flat sheet of Perspex that allowed only the stem of the plant to pass through. The opening that allowed the stem of the plant to pass through was covered with a small piece of Perspex that was built to fit perfectly in the opening. The upper part (14 × 14 × 12 cm) was the collection chamber that confined the foliage of the plant. Control samples were collected and analyzed to detect volatile chemicals released by the apparatus.

Plant Treatments. Volatiles released by plants were collected for 22 hr. After collecting volatiles from an intact plant, the same plant was mechanically damaged and the volatiles were collected for a further 22 hr. Mechanical damage was inflicted on leaves just before the commencement of a test by removing small squares (1 cm^2) using a razor blade. The damage was evenly distributed on every fully expanded leaf. To collect volatiles from currently infested plants, cabbages were infested for 24 hr with 20 larvae (either second and third instars of *P. rapae* or fourth instars of *P. xylostella*) and volatiles were then collected for 22 hr. The larvae and their by-products were then removed, the plant was washed with water, and the volatiles were collected for a further 22 hr to obtain samples from previously infested plants.

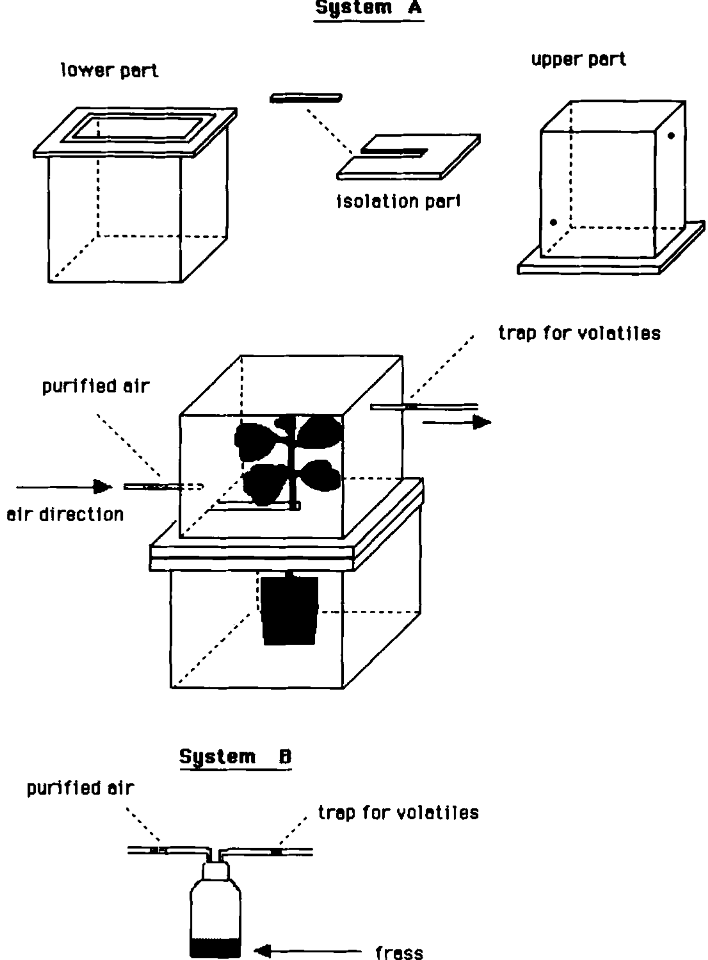

FIG. 1. Apparatus for collecting volatiles from plants (A) and frass (B).

Collection of Volatiles from Frass. The frass was obtained by placing paper sheets underneath cabbages infested by *P. rapae* or *P. xylostella* for 24 hr. During collection of volatiles released by frass, the frass was held in a 100-ml Schott beaker (Figure 1). Volatiles from 500 mg of frass of *P. rapae* or *P. xylostella* were collected for a period of 7 hr. Volatiles released from the apparatus itself were collected during control samples.

Experimental Procedure. For each treatment used in this study, at least

four samples were collected. Air was sucked through the system with a dual-head pump (Air Cadet, model 7530-65), and an air flowmeter (model 10A3265NXB11A, Fisher & Porter, Pty. Ltd.) controlled the air flow at 500 ml/min. The air inside the collection chamber was replaced every 4.7 min. Volatiles were trapped in a 12-cm-long × 1-cm-diameter glass tube, sealed in the middle with a glass frit, and 150 mg of Porapak Q (80–100 mesh) (Alltech) was placed on the top of the frit and kept in place with a small plug of glass wool. Purification of the air entering the system was achieved by using a 15-cm-long glass tube filled with 1 g of Porapak Q. The trap was washed with 1.5 ml of methylene chloride of HPLC quality to extract the volatiles. The samples containing the volatiles were concentrated through evaporation and 5 μl were injected into a gas chromatograph–mass spectrometer. Internal standards were not added since this was a qualitative study only. The GC model was a Varian 3400 gas chromatograph and the column used was J & W DB-1701 (30 m × 0.25 mm ID, 0.25-μm film thickness). The temperature conditions were 5 min at 40°C, increased 3°C/min to 200°C, and held for 20 min. The carrier gas was helium. Each peak from the GC was analyzed by a mass spectrometer (TSQ 70), with an electron impact ionization of 70 eV. The test samples were compared to the control samples from the apparatus of collection to ascertain which chemicals were specific to each treatment.

The identification of the natural compounds, *n*-hexyl acetate, *cis*-3-hexen-1-yl acetate, *trans*-2-hexenal, α-pinene, β-pinene, myrcene, 1,8-cineole, allyl isothiocyanate, and methyl isothiocyanate, was achieved by comparing their spectra with those of synthetic compounds obtained commercially from Sigma-Aldrich Corporation. The rest of the compounds were identified from the data base that was installed in the MS-computer (NIST Mass Spectral Database) and by comparing their spectra to those already published (Kjaer et al., 1963; Spencer and Daxenbichler, 1980).

RESULTS

Volatiles Emitted by Differently Treated Cabbages. The blends of volatile compounds emitted by differently treated cabbages belong to four chemical groups (Table 1). The monoterpenes α-pinene, β-pinene, sabinene, myrcene, and 1,8-cineole are isoprenoids. The isothiocyanates (methyl- and allyl-) and the thiol derivatives (methyl propyl sulfide, dimethyl trisulfide, and *S*-methyl methane thiosulfinate) are compounds that contain sulfur. The aldehydes (*trans*-2-hexenal, 4-methyl-3-pentenal, the ketone, 1-methoxy-3-methylene-2-pentanone) and the acetates (*n*-hexyl and *cis*-3-hexen-1-yl) are fatty-acid derivatives. 2,3-Dihydro-4-methyl furan is a furanoid.

The compounds emitted by intact plants of *B. oleracea capitata* cv. Green

TABLE 1. VOLATILE COMPOUNDS RELEASED BY DIFFERENTLY TREATED CABBAGES AND FRASS OF *P. rapae* AND *P. xylostella* (*n* = NUMBER OF SAMPLES COLLECTED FROM ITS TREATMENT, + = PRESENCE OF COMPOUNDS IN ONE ASSAY).

Identified compounds	Cabbage infested by *P. rapae* (*N* = 4)	Cabbage infested by *P. xylostella* *N* = 6	Cabbage previously infested by *P. rapae* (*N* = 4)	Cabbage previously infested by *P. xylostella* (*N* = 4)	Cabbage damaged by mechanical means (*N* = 4)	Intact cabbage (*N* = 5)	Frass of *P. rapae* (*N* = 4)	Frass of *P. xylostella* (*N* = 4)
α-Pinene	++++	+++++	++++	+++	+++	++++	—	—
β-Pinene	++++	++++	++++	++++	++++	+++	—	—
Sabinene	+++	++++	+++	++++	+++	+++	—	—
Myrcene	+++	+++	+++	+++	+++	++	—	—
1,8-Cineole	++++	++++++	++++	++++	+++	++++	—	—
cis-3-Hexen-1-yl acetate	++++	++++++	++++	++++	++++	+++++	—	—
n-Hexyl acetate	++++	++++++	++++	++++	++++	+++++	—	—
trans-2-Hexenal	+++	++++	+++	++	++	—	++	—
1-Methoxy-3-methylene-2-pentanone	+++	++	+++	++	++	—	—	—
4-Methyl-3-pentenal	++	—	—	—	—	—	++	—
Allyl isothiocyanate	+++	++	—	—	—	—	++++	—
Methyl isothiocyanate	—	—	—	—	—	—	+++	—
Dimethyl trisulphide	++++	++++	+++	+++	++++	++++	++++	++++
Methyl propyl sulphide	—	—	—	—	—	—	++	—
S-Methyl methane thiosulphinate	—	—	—	—	—	—	+++	++
2,3-Dihydro-4-methyl furan	—	—	—	—	—	—	++	—

Coronet at the stage of seven leaves (5 weeks old) were α-pinene, β-pinene, sabinene, myrcene, 1,8-cineole, *cis*-3-hexen-1-yl acetate, *n*-hexyl acetate, and dimethyl trisulfide. When mechanical damage was inflicted on the cabbage, the volatile profile changed. Two new compounds, *trans*-2-hexenal and 1-methoxy-3-methylene-2-pentanone, appeared in addition to those released by intact plants. In addition to the volatiles emitted by a mechanically damaged cabbage, cabbage currently infested by *P. rapae* emitted two new compounds, 4-methyl-3-pentenal and allyl isothiocyanate. The volatile profile emitted by a cabbage currently infested by *P. xylostella* was the same as that of cabbage currently infested by *P. rapae* except for the compound 4-methyl-3-pentenal, which did not appear. Cabbage previously damaged by *P. rapae* or *P. xylostella* emitted the same volatiles as cabbage damaged by mechanical means.

Volatiles Emitted by Frass: In both treatments, the volatile profile of frass was dominated by compounds containing sulfur. None of the isoprenoids was present in frass. The frass of *P. rapae* emitted more volatile chemicals compared to that of *P. xylostella*. The predominant group was the sulfur-containing compounds, *allyl* isothiocyanate, methyl isothiocyanate, methyl propyl sulfide, dimethyl trisulfide, and *S*-methyl methane thiosulfinate. The two aliphatic aldehydes, 4-methyl-3-pentenal and *trans*-2-hexenal, present in the plant treatments, were also present in frass of *P. rapae*. 2,3-Dihydro-4-methyl furan was another compound that appeared only in the volatiles emitted by frass of *P. rapae*. The frass of *P. xylostella* emitted only two compounds in detectable amounts, dimethyl trisulfide and *S*-methyl methane thiosulfinate, which were also present in the frass of *P. rapae*.

Variation between the samples of the same treatment was observed. This may be due to intrinsic differences in the chemistry between individual cabbages.

DISCUSSION

Most of the compounds present in the volatile blend emitted by plant treatments and frass have been identified before in Brassicaceae species (Bailey et al., 1961; Buttery et al., 1976; Wallbank and Wheatley, 1976; Cole 1980a,b; Tollsten and Bergström, 1988; Evans and Allen-Williams, 1992). The groups of compounds identified here have a distribution in many plant families: isothiocyanates are present in 11 plant families with a predominant distribution in Brassicaceae (Kjaer, 1960); Thiosulfinates, and trisulfides are not only present in species of Brassicaceae but also in *Allium* spp. and in some species of Fabaceae (Leguminosae) (MacLeon and Forcen, 1992); Isoprenoids are very common among species of the families Lamiaceae, Poaceae, Lauraceae, Myrtaceae, and Myristicaceae (Nicholas, 1973). Fatty-acid derivatives, the so-called green leaf volatiles, are compounds found in all plant species. They are formed by

the oxidative degradation of plant lipids and are continuously released by plants during aging or when injury occurs (Visser and Avé, 1978). Furanoids are quite common in many plant species (Hedin et al., 1975; MacLeon and Forcen, 1992) including Brassicaceae (Buttery et al., 1976). The compound 2,3-dihydro-4-methyl furan has been collected from *Ceratiola ericoides*, a species of Empetraceae (Jordan et al., 1992), but not from Brassicaceae.

Factors Affecting Volatile Releases of Plants. The factors affecting the volatile blend released by plants are the taxonomic group to which the plant belongs (Wallbank and Wheatley, 1976; Cole, 1980a; Lui et al., 1988; Tollsten and Bergström, 1988; Hernandez et al., 1989), age (Cole, 1980b), daylight (Matile and Altenburger, 1988), mechanical wounding, wounding by herbivores, and presence of damage somewhere on the plant (Dicke et al., 1990a, 1993; Turlings et al., 1990, 1991, 1993; Whitman and Eller, 1990). Wounding crushes plant cells, resulting in emission of volatiles, and may simultaneously induce systemic changes such as the production of toxins and release of volatiles by the undamaged parts of the plant (Takabayashi et al., 1991; Turlings et al., 1993; Dicke et al., 1993). The way that damage is inflicted may also affect the volatile blend emitted by plants. In the case of herbivores, the size and shape of mandibles are important. In the case of mechanical damage, the method of inflicting damage, the location of damage (e.g., on the stem, on the leaves away from the main veins or including the main veins), the severity of damage (overall amount of damage inflicted on the plant), the distribution of damage (evenly or unevenly distributed on the plant), and the continuity of damage (period of time over which the damage takes place) are important influences on the emission of volatile compounds (Baldwin, 1988). After the damage has ceased, volatiles related to damage continue to be released over a postdamage period (Turlings et al., 1990).

Damage and Release of Volatiles by Brassica oleracea capitata. The volatile blend released by intact cabbages that was identified here provided only information about the identity of the compounds released by intact plants of that particular variety at that specific age. The volatile blend emitted by cabbages changed when damage (mechanical or damage caused by herbivores) was inflicted on the plant. These changes were present during the period that the damage was inflicted and continued to be present during a postdamage period. Mechanical damage and damage caused by herbivores also induced changes to volatiles emitted by intact corn seedlings and Lima bean plants and the emission of volatiles related to damage continued during a postdamage period (Dicke et al., 1990a,b; Turlings et al., 1990, 1991). The volatile blend emitted by cabbage during the 22-hr postdamage period was not affected by the original cause (mechanical or by the lepidopterans) and the period that the damage was inflicted (5 min mechanical damage or 48 hr damage caused by the lepidopterans). The postdamage blend was different from the blend emitted during current damage

by the lepidopterans. More compounds were released when larvae were currently feeding on the plant. The blend of volatiles emitted by cabbages currently infested by different species of Lepidoptera was slightly different. The compounds 4-methyl-3-pentenal and allyl isothiocyanate were released when larvae were feeding on the plant, but at present they cannot be classified as herbivore-induced synomones since volatiles released during continuous mechanical damage were not collected in our studies.

Volatiles Released by Frass. The volatiles released by frass are plant chemicals (Wallbank and Wheatley, 1976; Finch, 1978; Cole, 1980b; Tollsten and Bergström, 1988; Jordan et al., 1992). The blend of volatiles released by frass is herbivore-species-specific since frass from different species feeding on the same plant released different volatiles. At present, we do not know if the volatiles released by frass are: (1) plant chemicals that passed the alimentary canal of larvae unchanged, (2) the results of the digestive process of larvae, or (3) chemicals produced by microorganisms inhabiting the frass of the larvae that utilize the frass of larvae as food (Thibout et al., 1993). The plant origin of volatiles released by frass of Lepidoptera has also been demonstrated by Ramachandran et al. (1991) and Auger et al. (1989). Auger et al. (1989) identified the volatiles released by frass of *P. xylostella* that was feeding on cabbage, and they found that only compounds of the thiol derivatives group (dimethyl disulfide, methyl propyl disulfide, dipropyl disulfide, and dimethyl trisulfide) were present. This supports our results that frass of *P. xylostella* releases only thiol derivatives compounds (dimethyl trisulfide and *S*-methyl methane thiosulfinate) and not isothiocyanates. The differences between our results on the chemical composition of volatiles emitted by *P. xylostella* feeding on cabbage and those of Auger et al. (1989) could be due to differences between cultivars, age of the cabbage that *P. xylostella* consumed, or microorganisms.

Tritrophic Interactions. The damaged cabbage plant and the frass of larvae feeding on cabbage are two of the sources providing infochemicals used by the parasitoid *C. rubecula* to locate its hosts (Agelopoulos and Keller, 1994a,b). The females utilize the volatiles released by the feeding sites to land on damaged leaves. Plant damage is associated with the presence of hosts since feeding is essential for the development of larvae. Leaves having current feeding damage are preferred over leaves bearing old damage, but the latter are visisted as well since larvae of *P. rapae* do not feed continuously and old damage can be present simultaneously with hosts. The frass of larvae is another source providing infochemicals to females of *C. rubecula*. *C. rubecula* is a highly specialized parasitoid that attacks hosts mainly feeding on species of Brassicaceae. Since sulfur and nitrogen–sulfur-containing compounds are characteristic of Brassicaceae, it is highly likely that *C. rubecula* uses these chemicals in some stage of host location. Indications that such chemicals are involved in host selection come from the attraction of *C. rubecula* to frass of *P. xylostella*, which releases

only sulfur-containing compounds. Sources providing sulfur- and nitrogen–sulfur-containing compounds are the plant and the frass of larvae feeding on cabbage. Although the cabbage plant directly provides a few of these compounds, the frass of larvae is the main source providing such chemicals. The presence and concentrations of compounds such as dimethyl trisulfide, allyl and methyl isothiocyanate, methyl propyl sulfide and *S*-methyl methane thiosulfinate may play a decisive role in locating host-infested cabbages. Larvae of *P. rapae* exhibit four different types of behavior related to feeding damage and defecation, which could be adaptations to avoid natural enemies. They feed in short bouts, retreat from the feeding place, excrete outside the leaf, and remove the frass from the leaf surface (Jones, 1977; Lederhouse, 1990). *C. rubecula* has developed strategies to overcome the concealed life-style (retreating from feeding sites) of *P. rapae*. It has been observed that once on the leaf, females palpate the damaged and undamaged sites with their antennae (Nealis, 1986; our personal observations). If larvae are not encountered on the damaged sites, the search continues on the undamaged part of the leaf, so larvae resting away from the feeding sites can be located. This pattern of searching seems to be related to damage, since females exhibited the same behavior when on mechanically damaged plants, although the time spent searching was much less. The last two types of behavior employed by larvae of *P. rapae*, defecation outside the leaf and removal of frass, must be considered in conjunction with volatile chemicals released by frass. Frass releases volatile chemicals characteristic of current feeding by *P. rapae* (allyl isothiocyanate and 4-methyl-3-pentenal), characteristic of damage (*trans*-2-hexenal) and chemicals characteristic of the identity of the host plant (sulfur–nitrogen compounds). When the frass is discarded outside the leaf, it contaminates the lower leaves of the plant and the surrounding ground, misguiding natural enemies away from the feeding sites of *P. rapae*.

The costs and benefits of the strategies adapted by species of the tritrophic system and, in general, the study of interactions inside a tritrophic system cannot progress realistically unless all the trophic levels are considered. For example, until now the chemical information provided by plants is considered to be a measure employed by plants to guide the natural enemies of the herbivore, but the infochemicals released during damage and defecation probably have other effects besides attraction of natural enemies of larvae of *P. rapae*. The infochemicals released by an infested plant are available to all trophic levels. The same information used by the parasitoid to locate the herbivore may be utilized by hyperparasitoids, the fourth trophic level, to locate the parasitoids. Infochemicals from plants damaged by *P. rapae* may deter other conspecific or interspecific herbivores from searching on infested plants. Indications that the latter may be true come from a study by Landolt (1993), who showed that attraction of the moth *Trichoplusia ni* (Hübner) to cabbage was decreased when it was infested by conspecific larvae.

Although the chemical part of our work provided information about the qualitative composition of the blend of volatile infochemicals released by cabbage plants that are currently infested by *P. rapae* and *P. xylostella*, extended bioassays on the orientation behavior of *C. rubecula* have to be conducted to determine which chemicals (quality–quantity) of the identified blend are utilized to locate hosts.

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ADDITIONAL MALE MEDITERRANEAN FRUITFLY (*Ceratitis capitata* WIED.) ATTRACTANTS FROM ANGELICA SEED OIL (*Angelica archangelica* L.)

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Abstract—Two sesquiterpene hydrocarbons, β-copaene and β-ylangene, were isolated from bioactive fractions of angelica seed oil and were shown by field bioassays to be attractive to the male Mediterranean fruit fly. Their relative attractiveness, compared with the (+)- and (−)-α-copaene enantiomers, are: (+)-α-copaene > angelica β-copaene > angelica β-ylangene > (−)-α-copaene. The enantiomer ratios for the two compounds are: β-copaene, 61.4% (+), 38.6% (−); β-ylangene, 91.9% (+), 8.1% (−). *trans*-α-Bergamotene was also isolated from the same fractions, but in insufficient quantity for bioassay [enantiomer ratio: 95.7% (+), 4.3% (−)].

Key Words—Attractants, medfly, *Ceratitis capitata*, Diptera, Tephritidae, α-copaene, β-copaene, β-ylangene, *trans*-α-bergamotene, Angelica seed oil, *Angelica archangelica*, enantiomers, male lures.

INTRODUCTION

Angelica seed oil (*Angelica archangelica* L.) is attractive to the male Mediterranean fruitfly (*Ceratitis capitata* Wied.) (Steiner et al., 1957). More than 800 lb of this oil was employed in nearly 50,000 traps to monitor the progress of the 1956 fly eradication program in Florida. A group of Italian researchers (Fornasiero et al., 1969; Guiotto et al., 1972) subsequently fractionated Angel-

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ica seed oil and identified α-copaene and α-ylangene, two structurally related tricyclic sesquiterpenes, as the most effective attractive components of the oil. Jacobson et al. (1987) isolated α-copaene from both angelica seed oil and copaiba oil, identifying the angelica material as dextrorotatory α-copaene ($[\alpha]_D^{23}$ + 6.4°), and that from copaiba as levorotatory α-copaene ($[\alpha]_D^{23}$ − 6.3°). [In the following discussion, the terms "dextrorotatory" and "levorotatory" are used in reference to isolates having positive or negative specific rotations. The designations "(+)" and "(−)" are used when referring to specific enantiomers of a compound. The absolute stereochemical configurations of copaene and ylangene (+) enantiomers are shown in Figure 1.]

Because of the effectiveness of dextrorotatory angelica α-copaene as a male medfly attractant, considerable interest has been generated in finding an inexpensive and abundant source for the compound and/or in developing a simple, relatively high yield synthesis. Both approaches have been largely frustrated so far. The (−) enantiomer of α-copaene usually predominates in plant sources examined to date (Takeoka et al., 1990), and when a plant source is found in which (+)-α-copaene is the major enantiomer, the total α-copaene (both enantiomers) content is commonly low, usually less than 1% of an essential oil prepared from the plant. Similarly, published synthetic routes to α-copaene are not practical for production of the desired enantiomer in quantity (Heathcock, 1966; Heathcock et al., 1967; Corey and Watt, 1973).

Several years ago, the present authors initiated a search for male medfly

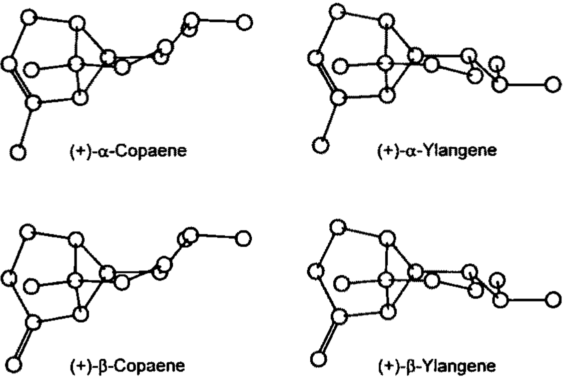

FIG. 1. Absolute configurations of copaene and ylangene (+) enantiomers. The absolute stereochemistry of (−)-α-copaene was previously reported by deMayo et al. (1965) and Kapadia et al. (1965), and that of (+)-α-ylangene by Ohta and Hirose (1969). The (+)-β-copaene and (+)-β-ylangene structures follow from double bond isomerization of dextrorotatory β-copaene and β-ylangene isolates to corresponding α- isomer products in which the (+) enantiomers are in excess (see Results and Discussion).

attractants other than α-copaene among the components of a group of essential oils shown by one of the authors (R.T.C.) to be attractive to the male medfly. Plant-derived essential oils have several advantages as potential sources of bioactive compounds. They contain components that fall into a relatively narrow and useful range of volatilities, particularly if the monoterpene hydrocarbons are removed. In addition, the components either occur naturally in plants or are derivatives of such components, formed during oil production. As such, they might be expected to have relatively low potential for being environmentally harmful. Offsetting such advantages, many essential oil components possess fairly complex structures, considering their molecular weight range, and may prove difficult to synthesize in quantity once they are identified as promising attractants. This is the situation at present with (+)-α-copaene. To have a reference against which the bioassay performance of candidate fractions and isolated components might be compared, a multigram supply of (+)-α-copaene was isolated from angelica seed oil. During this isolation process numerous fractions were generated, and many were submitted for field bioassay. In most instances the attractancy correlated well with the (+)-α-copaene content of the individual cut, but some active cuts were found to contain little if any α-copaene or α-ylangene. Several of these latter active fractions were further examined in the present study, in an attempt to identify the bioactive component(s).

METHODS AND MATERIALS

Angelica Seed Oil. The oil was purchased in kilogram quantities from R.W.B. Starke & Son, Eye, Suffolk, England. By capillary gas chromatography, the α-copaene content of the oil was 0.93%, and the α-ylangene content was 0.03%. An earlier sample, used in initial bioassay tests, was obtained from Polarome, New York, New York. It contained 0.6% α-copaene and less than 0.1% α-ylangene.

Trimedlure. Commercial product (UOP) was used in male medfly trapping.

Adsorbents. EM Silica gel 60 (170–250 mesh) was used for most gravity-feed liquid chromatography fractionations. Silver nitrate-impregnated (15% w/w) silica gel was used in efforts to isolate individual components from enriched fractions. Woelm neutral aluminum oxide (activity grade I) was employed to isomerize exocyclic double bonds to trisubstituted endocyclic double bonds.

Vacuum Fractional Distillation. A Perkin-Elmer model 251 Auto Annular Still (Teflon spinning band) maintained at 10 mm Hg was employed for vacuum fractionations.

Gas Chromatographic and GC-MS Instrumentation. Hewlett Packard 5830A and 5840A gas chromatographs, each fitted with a split/splitless injector and flame ionization detector (FID), were used for most analytical separations.

Hewlett Packard 5890 Series II GCs, similarly equipped with split/splitless injector and FID, were also employed for more recent analyses. The 5890s were interfaced with an HP 3365 Chemstation for data handling. A FinniganMAT 4500 quadrupole mass spectrometer/SuperINCOS data system provided mass spectra and library matching for component identification. Bonded and cross-linked fused silica columns (60 m × 0.32 mm ID, 0.25-μm film thickness) were installed in the units above. Both methylsilicone (DB-1; J & W Scientific) and Carbowax-type (DB-Wax; J & W) columns were employed. Enantiomeric separations were obtained with a 30-m × 0.32-mm-ID (0.25-μm film thickness) Cyclodex-B fused silica column (permethylated β-cyclodextrin in 1701 substrate; J & W). Typical GC operating conditions were: DB-1, 50°C to 250°C at 4°/min; DB-Wax, 50°C to 230°C at 4°C/min; Cyclodex B, 100°C for 2 min, 100°C to 150°C at 0.5°C/min. A Varian model 3700 GC with on-column injector and thermal conductivity detector was employed for component isolation when liquid chromatography and vacuum distillation were not adequate. Two glass columns were used with the preparative unit: 4.0 m × 4 mm ID, packed with 5% Carbowax 20 M on 80–100 mesh Chromosorb G; and 4.0 m × 4 mm ID, packed with 5% SF-96(50) (methylsilicone oil) + 0.1% Igepal 880 on 70/80 mesh silanized Chromosorb G. The Carbowax 20 M column was typically operated isothermally at 140°C, and the SF-96(50) at 150°C.

Fractionation Sequence. The sequence first used in fractionating the starting angelica seed oil is shown in Figure 2. The bold lines indicate the α-copaene flow through the sequence. In the final step, the Starke-derived dextrorotatory α-copaene (3.38 g, from 718.5 g of starting oil; 0.47%) was pooled with a smaller amount (1.92 g) isolated similarly from angelica seed oil obtained from Polarome Manufacturing Co. A second lot of Starke angelica seed oil was fractionated in a similar manner, to obtain additional quantities of active cuts for further fractionation and component isolation.

(+)-α-Copaene. Sample MFA1-54-4 (5.30 g; 95.3% α-copaene) is predominantly the (+) enantiomer, as determined by Cyclodex-B GC/FID and GC-MS analyses [enantiomeric ratio: 98.6% (+), 1.4% (−)]. The sample has a specific rotation of $[\alpha]_D$ +5.92° (*c* 2.13). Jacobson et al. (1987) reported a value of +6.4° (*c* 1.20). A sample of this material was included as a point of reference in each set of bioassay samples.

(−)-α-Copaene. A sample of (−)-α-copaene (MFA1-46-12; 6.3 g; 98.7%; enantiomeric ratio: 0.3% (+), 99.7% (−), based upon Cyclodex-B capillary GC analysis) was isolated from cubeb oil by a combination of fractional distillation and silver nitrate–silica chromatography. The sample has a specific rotation of $[\alpha]_D$ −5.39° (*c* 2.06). Jacobson et al. (1987) reported a value of −6.3° (*c* 1.20). A sample of this material was included for reference in some of the bioassay test sample sets.

Infrared Spectra. Infrared spectra of isolated compounds were measured

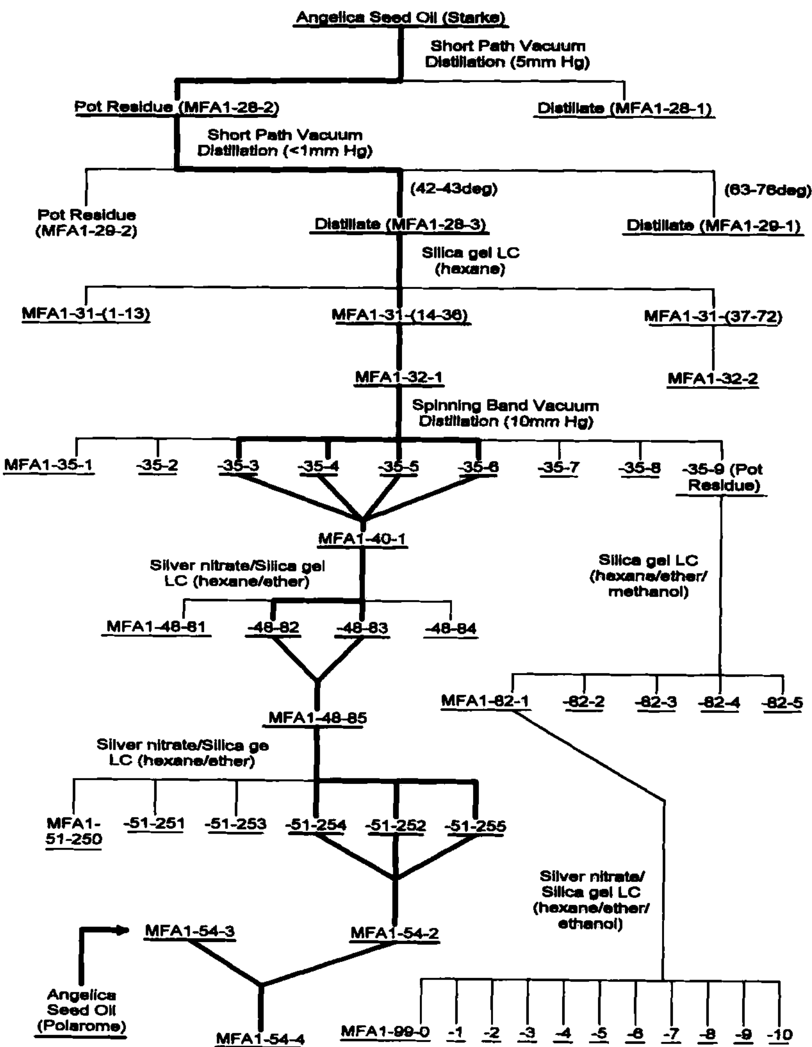

FIG. 2. Typical separation sequence used in isolation of dextrorotatory α-copaene from angelica seed oil. α-Copaene enrichment is shown by heavy line.

and plotted with a Perkin-Elmer model 237 infrared spectrophotometer. Samples were run as neat films on micro salt plates.

Specific Rotation. Rotations were determined with a Perkin-Elmer model

241 polarimeter, using a 1-ml microcell with a 1-dm path length. All samples were run in $CHCl_3$ solution at approx. 22°C, using the sodium D line.

Bioassay Insects. Flies were obtained from the mass-rearing colony at the USDA-ARS Tropical Fruit & Vegetable Research Laboratory in Honolulu, Hawaii. They had been sterilized by gamma irradiation one day before eclosion and were released into the test plot when they were 5–10 days old. The male–female ratio was approximately 1 : 1.

Bioassay. Test samples (0.010 or 0.020 ml; as noted in Tables 1–4 below) were pipetted onto cotton dental wicks [9.5 mm diameter × 6.4 mm long (3/8 in. × 1/4 in.); Johnson & Johnson No. 2] held in standard Jackson survey traps (Harris et al., 1971). Samples were replicated eight times (Tables 1 and 2 below) or five times (Tables 3 and 4 below). Traps were hung in a randomized field plot design (Tables 1, 2, and 4 below) or in a 4 × 4 balanced lattice plot design (Tables 3 below) on trees in a large macadamia nut orchard near Hilo, Hawaii. Each trap was 7.6 m or more from any other. A balanced lattice plot design was selected for the Table 3 field test to better accommodate the relatively large number of samples being evaluated. Laboratory-reared flies were released in a uniform fashion throughout the plot by allowing them to fly out of the holding cages as they were carried through the plot. At the time of the initial trap placement and immediately after each daily trap examination, 30,000–60,000 flies were released (1 : 1 sex ratio in total release) in the plots at about 0800 h Hawaii Standard Time. Long experience (R.T.C.) with testing lures against wild and laboratory flies has not shown any difference in the relative attraction of the two populations to various semiochemicals. The use of high populations of released flies in a relatively even spatial distribution reduces the coefficient of variation and increases the discriminatory power of the field plots over that which would be achievable with exposure to the wild populations in the field.

At each examination the sticky insert on the floor of the tent-shaped Jackson trap was replaced with a new one. The number of flies released was adjusted so as to avoid overloading the inserts on the most attractive treatments with trapped males. In general the flies die and/or leave the test plot rapidly (macadamia is not a breeding host), so that little resident population buildup occurs and most of the male fly captures are within one day of their release.

RESULTS AND DISCUSSION

The heavy line in the Figure 2 flow chart indicates the pathway originally followed to isolate a multigram quantity of dextrorotatory α-copaene from starting angelica seed oil. During this isolation process, other fractions containing little or no detectable amounts of α-copaene were obtained. These do not fall on the highlighted pathway in Figure 2. When submitted for bioassay, several

fractions were found to be more attractive to male medflies than might be expected, considering the absence or near-absence of any α-copaene. The present study is an investigation of these α-copaene-deficient samples, to find the basis for the activity.

One of the more promising samples produced during the fractionation sequence was MFA1-35-9, the pot residue remaining after vacuum distillation of seed oil material containing α-copaene (Figure 2). Table 1 lists typical results from a field bioassay. As reference points, samples of Trimedlure, angelica seed oil, (+)-α-copaene [angelica, 98.6% (+)] and (−)-α-copaene [cubeb; 99.7% (−)] were included among the test samples. MFA1-29-1 is a complex mixture of oxygenated and nonoxygenated material, mostly sesquiterpene-based. It showed relatively low continuing activity, and has not been examined further. The activity of MFA1-32-2 is consistent with its α-copaene content of 1.4–1.5%. MFA1-35-9 was found to be quite attractive, even though it contained 0.02% α-copaene at most. (MFA1-77-1, -78-1, and -79-1 are short-path distillation cuts prepared during fractionation of a second batch of Starke angelica seed oil. MFA1-77-1 was subsequently separated to provide additional α-copaene and MFA1-35-9-related components for isolation, bioassay, and identification.)

MFA1-35-9 was further fractionated by gravity feed preparative silica gel chromatography, producing five cuts (Figure 2), which were in turn bioassayed (Table 2). Trimedlure, angelica seed oil (Starke sample), and (+)-α-copaene were again included among the test samples. The bioassay results indicate that the bulk of the active material was concentrated in the first two cuts (MFA-1-82-1 and -82-2). The first of these was fractionated on silver nitrate-impregnated silica gel into 11 cuts (MFA1-99-0 through -99-10), which were submitted for bioassay (as noted above, 4 × 4 balanced lattice field plot design was used in this test, because of the relatively large number of samples being compared). The results are listed in Table 3. The activity was again found in the earlier cuts, with maximum activity in MFA1-99-3. By inspection of GC-FD and GC-MS data, three closely eluting sesquiterpene hydrocarbon components (A, B, and C) were found to have their highest concentrations in MFA1-99-2 or -99-3. They eluted after α-copaene on a DB-Wax capillary column, but preceded caryophyllene. Compounds B and C were readily identified as *trans*-α-bergamotene and β-copaene by mass spectral and retention index comparisons with those of authentic samples, but no satisfactory reference mass spectrum match could initially be found for that of compound A [*m/z* (%): 41 (19.9), 43 (8.2), 53 (7.4), 55 (14.6), 65 (7.7), 67 (12.3), 77 (25.9), 78 (8.2), 79 (29.5), 80 (5.2), 81 (25.2), 91 (56.4), 92 (13.0), 93 (28.7), 94 (8.4), 95 (8.2), 105 (61.1), 106 (15.0), 107 (22.0), 108 (6.4), 115 (6.9), 117 (10.2), 119 (2.7), *120 (100)*, 121 (17.2), 133 (19.3), 134 (5.1), 147 (11.1), 148 (6.4), 161 (96.2), 162 (14.6), 204 (7.3). A base peak of 120 is relatively uncommon among

TABLE 1. MALE FLY CATCH: ANGELICA SEED OIL FRACTIONS AND REFERENCE SAMPLES (8 REPLICATIONS)

Treatment (0.020 ml/wick)	α-Copaene concentration	Post treatment day (mean ± SE)[a]					
		Day 0	Day 1	Day 2	Day 3	Day 4	Day 7
Trimedlure		72.50 ± 14.17cd	80.62 ± 22.50bc	55.87 ± 5.86a	43.12 ± 6.07a	78.25 ± 14.14a	0.25 ± 0.16d
Angelica seed oil (Polarome)	<1%	65.25 ± 9.84cd	8.00 ± 4.63f	0.38 ± 0.26e	0 ± 0e	0 ± 0d	0.13 ± 0.13d
Angelica α-copaene-MFA1-54-4	95.3% (+/− = 98.6/1.4)	196.62 ± 23.05a	180.00 ± 16.49a	26.75 ± 8.01bc	25.50 ± 8.34b	34.00 ± 12.14b	24.38 ± 6.89a
Cubeb α-copaene-MFA1-46-12	98.7% (+/− = 0.3/99.7)	50.12 ± 16.41de	17.75 ± 6.67ef	0.62 ± 0.50e	0.12 ± 0.12e	0 ± 0d	0.38 ± 0.26d
High-boiling cut (Starke Angelica)-MFA1-29-1	0.00%	16.75 ± 4.41e	4.12 ± 3.43f	7.75 ± 3.42de	5.38 ± 2.66de	9.00 ± 4.66d	14.88 ± 3.50b
High-polarity LC cut, (Starke Angelica)-MFA1-32-2	1.4–1.5%	65.88 ± 17.76cd	40.75 ± 13.92de	14.25 ± 5.73cd	13.38 ± 5.07cd	2.75 ± 1.81d	0.25 ± 0.25d
Pot residue (Starke Angelica)-MFA1-35-9	0.02%	88.50 ± 9.28c	50.62 ± 14.67cd	26.88 ± 7.98bc	21.00 ± 6.16bc	28.38 ± 6.63bc	0.13 ± 0.13d
Low-boiling cut (Starke Angelica)-MFA1-77-1	10.2%	124.75 ± 25.95b	112.12 ± 23.34b	28.25 ± 9.06b	25.00 ± 6.62bc	6.25 ± 3.97d	0.63 ± 0.63cd
Intermediate cut (Starke Angelica)-MFA1-78-1	0.3%	48.62 ± 13.36de	14.75 ± 5.37ef	10.38 ± 1.78de	9.12 ± 2.73de	13.25 ± 3.90cd	6.88 ± 1.96c
High-boiling cut (Starke Angelica)-MFA1-79-1	<0.03%	15.62 ± 2.80e	0.50 ± 0.27f	0.38 ± 0.18e	0.25 ± 0.16e	2.00 ± 1.05d	4.75 ± 1.71cd
F(*df*)		15.084 (9, 63)	21.381 (9, 63)	12.458 (9, 63)	10.131 (9, 63)	12.596 (9, 63)	10.851 (9, 63)
EMS		1524.13	1262.85	198.93	160.20	378.84	49.886
kLSD		35.72	32.12	13.02	11.82	17.96	6.570

[a] Analysis of variance. Mean separation by Waller-Duncan (1969) kLSD test. Mean catches within a column followed by the same letter are not significantly different ($P < 0.05$).

TABLE 2. MALE FLY CATCH: ANGELICA SEED OIL CUTS (LOW α-COPAENE CONTENT) AND REFERENCE SAMPLES (8 REPLICATIONS)

Treatment (0.020 ml/wick)	α-Copaene concentration	Post treatment day (mean ± SE)[a]				
		Day 0	Day 1	Day 2	Day 3	Day 6
Trimedlure		96.75 ± 14.26b	151.75 ± 22.54b	90.38 ± 14.39a	21.50 ± 8.32ab	0.63 ± 0.26b
Angelica seed oil (Starke)	<1%	100.50 ± 12.75b	1.25 ± 1.11e	0 ± 0d	0.25 ± 0.25d	0.63 ± 0.32b
Angelica α-copaene-MFA1-54-4	95.3% (+/− = 98.6/1.4)	207.50 ± 17.75a	195.50 ± 30.80a	31.38 ± 6.06c	25.75 ± 4.70a	18.38 ± 6.04a
Pot residue (Starke Angelica) MFA1-35-9	0.02%	56.38 ± 14.23c	55.00 ± 8.48cd	35.38 ± 7.82bc	12.38 ± 5.86bc	0.88 ± 0.30b
LC cut (from MFA1-35-9)-MFA1-82-1	<0.1%	90.13 ± 8.80b	71.13 ± 7.77c	51.13 ± 10.03b	8.88 ± 4.37cd	0.63 ± 0.32b
LC cut (from MFA1-35-9)-MFA1-82-2	none detected	44.25 ± 11.78cd	29.63 ± 6.44de	12.88 ± 7.70d	2.63 ± 0.94cd	0.38 ± 0.18b
LC cut (from MFA1-35-9)-MFA1-82-3	none detected	22.13 ± 6.26de	0.38 ± 0.26e	0 ± 0d	0.88 ± 0.52cd	0.88 ± 0.52b
LC cut (from MFA1-35-9)-MFA1-82-4	none detected	8.38 ± 2.29e	0.25 ± 0.16e	0 ± 0d	1.00 ± 0.76cd	1.00 ± 0.63b
LC cut (from MFA1-35-9)-MFA1-82-5	none detected	7.50 ± 2.54e	0.50 ± 0.27e	0 ± 0d	0.50 ± 0.27d	0.38 ± 0.26b
$F(df)$		34.076 (8, 56)	32.243 (8, 56)	19.810 (8, 56)	5.577 (8, 56)	8.352 (8, 56)
EMS		933.223	1297.469	391.213	136.609	33.400
kLSD		27.327	32.252	17.917	11.504	5.469

[a] Analysis of variance. Mean separation by Waller-Duncan (1969) kLSD test. Mean catches within a column followed by the same letter are not significantly different ($P < 0.05$).

TABLE 3. MALE FLY CATCH: LOW α-COPAENE CONTENT ANGELICA SEED OIL CUT, SILICA GEL FRACTIONS, AND REFERENCE SAMPLES (5 REPLICATIONS)

Treatment (0.010 ml/wick)	α-Copaene concentration	Replication[a]					Total	Mean ± SE
		1	2	3	4	5		
Trimedlure		371	87	355	243	100	1156	231.20 ± 60.42c
Angelica seed oil (Starke)	<1%	84	23	168	126	86	487	97.40 ± 24.13efg
Angelica α-copaene-MFA1-54-4	95.3% (+/− = 98.6/1.4)	586	936	670	618	458	3268	653.60 ± 78.78a
Pot residue, Starke Angelica-MFA1-35-9	0.02%	96	474	165	292	111	1138	227.60 ± 70.60cd
LC cut (from MFA1-35-9)-MFA1-82-1	<0.1%	268	30	215	276	154	943	188.60 ± 45.27cde
LC cut (from MFA1-82-1)-MFA1-99-0[b]	none detected	14	4	74	217	15	324	64.80 ± 40.00fg
LC cut (from MFA1-82-1)-MFA1-99-1	0.44%	149	231	73	21	201	675	135.00 ± 39.12cdef
LC cut (from MFA1-82-1)-MFA1-99-2	0.12%	405	74	173	291	52	995	199.00 ± 66.64cde
LC cut (from MFA1-82-1)-MFA1-99-3	0.09%	431	256	471	434	224	1816	363.20 ± 51.04b
LC cut (from MFA1-82-1)-MFA1-99-4	none detected	163	21	315	83	16	598	119.60 ± 55.61defg
LC cut (from MFA1-81-1)-MFA1-99-5	none detected	31	73	81	73	29	287	57.40 ± 11.29fg
LC cut (from MFA1-82-1)-MFA1-99-6	none detected	26	31	40	20	19	136	27.20 ± 3.87fg
LC cut (from MFA1-82-1)-MFA1-99-7	none detected	27	38	28	37	19	149	29.80 ± 3.51fg
LC cut (from MFA1-82-1)-MFA1-99-8	none detected	27	28	15	14	30	114	22.80 ± 3.43g
LC cut (from MFA1-82-1)-MFA1-99-9	none detected	44	36	5	27	8	120	24.00 ± 7.65g
LC cut (from MFA1-82-1)-MFA1-99-10	none detected	27	34	75	6	11	153	30.60 ± 12.22fg
$F(df)$								15.124 (15, 60)
EMS								9054.755
kLSD								110.112

[a] 4 × 4 balanced lattice field plot design (Cochran and Cox, 1957); 5 replications; total catch for each replication, days 0–4 (no correction of error mean square called for in analysis of balanced lattice). Mean catches within a column followed by the same letter are not significantly different [$P < 0.05$; Waller and Duncan (1969) kLSD test].

[b] Insufficient sample available for five replications.

the sesquiterpene hydrocarbons.] β-Copaene was previously found in angelica root oil by Taskinen and Nykänen (1975). Because the unknown behaved similarly to β-copaene throughout the fractionation procedure, and because of some preliminary retention index-based peak assignments arrived at by one of the authors (T.R.M.) in analyses of other essential oils, compound A was tentatively identified as β-ylangene. In their paper identifying the structure of β-ylangene, Hunter and Brogden (1964) also provided infrared and mass spectra for the compound, which they isolated from cold-pressed orange oil. The relative intensities of the fragment ions in their mass spectrum differ considerably from those in our compound A mass spectrum, but the 120 fragment abundance does appear to be higher than that of the 119. Hirose (1967), in his discussion of sesquiterpene mass spectra, listed 120 as a "key" fragment for β-ylangene. Schreier et al. (1976) reported finding β-ylangene in grapes, but the mass spectrum they listed for β-ylangene does not include a 120 fragment.

In order to measure specific rotations, determine enantiomeric ratios, acquire infrared spectra, and assess the relative attractiveness of each of the three sesquiterpenes, enriched fractions in which the three compounds predominated were fractionated with the preparative gas chromatograph, in order to purify samples of each. By repeated chromatography on methylsilicone and polyethyleneglycol phases, samples of β-copaene (96.0%; MFA1-158-1, 154 mg) and the tentatively-identified β-ylangene (97.3%, MFA1-158-2, 108 mg) were isolated. *trans*-α-Bergamotene could not be freed from residual β-copaene and β-ylangene, even after repeated prepping.

Infrared spectra of the isolated angelica seed oil samples were very good matches with published spectra of β-copaene (Westfelt, 1967) and β-ylangene (Hunter and Brogden, 1964), validating the β-ylangene identification. The angelica β-copaene spectrum has prominent bands at 1638, 1461, 1382, 1373, 1363 and 867 cm^{-1}. The infrared spectrum of angelica β-ylangene is nearly superimposible on that of the β-copaene, with major bands at 1642, 1463, 1380, 1375, 1367 and 870 cm^{-1}.

Specific rotations for the purified samples were $[\alpha]_D$ +0.10° (*c* 1.56) for α-copaene and $[\alpha]_D$ +46.47° (*c* 1.01) for β-ylangene [the observed rotation with the α-copaene solution (0.001–0.002°) approached the readout limits of the polarimeter (0.000°), so the calculated specific rotation is only a tentative value]. Literature values of −7.6° and +19.3°, respectively, have been reported (Westfelt, 1966). Attempts to resolve enantiomer constituent peaks with the β-cyclodextrin-based capillary column were largely unsuccessful. At best, only the β-copaene sample could be partly resolved (60% valley) into two enantiomer peaks. However, the corresponding α- double bond isomers of both compounds are readily resolved on this column, with two well-separated peaks, representing the (+) and (−) enantiomers, appearing in the resulting chromatogram. Westfelt had noted in his 1967 paper that, on standing in contact with neutral or basic

alumina overnight, β-copaene was almost quantitatively isomerized to α-copaene. This conversion was used by us to isomerize isolated samples of the angelica β-copaene and β-ylangene to the corresponding α-isomers. GC-FID and GC-MS analyses of the respective products indicated that the conversions were indeed nearly quantitative; by-products and unchanged impurities from the starting β-isomer mixtures represent less than seven percent of each of the two product mixtures. The α-copaene produced was separated into two enantiomeric peaks on the Cyclodex-B column: 61.4% (+) and 38.6% (−) [enantiomer excess = 22.8% (+)]. These values therefore also apply to the original β-copaene isolated from the bioactive angelica seed oil cuts. Surprisingly, this β-copaene enantiomer ratio is considerably different from the ratio determined for α-copaene isolated directly from angelica [98.6% (+), 1.4% (−)]. The α-ylangene product from β-ylangene isomerization was also run on the Cyclodex-B column, yielding two enantiomer peaks: 91.9% (+) and 8.1% (−) [enantiomeric excess = 82.8% (+)]. Again, these values also apply to the starting β-ylangene isolated from the angelica cuts. α-Ylangene was not isolated by us from the angelica seed oil, but enantiomer estimates of 85% (+) and 15% (−) were made by analyzing several α-ylangene-enriched fractions on the Cyclodex-B column. These estimates suggest that the angelica α-ylangene enantiomer ratios differs from that of the angelica β-ylangene, but the difference is not as pronounced as it is for the corresponding angelica α- and β-copaenes.

The isomerization process, converting β-isomers to α-isomers, provided a path to purification of the angelica *trans*-α-bergamotene, which could not be freed from residual β-copaene and β-ylangene. The sample was treated overnight with neutral alumina, converting the contaminants to the α-isomers, which have retention indices differing significantly from that of unchanged *trans*-α-bergamotene on both methylsilicone and polyethyleneglycol stationary phases. The sample was then reprepped, yielding 98.5% pure *trans*-α-bergamotene (T45-137-4, 30 mg). The infrared spectrum of this product is a very good match with the spectrum of *trans*-α-bergamotene (Wenninger et al., 1967; Wenninger and Yates, 1970). A specific rotation of $[\alpha]_D$ +49.57° (*c* 1.70) was determined. Krishnappa and Dev (1973) reported a specific rotation of $[\alpha]_D$ −51° for levorotatory *trans*-α-bergamotene isolated from *Lansium anamalayanum* wood. The component enantiomers were well-separated on the Cyclodex B column: 95.7% (+) and 4.3% (−) [enantiomeric excess = 91.4% (+)]. The (−) enantiomer eluted first, in contrast with the elution order in the α-copaene and α-ylangene enantiomer separations, where the (+) enantiomer appeared before the (−).

The three sesquiterpenes isolated were not fully resolved from adjacent components in capillary GC-FID analyses of the starting angelica seed oil, but separations on both DB-1 and DB-Wax columns permitted us to estimate percent concentrations: β-ylangene—0.11%; *trans*-α-bergamotene—0.12%; and

β-copaene—0.27%. These values may be compared with those noted above for α-copaene (0.93%) and α-ylangene (0.03%).

Samples of isolated angelica β-copaene and β-ylangene were submitted for field bioassay. Unfortunately, insufficient purified *trans*-α-bergamotene was available for field testing. Results are summarized in Table 4. Both isolated angelica components, β-copaene [enantiomer ratio: 61.4% (+), 38.6% (−)] and β-ylangene [enantiomer ratio: 91.9% (+), 8.1% (−)], are attractive to male medflies. The β-copaene isolate appears to be somewhat more attractive than β-ylangene, but neither shows nearly the activity displayed by (+)-α-copaene. The cubeb (−)-α-copaene [enantiomer ratio: 0.3% (+), 99.7% (−)] is the least attractive of the four test samples (The cyclocopacamphene and cyclosativene samples were included as part of a separate ongoing study of α-copaene analog attractiveness).

It would be very useful to have clean samples of each of the β-copaene and β-ylangene enantiomers and both α-ylangene enantiomers available for bioassay comparisons. Unfortunately, little information is available in the literature on the enantiomeric composition of the copaenes and ylangenes found by researchers in various plant and essential oil substrates, so rational selection of potential sources containing only one enantiomer of a given copaene or ylangene is nearly impossible. The two β-copaene enantiomers can be produced by chemical conversion of the corresponding α- enantiomers (Westfelt, 1967), but production of the β-ylangenes by the same pathway is limited by the availability of good sources for enantiomerically pure α-ylangenes. Motl et al. (1963, 1965) isolated "a large amount" of a sesquiterpene that they identified as (+)-α-ylangene ($[\alpha]_D$ +50.5°) from the essential oil of *Schizandra chinensis* (Turcz.) Baill. fruits. Ohta et al. (1968) subsequently used the same oil as a source of (+)-α-ylangene ($[\alpha]_D$ + 55.6°). A sample identified as *S. chinensis* fruit oil was provided to us (Teranishi, 1990, personal communication; sample originally a gift from Mr. Liu, Peoples Republic of China), but only traces of α-ylangene and less than 4% α-copaene were found in this lot by GC-FID and GC-MS analysis.

In summary, of the angelica seed oil sesquiterpenes isolated in this study (as mixtures of enantiomers) and tested in field bioassays, α-copaene [enantiomer ratio: 98.6% (+), 1.4% (−)] remains the component most attractive to the male medfly. β-Copaene [enantiomer ratio: 61.4% (+), 38.6% (−)] is of intermediate attractiveness, and β-ylangene [enantiomer ratio: 91.9% (+), 8.1% (−)] is least attractive of the three, although still active. Guiotto et al. (1972) isolated both α-copaene (dextrorotatory; Jacobson et al., 1987) and α-ylangene from angelica seed oil and stated that laboratory bioassays showed both to be equally attractive to male medflies. Since α-ylangene was not isolated in the present study, we cannot verify this assessment by field bioassays. Neither the

TABLE 4. MALE FLY CATCH: REFERENCE GROUP PLUS COPAENE-RELATED COMPOUNDS (5 REPLICATIONS)

Treatment (0.010 ml/wick)	Purity[a] (%)	Enantiomer ratio (+)	Enantiomer ratio (−)	Post treatment day (mean ± SE)[b] Day 0 (mean ± SE)	Day 1 (mean ± SE)	Day 2 (mean ± SE)	Day 3 (mean ± SE)	Day 4 (mean ± SE)
Trimedlure				34.00 ± 5.81de	192.00 ± 28.33a	164.00 ± 29.49a	0.40 ± 0.40b	0 ± 0b
Angelica α-copaene-MFA1-54-4	95.3	98.6	1.4	269.60 ± 11.07a	238.00 ± 14.43a	125.60 ± 28.44a	51.00 ± 14.71a	59.20 ± 22.32a
Cubeb α-copaene-MFA1-46-12	98.7	0.3	99.7	41.40 ± 17.86cde	3.00 ± 1.41d	1.60 ± 0.60b	0.20 ± 0.20b	0.80 ± 0.37b
Angelica β-copaene-MFA1-158-1	96.0	61.4	38.6	98.00 ± 21.69b	138.20 ± 35.30b	33.40 ± 11.56b	0.20 ± 0.20b	0.60 ± 0.40b
Angelica β-ylangene-MFA1-158-2	97.3	91.9	8.1	50.00 ± 10.03cd	79.60 ± 13.86c	4.00 ± 2.55b	0.20 ± 0.20b	0.20 ± 0.20b
Orange β-copaene-MFA1-158-3[c]	97.8	95.4	4.6	70.20 ± 18.22bc	89.40 ± 39.96bc	18.40 ± 12.52b	0.40 ± 0.24b	0.20 ± 0.20b
Orange cyclocopacamphene-MFA1-158-4	99.4	not resolved		9.40 ± 1.03e	2.80 ± 2.31d	0 ± 0b	0 ± 0b	0 ± 0b
P. longifolia cyclosativene-MFA1-158-5	99.5	not resolved		16.60 ± 4.79de	0.60 ± 0.40d	1.00 ± 0.45b	0.20 ± 0.20b	0.80 ± 0.58b
$F(df)$				39.625 (7, 28)	21.261 (7, 28)	18.132 (7, 28)	6.668 (7, 28)	6.894 (7, 28)
EMS				893.304	1943.064	1142.680	241.614	313.816
kLSD				34.803	52.074	40.130	19.627	22.292

[a] Determined with DB-1 column.
[b] Analysis of variance. Mean separation by Waller-Duncan (1969) kLSD test. Mean catches within a column followed by the same letter are not significantly different ($P < 0.05$).
[c] Sample-limited; one (of five) replications slightly underdosed (<0.010 ml).

specific rotation nor the enantiomeric makeup of this angelica seed oil component have been reported in the literature.

The only other comparison of the relative attractiveness of (+)- and of (−)-α-copaene appearing in the literature is that of Warthen and McInnis (1989). They used a laboratory bioassay to compare the short-range attraction/feeding stimulation of male medflies. Using (+)-α-copaene from angelica seed oil and (−)-α-copaene from copaiba oil (Jacobson et al., 1987), they found no difference in intensity of response. Several copaiba oil samples from our files were examined with the Cyclodex-B column and the α-copaene present was found to be 99+% (−) enantiomer, so their test samples were probably similar in enantiomeric purity to those used by us in the field bioassays noted above. Their bioassay design may have exposed their test insects to sufficiently high levels of test samples that differences in attractiveness were masked. To some extent, similar effects might be deduced from the field bioassay data summarized in Tables 1–4 above. Typically, traps collected considerable numbers of male flies on the first day of exposure, no matter what the test sample. The release rate of any sample from the cotton dental wick would be highest on the first day, and first-day catches may reflect a relatively nonselective response on the part of the medflies to these volatiles plumes. The release rate of a given sample would drop rapidly on successive days, so comparisons of trap catches after the first day are likely better indicators of a test sample's effectiveness, relative to other test samples, than are first day counts.

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TANNIN SENSITIVITY IN LARVAE OF *Malacosoma disstria* (LEPIDOPTERA): ROLES OF THE PERITROPHIC ENVELOPE AND MIDGUT OXIDATION

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Abstract—Final-instar *Malacosoma disstria* fed artificial diets containing tannic acid develop lethal pupal deformities. We examined some of the factors potentially underlying tannin sensitivity in this species, including the permeability of the peritrophic envelope to tannic acid and the chemical fate of tannic acid in the gut. Tannic acid does not penetrate the peritrophic envelope of *M. disstria*, demonstrating that the containment of tannic acid within the endoperitrophic space is not sufficient to protect an insect herbivore from the adverse effects of ingested tannins. Ingested tannic acid undergoes extensive chemical modification in the midgut. Only 19–21% of the high molecular weight components of the tannic acid ingested was recovered in the frass. Of two possible chemical fates of ingested tannic acid, oxidation is the predominant chemical transformation, whereas little hydrolysis occurs. Measurements of gut redox parameters showed that conditions in the midgut favor the oxidation of phenols. However, similar conditions occur in the midguts of *Orgyia leucostigma*, in which no oxidation occurs. Therefore, oxidizing gut redox conditions do not necessarily lead to polyphenol oxidation in lepidopteran larvae. We conclude that the sensitivity of *M. disstria* to ingested tannins is a consequence of their oxidation in the midgut.

Key Words—Lepidoptera, Lasiocampidae, *Malacosoma disstria*, *Orgyia leucostigma*, larva, tannin, tannic acid, peritrophic membrane, oxidation.

INTRODUCTION

Insect herbivores vary greatly in their sensitivity to ingested tannins. In tannin-sensitive species, low levels of dietary tannins can have a variety of adverse

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consequences, ranging from reduced growth and abnormal development to death. Whereas at one time it was believed that the adverse effects of tannins resulted from their proclivity to bind with proteins in the gut and inhibit protein digestion (Feeny, 1976; Rhoades and Cates, 1976), it is now recognized that tannins and other polyphenols can act by a variety of different mechanisms in insects, including inhibition of feeding (Schultz, 1989; Hagerman and Butler, 1991), reduction in the efficiency of utilization of nutrients (Felton et al., 1989), and formation of lesions in the epithelial layer of the midgut (Bernays et al., 1980; Steinly and Berenbaum, 1985). It seems likely that in many cases the agent responsible for these effects is an oxidation product (e.g., a quinone) or a by-product of oxidation (e.g., superoxide anion radicals, hydroxyl radicals, or hydrogen peroxide) rather than the polyphenol itself (Appel and Martin, 1990; Appel, 1993).

Two factors are likely to be important in determining whether an insect will be adversely affected by ingested tannins: (1) the permeability of the peritrophic envelope (multiple layers of peritrophic membranes) to tannins or cytotoxic transformation products of tannins, and (2) the chemical conditions in the midgut that determine the chemical fate of ingested tannins.

We have recently reported a study of the permeability of the peritrophic envelope to tannic acid in tannin-tolerant larvae of the white-marked tussock moth, *Orgyia leucostigma* (Lymantriidae), and of the chemical fate of ingested tannic acid in this species (Barbehenn and Martin, 1992). We determined that in these larvae the peritrophic envelope is impermeable to tannic acid and that virtually all (90–100%) ingested tannic acid is egested unchanged in the frass. Thus, tannin tolerance was associated with the effective containment of tannic acid within the endoperitrophic space and a lack of oxidation of tannic acid in the gut. In his seminal study of tannins and the oak moth, *Operophtera brumata*, Feeny (1970) also presented evidence suggesting that the peritrophic envelopes of *O. brumata* larvae are impermeable to tannins.

In the present study we have undertaken a similar exploration of the fate of ingested tannic acid in the tannin-sensitive larvae of the forest tent caterpillar, *Malacosoma disstria* (Lasiocampidae), a polyphagous tree-feeder (Stehr and Cook, 1968). Despite a wide host range that includes many tannin-producing trees (e.g., *Quercus* spp.), *M. disstria* larvae are highly sensitive to dietary tannins. When as little as 0.5% (dry weight) tannic acid is included in an artificial diet, early-instar larvae are unable to complete development, and larvae fed such a diet during the final instar develop lethal pupal deformities (Karowe, 1989). Like many oak-feeding Lepidoptera, this species feeds on its hosts during the time of leaf expansion, when tannins are present at their lowest levels (Feeny, 1968).

In this study, our experiments address the following questions: (1) Is the peritrophic envelope of *M. disstria* larvae permeable to tannic acid? (2) Is tannic acid chemically transformed during its passage through the gut? (3) If chemical

transformation occurs, in what region(s) of the gut does this process take place? (4) By what process(es) are tannins chemically transformed? and (5) Are there differences in the redox conditions in the midguts of the tannin-sensitive larvae of *M. disstria* and the tannin-tolerant larvae of *O. leucostigma*?

METHODS AND MATERIALS

Insects and Diets. M. disstria and *O. leucostigma* eggs were obtained from the Forest Pest Management Institute (Sault Ste. Marie, Ontario). Larvae were reared at 22°C under a 16-hr light–8-hr dark photoperiod at 70% average relative humidity, unless otherwise noted. *M. disstria* larvae were fed an artificial diet (Addy, 1969), modified by substituting 7.0 g of agar for sodium alginate and adding 4.0 g of methyl paraben (Sigma) per 605 g of dry diet. *O. leucostigma* larvae were reared on *O. leucostigma* diet (Bio-Serv Inc., Frenchtown, New Jersey) unless otherwise indicated.

Permeability of Peritrophic Envelope to Tannic Acid. Mid-fifth-instar (final-instar) larvae ($N = 11$) were fed freshly prepared diet containing 3% dry wt tannic acid (Sigma lot No. 64F-0049) for two days. Intact guts were dissected from chilled (−20°C, 7 min) larvae, the fore- and hindguts were ligated with silk sutures (size 6-0), and a small hole (average area 1.5 $mm^2 \pm 0.1$ SE) was cut through the mid-midgut wall to expose the peritrophic envelope (Barbehenn and Martin, 1992). Dissected gut preparations were rinsed in three saline solutions (227 mM KCl, 3 mM NaCl, 300 mM fructose, pH 6.1) for 30–60 sec and incubated in 2.0 ml of the same solution in a shaker bath (2 hr, 23°C). Aliquots (1.5 ml) of incubating solution were lyophilized, redissolved in 0.75 ml of mobile phase (20% (v/v) aqueous acetonitrile and 1% (v/v) acetic acid) and filtered (0.45 μm, Gelman Acrodisc). Tannic acid in these solutions was quantified using HPLC (10-μl injection; Waters 10-μm C-18 column, 4.6 × 250 mm; flow rate 1.0 ml/min; Shimadzu UV detector, 280 nm, 0.002 AU). Peak areas were integrated with a Shimadzu C-R4A Chromatopac. Control larvae fed a tannin-free diet ($N = 3$) were prepared similarly to determine the presence of interfering peaks in the HPLC chromatograms. The midgut contents of each larva were dried (60°C, two days) and weighed to calculate the amount of tannic acid initially present in each midgut.

In a second test of the permeability of the peritrophic envelope to tannic acid, the above procedure was repeated with the following modifications: final-instar larvae ($N = 11$) were fed artificial diet containing 8% (dry wt) tannic acid or control diet ($N = 2$), holes were cut through the anterior midgut wall, gut preparations were incubated in a pH 7.5 buffered saline solution containing 3 mM sodium chloride, 228 mM potassium chloride, 41 mM magnesium sulfate, 20 mM calcium chloride, 52 mM Tris (hydroxymethyl) aminomethane (Sigma),

7.5 mM Tris(hydroxymethyl)aminomethane hydrochloride, and 440 mM fructose, and 30-μl samples were analyzed using HPLC.

Permeability of Peritrophic Envelope to Brown Pigments. The oxidation of tannins and other polyphenols produces brown pigments that can be quantified by measuring their absorbance at 420 nm (Cilliers and Singleton, 1989). We observed that brown pigments leached out of the fenestrated midguts of *M. disstria* that contained tannic acid. To quantify the amounts of brown pigments that permeated the peritrophic envelope, the incubating solutions from the second experiment described in the previous section (8% tannic acid) were analyzed with HPLC as described above, with the exception that the absorbance of the eluate was measured at 420 nm.

Tannic Acid Budget. Final-instar larvae ($N = 13$) were placed individually in 30-ml plastic cups with ventilated lids. Larvae were fed artificial diet containing 3% (dry wt) tannic acid for three days, following which they were fed a tannin-free diet until all dark-colored (tannin-containing) frass had been collected. Larvae fed the tannin-free rearing diet ($N = 4$) served as controls to measure the presence of any substances in the frass that interfere with the measurement of egested tannic acid. Food and frass samples were collected daily, frozen (−20°C), and lyophilized when the collection was completed. The amount of food consumed was calculated from the amount of frass produced and the approximate digestibility of the diet: (mg dry wt frass)/(1 − 0.555), where 0.555 is the value for the approximate digestibility (AD) for final-instar *M. disstria* larvae fed artificial diet containing 2% tannic acid (Karowe, 1989). The percent of ingested tannic acid remaining in the frass was calculated using the formula: (mg frass × % TA in frass)/(mg ingested diet × % TA in diet).

To prepare samples for tannic acid analysis, lyophilized samples (3–7 mg) of control diet ($N = 3$), frass from larvae fed the control diet ($N = 4$), 3% tannic acid diet ($N = 4$), and frass from larvae fed the tannin-containing diet ($N = 13$) were pulverized with a glass rod in screw-cap centrifuge tubes (1.5 ml) and extracted in 70% acetone (2 × 1.0 ml, 50°C, 45 min) in a shaker. Following centrifugation (13,500*g*, 15 min), supernatant solutions were pooled within samples, concentrated to 1.0 ml in a stream of nitrogen, lyophilized, redissolved in 1.0 ml of 20% (v/v) aqueous acetonitrile containing 1% (v/v) acetic acid, and filtered (0.45 μm). Filtered samples were analyzed by HPLC as described above. A tannic acid standard curve was constructed from a series of tannic acid solutions prepared by serial dilution. Artificial diet contained nontannin compounds that coeluted with the lower-molecular-weight components of tannic acid (peaks a and b, Figure 1A below). In order to quantify higher-molecular-weight tannins (peaks c and d, Figure 1A below), areas of nontannin peaks (identified in control samples) with retention times greater than 4.0 min were subtracted from corresponding peak areas in chromatograms of extracts of tannin-containing samples.

Tannic Acid Levels Along the Gut. We dissected samples of the contents of foreguts (0.2–4.0 mg dry wt), anterior midguts (0.8–1.4 mg dry wt), posterior midguts (0.7–1.8 mg dry wt), and hindguts (0.4–2.2 mg dry wt) of chilled (−20°C, 7 min) larvae that had been fed either a diet containing 3% tannic acid ($N = 11$) or a tannin-free diet ($N = 3$). Fresh weight-to-dry weight conversion factors for foregut, midgut, and hindgut samples were calculated from control larvae. Samples of gut contents were extracted in 70% acetone (1.0 ml) and centrifuged (10,000*g*, 10 min). Supernatant solutions (0.5 ml) were partially evaporated in a stream of nitrogen, lyophilized, redissolved in mobile phase (1.0 ml), filtered (0.45 μm), and the high-molecular-weight component d was quantified using HPLC, as described above.

Gallic Acid Budgets. Two- to 3-day-old final-instar *M. disstria* larvae ($N = 13$), reared at 22° and 24°C, were fed artificial diet containing 2% dry wt gallic acid (Sigma) for three days followed by gallic acid-free diet. Egested gallic acid was recovered by collecting all dark brown or black frass. Frass and diet samples were collected daily, frozen (−20°C), and lyophilized. Control larvae ($N = 5$) were maintained on the gallic acid-free rearing diet. Control and gallic acid-containing diet (25–30 mg) and frass (5–30 mg) samples were extracted in 50% methanol (3 × 1.0 ml, 50°C, 30 min) and analyzed for gallic acid using the rhodanine assay (Inoue and Hagerman, 1988) as described in Barbehenn and Martin (1992). The percent of ingested gallic acid remaining in the frass was calculated using the formula: (mg frass × % GA in frass)/mg ingested diet × % GA in diet), with the amount of ingested diet calculated as above assuming an AD of 55.5%.

Diet and frass samples collected for the measurement of a tannic acid budget (above) were also analyzed for gallic acid to determine the extent to which tannic acid was hydrolyzed by *M. disstria*. All samples were assayed for gallic acid using the rhodanine assay and a budget was calculated as described above.

Quantification of Phenol Oxidation. To compare the extent to which tannic acid is oxidized in larvae of *M. disstria* and *O. leucostigma*, the amount of brown pigment (oxidation products) that could be extracted from the frass of larvae consuming tannin-free and tannin-containing diets was determined. Final-instar larvae were assigned at random to a control (tannin-free) diet ($N = 24$ *M. disstria*, $N = 10$ *O. leucostigma*) or tannin-containing (1% tannic acid) diet ($N = 27$ *M. disstria*, $N = 10$ *O. leucostigma*). Food and frass samples were collected and dried (60°C, >5 days) daily during the entire instar. After grinding with a mortar and pestle, 10 to 15 mg subsamples were extracted in 70% acetone (1.5 ml, 50°C, 1 hr) in a shaker. Samples were centrifuged (13,500*g*, 5 min) and the absorbance was measured with a Zeiss spectrophotometer (420 nm) (Cilliers and Singleton, 1989). The background absorbance (A_{420}/mg sample) from each diet extract was subtracted from each frass extract to give a net

production of brown pigment per milligram of frass. Means were compared by Mann-Whitney U-tests.

The formation of brown pigments (A_{420}/mg sample) was also measured in frass of final-instar *M. disstria* larvae fed 2% gallic acid-containing diet. Methanolic extracts used for the measurement of gallic acid were diluted with 50% methanol (3.5 ml), and the absorbance of each solution was measured with a spectrophotometer. Measurements of background absorbance in control and gallic acid-containing diets were made and used to correct levels of brown pigments extracted from frass. Measurements of A_{420} per milligram of frass were multiplied by 4.33 (dilution factor) to allow their comparison with measurements of tannic acid oxidation.

Gut Acid-Base and Redox Conditions. To compare the acid–base and redox conditions in the guts of *M. disstria* and *O. leucostigma*, pH and redox potentials were measured in the foreguts and midguts of larvae that had been chilled (−20°C, 7 min) prior to dissection. pH was measured using a microneedle pH electrode (Microelectrodes MI-408P) and a silver–silver chloride reference electrode (Microelectrodes MI-401). Redox potentials were measured using a 0.02-in. platinum electrode (Microelectrodes MI-800) and a silver–silver chloride reference electrode. To minimize exposure of the gut contents to air, the microelectrodes were inserted together through small adjacent holes cut through the gut wall. Because of the greater stability of the redox potential measurements, pH measurements were made first. All measurements were made using a Metrohm/Brinkman (model 103) millivolt and pH meter. Observed redox potentials were converted to standard redox potentials (E_h) by adding 200 mV, and pe (electron availability) was calculated as $E_h/59.2$ (Appel and Martin, 1990). Overall redox conditions were summarized by the "redox parameter" (pH + pe) (Appel and Martin, 1990).

RESULTS

In order to test the permeability of the peritrophic envelope of *M. disstria* to tannic acid, we fed larvae a diet containing tannic acid and tested for the presence of tannic acid in the incubating solutions surrounding excised guts in which small holes had been cut to expose the peritrophic envelope. A chromatogram of the tannic acid included in the larval diet is illustrated in Figure 1A. Commercial tannic acid is composed of galloyl esters of glucose along with some unesterified gallic acid. Peak a is gallic acid; peaks b–e are galloyl esters of glucose, which differ in the number of galloyl groups attached to the glucose moiety. None of the higher-molecular-weight components of tannic acid (peaks c–e) could be detected in the incubation solution after a 2-hr incubation period (Figure 1C). Indeed, chromatograms of incubating solutions surrounding the

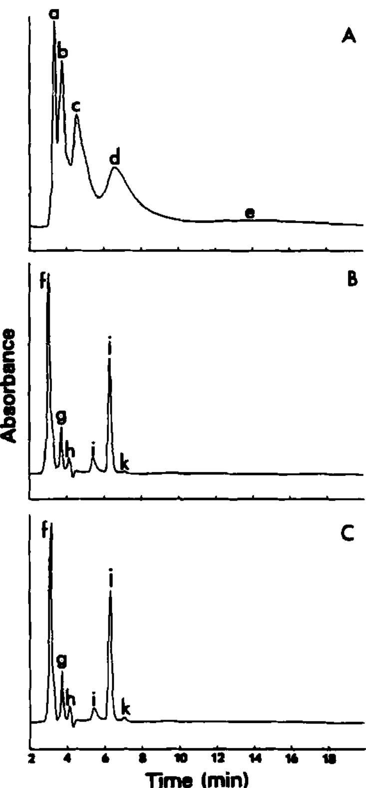

FIG. 1. *Malacosoma disstria* peritrophic envelope permeability to ingested tannic acid. (A) Chromatogram of tannic acid; (B) representative chromatogram of the incubating solution surrounding the gut of a larva fed a tannin-free diet ($N = 3$); (C) representative chromatogram of the incubating solution surrounding the gut of a larva fed a tannic acid-containing diet ($N = 11$). No compounds eluted before 2 min.

excised guts of larvae fed control (Figure 1B) and tannin-containing diets (Figure 1C) were virtually identical. The midguts of larvae fed the 3% tannic acid diet contained an average of 50 μg of peaks c and d of tannic acid. The sensitivity of the analytical method was such that we could have detected 0.00125 μg of tannic acid per microliter of incubating solution. Thus, we calculate that no more than 5% of the ingested tannic acid penetrated the peritrophic envelope during the 2-hr incubation period. Peaks f and g (Figure 1C) include compounds in the incubation solution but could also include small amounts of gallic acid (peak a) and a low-molecular-weight polyphenol (peak b) from ingested tannic acid.

This experiment was repeated using procedures that would ensure the presence of larger amounts of tannic acid in the midgut and the detection of smaller quantities in the incubating solution. When larvae were fed an 8% tannic acid diet, none of the polyphenols c or d could be detected in the incubating solutions, indicating that less than 0.3% of these polyphenols in the midgut penetrated the peritrophic envelope.

We quantified the amount of polyphenols c and d in tannic acid (Figure 1A) in the food and frass of *M. disstria* larvae to construct a tannic acid budget. Tannic acid was stable in the artificial diet; the efficiency of recovery of tannic acid from diet changed from virtually 100% soon after it was prepared to 94% after it was incubated for 24 hr under the conditions of the feeding experiment. Of the ingested polyphenols, only 21% ($\pm$2.4 SE) could be accounted for in the frass (Table 1). The remaining 79% was chemically transformed into substances that could not be detected using HPLC analysis.

The region of the digestive tract of *M. disstria* larvae in which the chemical transformation of tannic acid is most extensive was determined by a comparison of the percent of polyphenol d (Figure 1A) in the contents of the foregut, anterior midgut, posterior midgut, and hindgut (Table 2). There is a steady decrease in the percent of this tannic acid component as food passes from the foregut to the hindgut. Assuming that no food is assimilated from the foregut (Dow, 1986), but that 55.5% is assimilated during its passage through the midgut (Karowe, 1989), it can be calculated from the data summarized in Table 2 that only 29.0% of the tannic acid present in the food is still present in the posterior midgut (containing 0.27% polyphenol d). In the hindgut, only 18.7% of the tannic acid present in the food is still present (Table 1). These results clearly identify the midgut as the region in which the transformation of most of the tannic acid occurs.

Hydrolysis and oxidation are two chemical transformations that might reduce the levels of tannic acid in the guts of *M. disstria*. Although the measurement of gallic acid in the frass of an insect fed tannic acid might provide evidence for the hydrolysis of tannic acid, the amount of gallic acid present may be affected by either assimilation (Bernays et al., 1983; Kato, 1978) or oxidation (this study). Nonetheless, the results summarized in Table 1 suggest that hydrolysis accounts for the disappearance of only a small fraction of the tannic acid ingested by *M. disstria*. The tannic acid used in our experiments contained free gallic acid as an impurity (7%). Given that the larvae, therefore, ingested 0.14 mg of free gallic acid and that the hydrolysis of pure tannic acid generates 70% of its weight in gallic acid, and assuming that gallic acid is assimilated or oxidized with an efficiency of 93.6% whether it is ingested or produced by hydrolysis, then the 0.012 mg of gallic acid present in the frass can be accounted for by the hydrolysis of only 4% of the tannic acid ingested.

Phenol oxidation generates brown pigments (Hathway and Seakins, 1957;

TABLE 1. DRY MASS BUDGETS FOR TANNIC ACID AND GALLIC ACID INGESTED BY FINAL-INSTAR *Malacosoma disstria* LARVAE

	Tannic acid			Gallic Acid		
Diet (*N*)	Ingested	Egested	Remaining (%)	Ingested	Egested	Remaining (%)
3% TA (13)	1.05 ± 0.17[a]	0.25 ± 0.05[a]	21.0 ± 2.4	0.14 ± 0.02[c]	0.012 ± 0.002[c]	7.9 ± 0.8
3% TA (11)	0.030 ± 0.004[b]	0.006 ± 0.001[b]	18.7 ± 4.1			
2% GA (11)				2.52 ± 0.15	0.16 ± 0.02	6.4 ± 0.7

[a] Tannic acid peaks c and d; the total amount of tannic acid ingested (peaks b–e) is 1.72 mg. Data are presented as mean ± SE.
[b] Tannic acid peak d in fresh samples of diet and frass pellets dissected from the hindgut.
[c] Free gallic acid present as an impurity (7%) in tannic acid.

Thomson, 1962; Leatham et al., 1980; Igarashi and Yasui, 1985; Cilliers and Singleton, 1989). In *M. disstria* larvae fed a tannin-containing diet, the contents of the mid- and posterior midgut change from light tan to dark brown or green-brown, suggesting the oxidation of tannins. Pigment formation in the guts of tannin-fed larvae was quantified by measuring the absorbance at 420 nm of extracts of the frass of *M. disstria* larvae fed tannin-free and tannin-containing artificial diets. The amount of brown pigment in the frass of *M. disstria* larvae fed a diet containing tannic acid is significantly greater ($P < 0.0001$) than the amount in the frass of larvae fed a tannin-free control diet (Figure 2). Brown pigments were present in larger amounts in the frass of male than of female

TABLE 2. PERCENT TANNIC ACID IN DIET AND GUTS OF FINAL-INSTAR *Malacosoma disstria* LARVAE[a]

Sample location	Percent tannic acid (*N*)
Artificial diet	0.99 ± 0.07 (4)
Foregut	0.86 ± 0.08 (11)
Anterior midgut	0.80 ± 0.11 (11)
Posterior midgut	0.64 ± 0.09 (11)
Hindgut	0.42 ± 0.09 (11)

[a]Tannic acid component d (Figure 1A). Data are presented as mean ± SE.

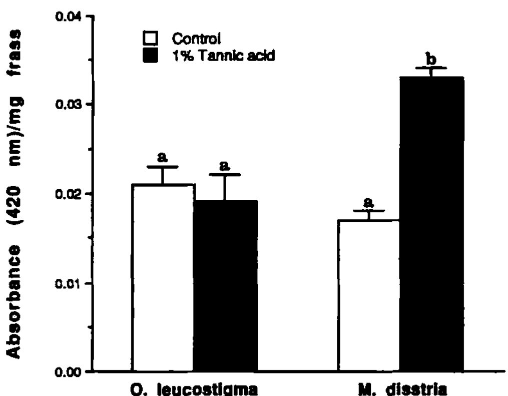

FIG. 2. Tannic acid oxidation in *Malacosoma disstria* (tannin sensitive) and *Orgyia leucostigma* (tannin tolerant). Data from male and female larvae are combined. Means within species were compared by Mann-Whitney U-tests. Different letters above bars denote $P < 0.0001$.

larvae fed both control ($P < 0.0005$) and tannic acid-containing ($P < 0.0004$) diets. Frass extracts from males and females fed control diets averaged 0.019 AU/mg (±0.001) and 0.014 AU/mg (±0.001), respectively. Frass extracts from males and females fed tannic acid-containing diets averaged 0.036 AU/mg (±0.002) and 0.027 AU/mg (±0.001), respectively. Ingested gallic acid was also oxidized by *M. disstria* larvae (Figure 3). Oxidation products (measured as absorbance at 420 nm/mg frass) are elevated eightfold compared to control frass. These measurements cannot be used to calculate the actual amount of phenol oxidized, since the relationship between the amount of absorption at 420 nm by the pigments formed and the amount of phenol oxidized has not been determined.

The concentration of brown pigments that leached through the peritrophic envelopes of *M. disstria* larvae containing tannic acid was elevated twofold compared with their concentration in control solutions (4200 ± 230 AU/μl (mean ± SE) and 2220 ± 70 AU/μl, respectively). Most of the brown compounds eluted between 3.0 and 4.5 min. However, the HPLC procedure we employed did not result in sufficient resolution of peaks to allow us to identify any new compounds generated during the oxidation of tannic acid.

In contrast to the production of brown pigments by *M. disstria* larvae fed a tannin-containing diet, when *O. leucostigma* larvae are fed a diet containing tannic acid their frass does not contain brown pigments in excess of those produced by larvae that consume a tannin-free diet (Figure 2). As noted earlier, tannic acid passes through the guts of *O. leucostigma* larvae without any chem-

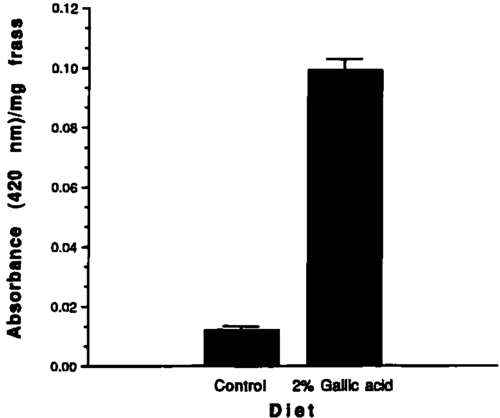

FIG. 3. Gallic acid oxidation in *Malacosoma disstria* larvae. Final-instar larvae were fed artificial diet containing 2% dry wt gallic acid or control diet.

ical modification (Barbehenn and Martin, 1992). These experiments show that phenols are oxidized in the guts of *M. disstria*, but not in *O. leucostigma*.

In seeking an explanation for the occurrence of tannic acid oxidation in the guts of *M. disstria* larvae and the absence of oxidation in the guts of *O. leucostigma* larvae, we compared the redox conditions in their gut lumens. The pH and redox conditions in the guts of *M. disstria* and *O. leucostigma* are similar (Table 3). The midgut pH in both species is close to 10, and as a consequence, ingested polyphenols are ionized. Likewise, the values of pe, a parameter that reflects electron availability (Lindsay, 1979; Appel and Martin, 1990), are similar in both the foreguts and midguts of the two species. As a consequence, the redox parameter (pH + pe), which is a composite measure of the redox status of a complex aqueous system giving equal weight to acid–base status and electron availability (Lindsay, 1979; Appel and Martin, 1990), is also similar in both species. *M. disstria* and *O. leucostigma* maintain weakly oxidizing conditions in their foreguts and strongly oxidizing conditions in their midguts. Thus, while these results are compatible with our observation of the occurrence of oxidation in the guts of *M. disstria* larvae, they provide no explanation for the absence of oxidation in *O. leucostigma*.

DISCUSSION

In this study we have demonstrated that the peritrophic envelope of the tannin-sensitive larvae of *M. disstria*, like the peritrophic envelope of the tannin-tolerant larvae of *O. leucostigma*, is impermeable to tannic acid. Clearly, the mere containment of tannic acid within the endoperitrophic space is insufficient to protect an insect herbivore from the potentially adverse effects of ingested tannins.

The impermeability of the peritrophic envelopes of *M. disstria* and *O.*

TABLE 3. pH AND REDOX CONDITIONS OF FOREGUTS AND MIDGUTS OF *Malacosoma disstria* AND *Orgyia leucostigma*[a]

	pH	Eh (mV)	pe	pH + pe
M. disstria				
Foregut (n = 6)	6.06 ± 0.10	96 ± 6	1.61 ± 0.11	7.67 ± 0.13
Midgut (n = 10)	10.23 ± 0.07	−30 ± 12	−0.51 ± 0.21	9.97 ± 0.18
O. leucostigma				
Foregut (n = 6)	5.58 ± 0.27	115 ± 6	1.94 ± 0.11	7.51 ± 0.30
Midgut (n = 14)	9.80 ± 0.11	−3 ± 11	−0.05 ± 0.19	9.75 ± 0.18

[a] Data are presented as mean ± SE.

leucostigma to the polyphenolic components of tannic acid presents an apparent dilemma, given that these molecules (approx. 3.5–4.7 nm diameter; Barbehenn and Martin, 1992) are smaller than the pore diameters in the peritrophic membranes of Lepidoptera, which have been reported to be greater than 7 nm (Adang and Spence, 1983; Santos and Terra, 1986; Wolfersberger et al., 1986). Two mechanisms might account for the containment of tannic acid within the endoperitrophic spaces of lepidopteran larvae: (1) binding by tannin-binding substances and (2) electrostatic exclusion. The binding of tannins by proteins or other substances (De Veau and Schultz, 1992; Ikeda et al., 1992) has the potential to form complexes with molecular dimensions that exceed the diameters of the pores of peritrophic membranes. Although the presence of tannin-binding proteins has not been examined in insects, they are produced by tannin-adapted vertebrate folivores (Austin et al., 1989). Secondly, the glycosaminoglycan component of the peritrophic membrane is ionized at physiological pH, forming a dense, negatively charged barrier (Miller and Lehane, 1993). Phenolic hydroxyl groups would also be ionized under the strongly basic conditions that prevail in the midguts of *M. disstria* and *O. leucostigma* (Hagerman, 1989). It is possible that electrostatic repulsion between anionic sites in peritrophic membranes and negatively charged polyphenolate ions prevent the diffusion of polyphenols through the peritrophic envelope. The permeability of other extracellular matrices containing glycosaminoglycans has been found to be reduced for dextrans that bear negatively charged groups (Chang et al., 1975).

Our results provide support for the hypothesis that the primary determinant of the effect of ingested tannins on insect herbivores is their chemical transformation in the gut (Appel and Martin, 1990; Appel, 1993). The essential difference between the larvae of *M. disstria* and *O. leucostigma* that explains their tannin sensitivity and tannin tolerance, respectively, appears to be the occurrence of tannin oxidation in *M. disstria*. Whereas 90–100% of the tannic acid ingested by *O. leucostigma* is recoverable (Barbehenn and Martin, 1992), only 19–21% of the tannic acid ingested by *M. disstria* can be recovered from the frass. Three observations suggest that tannic acid ingested by *M. disstria* is oxidized: (1) approximately 80% of ingested tannic acid is chemically transformed, (2) hydrolysis cannot account for the chemical modification of more than 4% of the ingested tannic acid, and (3) tannic acid and gallic acid produce brown pigments in the midguts of *M. disstria*, but not in *O. leucostigma*.

Our results do not support the hypothesis that thermodynamic indicators of gut redox conditions, such as the redox potential (E_h) or the redox parameter (pe ± pH), are sufficient to predict the oxidation of phenols in lepidopteran larvae (Appel and Martin, 1990; Appel, 1993). Both *M. disstria* and *O. leucostigma* maintain strongly oxidizing redox conditions in their midguts, yet tannic acid is extensively oxidized in the former species but not in the latter. The thermodynamic favorability of oxidation (determined by oxidizing redox

conditions) does not necessarily imply that oxidation is kinetically favorable, i.e., that phenol oxidation will proceed at an observable rate. The absence of phenol oxidation under conditions in which oxidation is favored thermodynamically could be explained by the absence of necessary catalysts (e.g., polyphenol oxidase, laccase) (Felton et al., 1989), the presence of antioxidant systems (e.g., catalase, dehydroascorbic acid reductase, ascorbic acid, α-tocopherol) (Larson, 1988; Felton and Duffey, 1991, 1992; Summers and Felton, 1993) and/or low levels of essential oxidants (e.g., oxygen, hydrogen peroxide).

Although we are currently unable to distinguish among these potential mechanisms, we have made one observation consistent with the hypothesis that low oxygen levels in *O. leucostigma* may explain the absence of phenol oxidation in this species. The contents of the midguts of *O. leucostigma*, which are normally the same color as the diet (tan), turn dark brown in a few minutes after the gut is opened and its contents exposed to air. Oxygen tensions in the guts of insect herbivores have not been measured, to our knowledge. Such measurements might reveal important differences between tannin-sensitive and tannin-tolerant species.

The adverse effects of dietary tannic acid on *M. disstria* are different in early- and late-instar larvae. Early-instar larvae fed tannin-containing diets have low consumption rates, resulting in greatly prolonged development times and the failure to develop beyond the third or fourth instar (Karowe, personal communication; Barbehenn, unpublished). Feeding deterrence by polyphenols in early-instar lepidopteran larvae appears to be common (Isman and Duffey, 1982; Klocke and Chan, 1982; Manuwoto et al., 1985; Manuwoto and Scriber, 1986) and is even exhibited by early-instar larvae of the "tannin-tolerant" species *O. leucostigma* (Barbehenn, unpublished).

By contrast, when late-instar larvae of *M. disstria* are fed tannic acid, consumption and growth rates may be reduced, but little larval mortality occurs (Karowe, 1989). Instead, mortality occurs during the pupal stage and during the eclosion of adults (Karowe, 1989; Barbehenn, unpublished) and is associated with severe deformities in the wings of pupae and adults. Wings are commonly shortened to stubs in pupae (see Karowe, 1989) and are twisted and unexpanded in adults that are able to eclose. Similar wing deformities are the primary symptoms observed when many larval Lepidoptera are fed diets containing inadequate amounts of essential fatty acids (linolenic and/or linoleic acid) (Chippendale et al., 1964; Kato, 1978; Dadd, 1981). We hypothesize that since these polyunsaturated fatty acids are easily destroyed by oxidation (Lea, 1962), tannic acid acts as a prooxidant in *M. disstria*, generating reactive radical oxidants (e.g., superoxide and hydroxyl radicals) that bring about the oxidation of essential fatty acids. We note that, in contrast to *M. disstria*, *O. leucostigma* larvae fed *M. disstria* diet containing 3% tannic acid diet develop normal wings (Barbe-

henn, unpublished). This result demonstrates the adequacy of the *M. disstria* diet for wing formation when the tannic acid remains unoxidized.

Wing deformities have also been observed when *Spodoptera* (*Prodenia*) *eridania* and *Manduca sexta* larvae are fed diets containing L-dopa or L-canavanine, respectively (Rehr et al., 1973; Rosenthal, 1977). In *S. eridania*, the wing deformities closely resemble those observed in *M. disstria* fed tannic acid. The authors postulated that L-dopa may interfere with tyrosinase, resulting in incomplete cuticle hardening and darkening. However, the observations reported Rehr et al. (1973) do not rule out the possibility that the adverse effects of ingested L-dopa are a consequence of its potential to act as a prooxidant that produces a deficiency in an oxidizable nutrient. Although our nutrient deficiency hypothesis remains untested, it is clear that the nature of the detrimental effect of tannins on late-instar *M. disstria* is different from that described previously in other tannin-sensitive insects, in which lesions in the midgut epithelium result in death during the feeding period (Bernays et al., 1980; Berenbaum, 1983; Steinly and Berenbaum, 1985).

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CELLULOSE DIGESTION IN PRIMITIVE HEXAPODS: EFFECT OF INGESTED ANTIBIOTICS ON GUT MICROBIAL POPULATIONS AND GUT CELLULASE LEVELS IN THE FIREBRAT, *Thermobia domestica* (ZYGENTOMA, LEPISMATIDAE)

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Abstract—Antibiotic feeding studies were conducted on the firebrat, *Thermobia domestica* (Zygentoma, Lepismatidae) to determine if the insect's gut cellulases were of insect or microbial origin. Firebrats were fed diets containing either nystatin, metronidazole, streptomycin, tetracycline, or an antibiotic cocktail consisting of all four antibiotics, and then their gut microbial populations and gut cellulase levels were monitored and compared with the gut microbial populations and gut cellulase levels in firebrats feeding on antibiotic-free diets. Each antibiotic significantly reduced the firebrat's gut microflora. Nystatin reduced the firebrat's viable gut fungi by 89%. Tetracycline and the antibiotic cocktail reduced the firebrat's viable gut bacteria by 81% and 67%, respectively, and metronidazole, streptomycin, tetracycline, and the antibiotic cocktail reduced the firebrat's total gut flora by 35%, 32%, 55%, and 64%, respectively. Although antibiotics significantly reduced the firebrat's viable and total gut flora, gut cellulase levels in firebrats fed antibiotics were not significantly different from those in firebrats on an antibiotic-free diet. Furthermore, microbial populations in the firebrat's gut decreased significantly over time, even in firebrats feeding on the antibiotic-free diet, without corresponding decreases in gut cellulase levels. Based on this evidence, we conclude that the gut cellulases of firebrats are of insect origin. This conclusion implies that symbiont-independent cellulose digestion is a primitive trait in insects and that symbiont-mediated cellulose digestion is a derived condition.

Key Words—Cellulose digestion, cellulases, firebrats, *Thermobia domestica*, Lepismatidae, Zygentoma, Thysanura.

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INTRODUCTION

Of all living insect groups, the zygentomate families Lepidotrichidae and Lepismatidae (formerly in the order Thysanura) are probably nearest to the ancestral stock from which the higher insects evolved (Boudreaux, 1979; Kristenson, 1981). The family Lepismatidae includes firebrats and silverfish. The natural diets of firebrats and silverfish include lichens, terrestrial algae, fungal hyphae, fungal spores, yeasts, pollen, detritus, and small invertebrates (Lindsay, 1940; Smith, 1970; Wygodzinsky, 1972; Kaestner, 1973). In their feeding habits, therefore, they resemble other highly omnivorous scavengers and detritus-feeders, such as roaches. Cellulolytic capacity has been demonstrated in five species of Zygentoma: *Ctenolepisma longicaudata* (Lindsay, 1940), *C. lineata* (Lasker and Giese, 1956), *Acrotelsa collaris* (Modder, 1964, 1975), *Lepisma saccharinum* (Zinkler, 1983), and *Thermobia domestica* (Zinkler et al., 1986; Zinkler and Götze, 1987). Cellulolytic enzymes have been detected in both the crops and midguts of silverfish and firebrats. The crop is the most prominent section of the gut, often extending more than half of the animal's body length (Lindsay, 1940; Lasker and Giese, 1956; Barnhart, 1961; Zinkler et al., 1986; Zinkler and Götze, 1987). The hindgut is very small.

It has generally been asserted that the participation of gut microbes is not required for cellulose digestion in these primitive insects. The carefully designed study of cellulose digestion in the silverfish *C. lineata* by Lasker and Giese (1956) is regularly cited as a definitive demonstration of symbiont-independent cellulose digestion in the "Thysanura." In this study, the authors produced aposymbiotic (symbiont-free) animals and observed that the activity of the midgut fluid toward cellulose and the ability of the silverfish to degrade ^{14}C-labeled cellulose were undiminished in the aposymbiotic animals. From this result they concluded that cellulose digestion must have been accomplished by enzymes produced by the insects. Unfortunately, this conclusion is somewhat weakened by two methodological ambiguities that would not have been evident in 1956 when this study was performed. For example, there is no indication that the purity of the labeled cellulose was checked before feeding it to the silverfish, and it is now known that preparations of labeled cellulose often contain significant amounts of labeled noncellulosic impurities. Indeed, it is not uncommon to find that as much as 10% of the counts in some commercial preparations can be extracted with water and an additional 15–20% can be extracted with base. Thus, the possibility cannot be ruled out that an impurity, not cellulose, was the source of the labeled carbon dioxide in the respired gasses collected from the aposymbiotic nymphs. Furthermore, in assaying cellulolytic activity in the gut fluid, Lasker and Giese used "regenerated cellulose" as the substrate. The preparation of "regenerated cellulose" (Trager, 1932) results in significant loss of crystallinity, making it much more easily degraded than native cellulose.

Indeed, it is now known that the digestion of amorphous cellulose can be accomplished by endocellulases acting alone, whereas the efficient digestion of crystalline cellulose requires the synergistic action of a mixture of enzymes with different specificities (e.g., endocellulases, exocellulases, and cellobiases) (Coughlan and Ljungdahl, 1988). Consequently, finding undiminished activity toward "regenerated cellulose" in the guts of aposymbiotic animals does not establish that they are still able to digest native cellulose, but only that they secrete an endocellulase of unknown activity toward crystalline cellulose. Of course, these criticisms do not necessarily mean that Lasker and Giese are wrong in their conclusion that symbiont-independent cellulose digestion occurs in *C. lineata*, but only that evidence in favor of that conclusion is not compelling.

In this study we explore the origin of the soluble cellulases in the gut fluids of the common firebrat, *Thermobia domestica* (Zygentoma, Lepismatidae), by assessing the effects of ingested antibiotics on gut cellulase activity. The study is based upon the assumption that the level of gut cellulase activity should be reduced by the ingestion of antibiotics that effectively reduce the firebrat's gut microbial populations, if these populations are the source of the enzymes. We have used four antibiotics (nystatin, metronidazole, streptomycin, and tetracycline), each with activity against a different group of microbes, that have been shown to reduce the gut flora of other insects (Bracke et al., 1978; Jarosz, 1979; Taylor, 1982; Gilliam et al., 1988, Scrivener et al., 1989). We have determined the effects of the ingested antibiotics, singly and in combination, on consumption and survival, on the sizes of the microbial populations in the gut, and on the activity of the gut fluid toward microcrystalline cellulose.

In their study of *T. domestica*, Zinkler and Götze (1987) reported that "preliminary experiments with a diet containing a spectrum of antibiotics did not alter the cellulolytic enzyme activities significantly," but these authors included no description of, or data from, these "preliminary experiments," and we have been unable to find any subsequent publication describing this important study.

METHODS AND MATERIALS

Insect Collection and Maintenance. Firebrats were collected from buildings on the University of Michigan-Ann Arbor campus. Laboratory colonies were maintained at 35°C in 2.2-gallon Servin' Saver plastic containers in which were placed a beaker of water to maintain humidity and paper towels to provide places for the insects to hide. Firebrats were fed a diet of rolled oats (Lasker and Giese, 1956; Zinkler and Götze, 1987).

Antibiotics. Nystatin, tetracycline, streptomycin, and metronidazole were purchased from the Sigma Chemical Company (St. Louis, Missouri). Antibiotic

concentrations used in the feeding studies were determined by the minimum inhibitory concentration (MIC) method (Davis et al., 1990). Antibiotics were incorporated into nutrient agar (Difco) (for antibacterial testing) or potato dextrose agar (Difco) (for antifungal testing) at various concentrations. Firebrat gut homogenate in 0.5-ml aliquots of nutrient broth was spread onto the plates and incubated at 35°C for three days. The lowest concentration of antibiotic resulting in no growth of bacteria or fungi was the concentration chosen for the diets used in the feeding studies.

Diet Preparation for Antibiotic Feeding Studies. Lightly ground rolled oats were sterilized in an autoclave (20 min, 121°C), cooled, and mixed with an aqueous solution of a single antibiotic or the antibiotic mixture. The rolled oat/antibiotic slurry was then mixed thoroughly by hand and frozen at −4°C. Frozen diet was lyophilized (8 hr) and then stored desiccated at 4°C. Control diet (antibiotic-free) was prepared as above, substituting distilled water for the antibiotic solutions. Antibiotics were incorporated into the rolled oats at the following concentrations: nystatin, 200 units/g; metronidazole, 500 μg/g; streptomycin, 100 μg/g; tetracycline, 200 μg/g; antibiotic-cocktail (nystatin, 500 units/g; metronidazole, 1000 μg/g; streptomycin, 1000 μg/g; tetracycline, 1000 μg/g).

Antibiotic Feeding Experiments. Insects used for feeding experiments were starved three days prior to the start of each experiment and then offered the rolled oat diet with (experimental treatment) or without (control treatment) antibiotics. Fresh diet was offered every three days for the duration of each experiment. Paper towels normally present in the cages for hiding were replaced with plastic strips. Consumption (milligrams consumed per firebrat) was determined by subtracting the weight of the freeze-dried oat diet remaining after a three-day feeding period from the original dry weight of the diet offered. Firebrat survival was measured at three-day intervals. Viable gut bacteria, total gut microbes, and gut cellulase levels were measured at three-day intervals in the metronidazole, streptomycin, and tetracycline feeding studies, at weekly intervals in the nystatin feeding study, and after three and 12 days in the antibiotic-cocktail feeding study.

Preparation of Crude Cellulase Extracts. Firebrats were immobilized by cooling (1 min, −4°C) and dissected in 0.1 M sodium acetate buffer (pH 5.0). Whole guts were removed by making a dorsal longitudinal incision in the insect body and teasing the gut away from the carcass and then homogenized individually by hand in 0.6 ml of buffer for 1 min using a 15-ml glass homogenizer. The homogenate was centrifuged (20 min, 4°C, 10,000 rpm/Sorvall SA-600 rotor) and the supernatant solution decanted. The pellet was resuspended in 0.6 ml of buffer that was used to rinse the homogenizer pestle and centrifuged as before. Supernatant solutions were pooled for column chromatography.

Column Chromatography and Extract Concentration. An aliquot (1.0 ml) of the pooled supernatant solutions from a single firebrat gut extract was placed

onto a Sephadex G-25M column (1.2 × 2.0 cm, Pharmacia) and eluted with 0.1 M sodium acetate buffer (pH 5.0). The first 3.5 ml of eluant was collected, frozen using liquid nitrogen, and lyophilized (8 hr). Cellulase assays were conducted on 0.05-ml aliquots of a solution prepared by redissolving the freeze-dried material in 0.22 ml of acetate buffer (pH 5.0).

Cellulase Assay. Activity of the firebrat cellulase complex was determined by measuring the amount of reducing sugar released upon incubation of partially purified firebrat gut extract with microcrystalline cellulose (Dubois et al., 1956). An aliquot (0.95 ml) of a microcrystalline cellulose suspension (0.5%) in 0.1 M sodium acetate buffer (pH 5.0) was added to 0.05 ml of partially purified gut extract. In controls, 0.05 ml of thermally denatured (110°C, 10 min) gut extract replaced the active gut extract. Following incubation (2 hr, 45°C), the cellulose/gut extract suspensions were centrifuged (3500*g*, 2 min), and an aliquot (0.5 ml) of the supernatant solution was assayed for reducing sugars by adding 0.5 ml of 5% phenol followed immediately by 2.5 ml of concentrated sulfuric acid. This mixture was vortexed briefly and placed in a 25°C water bath (20 min), after which absorbance was measured at 490 nm. Determinations were done in duplicate. Cellulase activity is expressed as micromoles of glucose equivalents liberated per gram whole gut (wet weight) per hour.

Viable Bacterial Counts and Viable Fungal Counts. Insects were weighed, immobilized at −4°C and surface-sterilized by submersion in 70% ethanol (1 min), submersion in 15% bleach (1 min), then rinsed twice in sterile distilled water (1 min each). As a test of the effectiveness of this sterilization method, whole surface-sterilized firebrats were placed onto nutrient agar and potato dextrose agar and incubated for three days at 35°C. Surface sterilized insects were dissected as described for cellulase assays, except that flame sterilized forceps were used for all transfer and dissections. Whole guts were placed in sterile tubes (20 mm × 125 mm) containing 0.5 ml of nutrient broth (Difco) and 5 sterile sintered glass beads (6 mm diameter). Homogenation of the gut was achieved by vortexing at high speed for 1 min, after which the gut homogenate was diluted with nutrient broth using 10-fold serial dilutions and then spread onto nutrient agar (viable gut bacteria) or potato dextrose agar (viable gut fungi). Determinations were conducted in duplicate. After a three-day incubation at 35°C, counts of bacterial colony-forming-units (CFU) and viable fungal counts were made. Only bacterial plates containing 30–300 colonies were counted. Measurements of viable bacteria are expressed as colony-forming-units per whole gut, and viable gut fungi are expressed as fungal counts per whole gut.

Total Microbial Counts. Total counts of microorganisms in the firebrat's gut were made using the acridine orange–epifuorescence technique (Francisco et al., 1973). Dilution tubes prepared for the estimation of viable bacterial and fungal counts were also used to estimate total gut microbes. A filter-sterilized solution of acridine orange (1.0 ml, 1% in 0.1 M sodium bicarbonate buffer,

pH 8.3) was mixed with an aliquot of gut homogenate that had been diluted with nutrient broth and made up to 10 ml with filtered 0.1 M sodium bicarbonate buffer, pH 8.3. Appropriate dilutions were made so that fields of view contained at least 30 cells. This solution was placed in a 10-ml disposable syringe and filtered through a 0.22-μm polycarbonate black membrane filter (Poretics, Livermore, California) using a 25-mm syringe filter (Gelman, Ann Arbor, Michigan). Following filtration, the membrane was quickly placed onto a glass slide, a drop of low-fluorescence immersion oil was added, and a cover slip was placed over the filter. Microbes were counted using an Olympus BH-2 microscope equipped with a Mercury 100 fluorescence unit, dichroic mirror B (DM-500 + O-515), excitor filter B (IF-490), and barrier filter O-530. Ten fields of view on the filter were counted using a 100 $\times$ Splan oil objective. This method proved effective in distinguishing bacterial or fungal cells from nonmicrobial material. Microbial cells typically fluoresced red or green. Total gut microbial counts are expressed as microbes per whole gut.

Statistics. All statistics were done using Systat (Wilkinson, 1987). Firebrat consumption of antibiotic-containing and antibiotic-free diets was compared using a paired Student's t test. Measurements of gut weights, gut cellulase levels, viable gut bacteria, viable gut fungi, and total gut bacteria were analyzed using a two-way analysis of variance (ANOVA) followed by contrasts with α adjusted for multiple comparisons by the Bonferoni method. To conform to assumptions underlying ANOVA, homogeneity of variances were tested using Bartlett's test ($\alpha = 0.05$) and normality was checked using rankit plots. Transformations were conducted on some data sets to pass Bartlett's test; viable gut bacteria were natural-log-transformed in the nystatin, streptomycin, and tetracycline feeding studies; total gut microbes were natural-log-transformed in the nystatin, metronidazole, and antibiotic-cocktail feeding studies; and viable gut fungi in the nystatin feeding study and gut cellulase levels in the tetracycline feeding study were square-root-transformed.

The streptomycin and tetracycline feeding experiments were carried out simultaneously and compared to one group of control firebrats in the laboratory but were compared to the control treatment independently during statistical analysis to negate interactions between the two antibiotic treatments.

RESULTS

Diet Consumption.

Antibiotic-containing and antibiotic-free diet consumption were monitored over the course of each feeding experiment to ensure that incorporation of antibiotics into the diet did not cause the firebrats to change their feeding behavior. Paired t test analysis showed that none of the antibiotics singly (nystatin, t =

0.21, $df = 4$, $P > 0.80$; metronidazole, $t = 2.18$, $df = 4$, $P > 0.05$; streptomycin, $t = 0.80$, $df = 4$, $P > 0.40$; tetracycline, $t = 0.18$, $df = 4$, $P > 0.80$) or in combination ($t = 1.15$, $df = 3$, $P > 0.30$) significantly affected firebrat consumption. During the feeding experiments, consumption for the antibiotic-free and antibiotic-containing diets average 1.53 ± 0.28 and 1.61 ± 0.28 mg diet/insect/three-day sampling period, respectively.

Survival

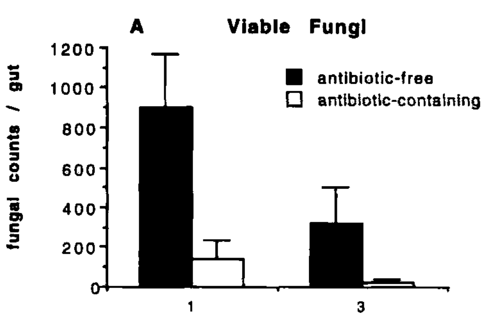
Firebrat survival was monitored over the course of each antibiotic feeding study to ensure that the concentrations of antibiotics used were not overly toxic. Except in the case of the antibiotic-cocktail feeding study, firebrats feeding on antibiotic-containing diets survived as well as firebrats feeding on the antibiotic-free diet. Losses at each sampling period for the nystatin, metronidazole, streptomycin, and tetracycline feeding studies ranged from zero to three insects, with the maximum difference between firebrat deaths for the control and test treatments in any one sampling period being only two insects. Fifteen firebrats from the antibiotic-containing treatment died during the first sampling period of the antibiotic-cocktail feeding study compared to only three from the control treatment. However, after these initial losses, surviving firebrats feeding on the antibiotic-containing diet survived as well as insects feeding on the antibiotic-free diet.

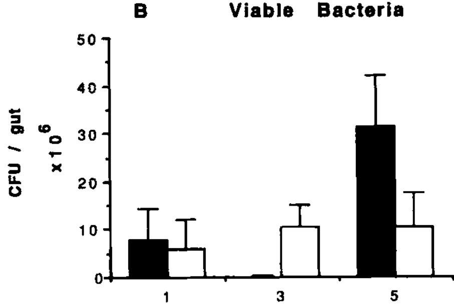
Gut Weights

Two-way ANOVA indicated that the gut weights for firebrats feeding on nystatin ($F = 1.06$, $df = 1,24$, $P > 0.30$), metronidazole ($F = 1.16$, $df = 1,40$, $P > 0.20$), streptomycin ($F = 1.21$, $df = 1,40$, $P > 0.20$), tetracycline ($F = 0.77$, $df = 1,40$, $P > 0.30$) and the antibiotic-cocktail ($F = 1.67$, $df = 1,30$, $P > 0.20$) were not significantly different from the gut weights of firebrats feeding on the antibiotic-free diet in each feeding experiment. Because there were no differences between gut weights in test and control animals, comparison of treatments involving viable gut bacteria and total gut microbes were made as counts per whole gut rather than per milligram of gut.

Effects of Antibiotics on Populations of Gut Flora

Nystatin. Firebrats feeding on the nystatin-containing diet had significantly fewer viable gut fungi compared to firebrats feeding on the nystatin-free diet ($F = 12.41$, $df = 1,16$, $P \leq 0.01$) (Figure 1A). Overall, nystatin removed 89% of the firebrat's viable gut fungi. While nystatin significantly reduced the





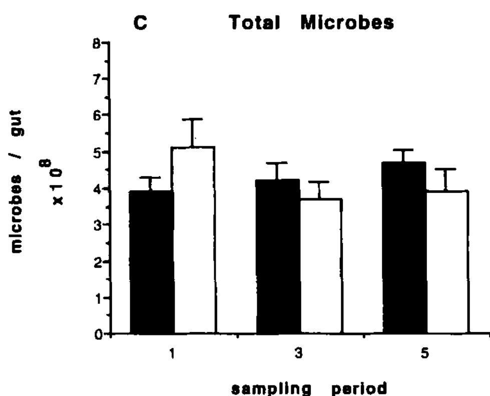


FIG. 1. Viable gut fungi (A), viable gut bacteria (B), and total gut microbes (C) measured during the nystatin feeding study. Sampling periods 1-3 are 7, 14, and 21 days, respectively. Bars are mean with SEM, $N = 5$.

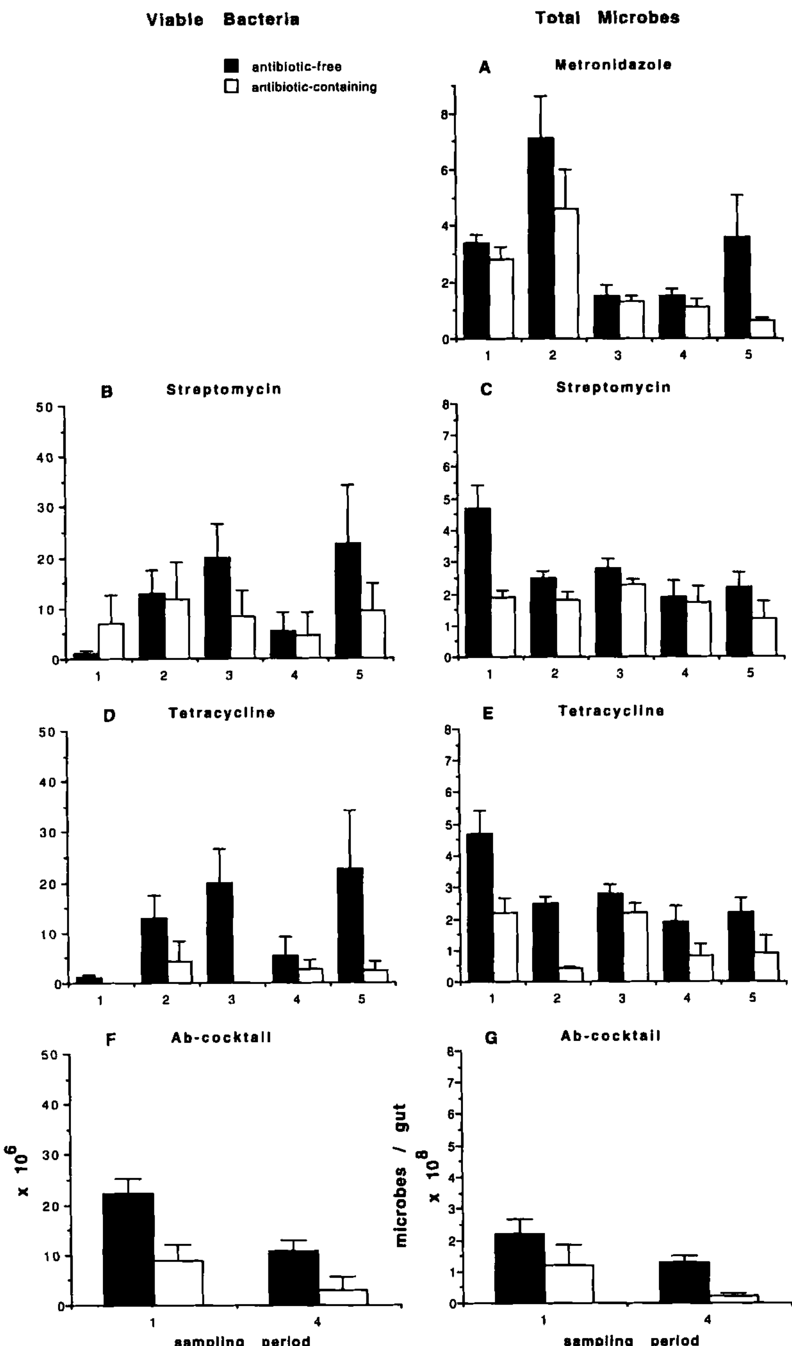
firebrat's viable gut fungi, viable gut bacteria ($F = 0.037$, $df = 1,23$, $P > 0.80$) (Figure 1B) and total gut microbes ($F = 0.069$, $df = 1,24$, $P > 0.70$) (Figure 1C) were not significantly affected.

Ingested fungal spores and yeast cells are the most likely inocula responsible for the fungal colonies counted in these experiments, but we cannot rule out the possibility that some colonies were generated from multicellular fragments of hyphae or mycelia that remained viable in the gut. In any event, it is clear that the total population of potential fungal colony-forming units in the gut, in whatever form they may be present, is significantly reduced by nystatin. It is also evident that fungi make up a very small component of the gut flora compared to bacteria, which are four orders of magnitude more abundant.

Metronidazole. Firebrats feeding on the metronidazole-containing diet had significantly fewer total gut microbes that firebrats feeding on the metronidazole-free diet ($F = 13.84$, $df = 1,40$, $P \leq 0.001$) (Figure 2A). Counts of total gut microbes were reduced at each sampling period by metronidazole, and overall metronidazole reduced the firebrat's total gut flora by 35%. The significant sampling period × treatment effect ($F = 2.61$, $df = 4,40$, $P \leq 0.05$) observed for counts of total microbes in this feeding study was due to the difference between treatments in sampling period 5. Counts of total gut microbes in firebrats fed metronidazole were reduced by 82% compared to counts in firebrats fed the antibiotic-free diet in sampling period 5, whereas the treatments differed by only 23% in the first four sampling periods.

Streptomycin. Counts of viable gut bacteria in firebrats feeding on the streptomycin-containing diet were not significantly different from counts in firebrats feeding on the antibiotic-free diet ($F = 2.18$, $df = 1,36$, $P \geq 0.05$) (Figure 2B). This nonsignificant difference is likely due to the high variability of the counts at each sampling period, and to sampling period 1, when counts in the antibiotic-containing treatment were higher than counts in the antibiotic-free treatment. After sampling period 1, viable counts in firebrats feeding on the antibiotic-containing diet were reduced compared to counts in firebrats feeding on the antibiotic-free diet.

Firebrats feeding on the streptomycin-containing diet had significantly fewer total gut microbes than firebrats feeding on the streptomycin-free diet ($F = 15.52$, $df = 1,40$, $P \leq 0.001$) (Figure 2C). The firebrat's total gut microbes were reduced by streptomycin at each sampling period, and overall streptomycin removed 32% of the firebrat's total gut microbes. The significant sampling period × treatment effect ($F = 2.92$, $df = 4,40$, $P \leq 0.05$) for counts of total gut microbes in this feeding study is due to the difference between treatments in sampling period 1. Counts of total microbes in firebrats fed streptomycin were reduced by 60% compared to counts in the control treatment in sampling period 1, whereas the test and control treatments differed by an average of only 26% in the last four sampling periods.



Viable Bacteria
Total Microbes
antibiotic-free
antibiotic-containing
A
Metronidazole
B
Streptomycin
C
Streptomycin
D
Tetracycline
E
Tetracycline
F
Ab-cocktail
G
Ab-cocktail
CFU / gut x 10^6
microbes / gut x 10^8
sampling period
sampling period

Tetracycline. Firebrats feeding on the tetracycline-containing diet had significantly fewer viable gut bacteria (F = 17.70, df = 1.32, $P \leq 0.001$) (Figure 2D), and significantly fewer total gut microbes (F = 30.85, df = 1.40, $P \leq 0.001$) (Figure 2E) than firebrats feeding on the tetracycline-free diet. Tetracycline reduced the firebrat's viable gut bacteria and total gut microbes at each sampling period, and overall tetracycline removed 81% of the firebrat's viable gut bacteria and 55% of the firebrat's total gut microbes.

Antibiotic Cocktail. Firebrats feeding on the antibiotic-cocktail diet had significantly fewer viable gut bacteria (F = 13.06, df = 1,29, $P \leq 0.001$) (Figure 2F) and significantly fewer total gut microbes (F = 19.43, df = 1,30, $P \leq 0.001$) (Figure 2G). The antibiotic cocktail reduced the firebrat's viable gut bacteria and total gut microbes at each sampling period, and overall, the antibiotic-cocktail reduced the firebrat's viable gut by 67% and reduced total gut flora by 64%.

Changes in Populations of Gut Flora Over Time

Nystatin. Viable gut fungi in both test and control treatments decreased significantly over time (F = 5.06, df = 1,16, $P \leq 0.05$) (Figure 1A), but viable gut bacteria (Figure 1B) and total gut microbes (Figure 1C) in both treatments showed no significant decreases over time (summary statistics; Table 2 below). Viable gut fungi were 67% lower in sampling period 2 than in sampling period 1.

Metronidazole. The significant time effect observed in counts of total gut microbes (F = 15,89, df = 4,40, $P \leq 0.001$) (Figure 2A) was due to decreases in the counts measured during sampling periods 3, 4, and 5. Counts of total gut

FIG. 2. Viable gut bacteria measured during the streptomycin (**B**), tetracycline (**D**) and Ab-cocktail (**F**) feeding studies and total gut microbes measured during the metronidazole (**A**), streptomycin (**C**), tetracycline (**E**), and Ab-cocktail (**G**) feeding studies. Sampling periods 1–5 are 3, 6, 9, 12, and 15 days, respectively. Bars are mean with SEM; N = 5 in the metronidazole, streptomycin, and tetracycline feeding studies except for viable gut bacteria in the streptomycin feeding study where N = 4 for the test treatment at sampling period 1 and 2, and viable gut bacteria in the tetracycline feeding study where N = 3 for sampling periods 1, 3, and 4 (test treatment) and N = 4 for sampling period 2 (control treatment). Sample sizes for viable gut bacteria in the Ab-cocktail feeding study at sampling period 1 (control treatment), sampling period 1 (test treatment), and sampling period 2 (both treatments) were N = 9, 10, and 7, respectively. Sample sizes for total gut microbes in the Ab-cocktail feeding study at sampling period 1 (both treatments) and sampling period 2 (both treatments) were N = 10 and 8, respectively.

microbes in sampling period 1 were significantly greater than those in sampling periods 3, 4, and 5 by 55% (F = 12.21, df = 1,40, $P \leq 0.001$), 58% (F = 16.66, df = 1,40, $P \leq 0.001$), and 32% (F = 13.89, df = 1,40, $P \leq 0.001$) respectively, but not significantly different from sampling period 2 (F = 5.26, df = 1.40, P = 0.027). Counts of total gut microbes measured in sampling period 2 were significantly greater than those measured in sampling periods 3, 4, and 5 by 76% (F = 33.39, df = 1,40, $P \leq 0.001$), 78% (F = 40.64, df = 1,40, $P \leq 0.001$) and 64% (F = 36.24, df = 1,40, $P \leq 0.001$). While counts of total microbes in the test treatment decreased consistently over the last three sampling periods, counts in the control treatment decreased only from sampling period 3 to sampling period 4 and then increased to initial levels at sampling period 5.

Streptomycin. Counts of viable gut bacteria in both test and control treatments fluctuated over the course of the streptomycin feeding study, and no significant time effect was detected (Figure 2B). The significant time effect observed for counts of total microbes in this feeding study (F = 4.66, df = 4,40, $P \leq 0.01$) (Figure 2C) was due to a decrease in the control treatment after sampling period 1. Counts of total gut microbes in sampling periods 2, 3, 4, and 5 were reduced by an average of 38% compared to counts in sampling period 1. Two-way ANOVA, excluding sampling period 1, showed no significant sampling period effect (F = 1.73, df = 3,32, P = 0.181), but still retained a significant treatment effect (F = 4.70, df = 1,32, $P \leq 0.05$).

Tetracycline. Counts of viable gut bacteria in both test and control treatments fluctuated over the course of this feeding study, and no significant decreases over time were detected (Figure 2D). The significant time effect observed for counts of total microbes in this feeding study (F = 8.88, df = 4,40, $P \leq 0.001$) (Figure 2E) was due to decreases in counts after sampling period 1. Sampling period 1 was significantly greater than sampling periods 2, 4, and 5 by 58% (F = 21.99, df = 1,40, $P \leq 0.001$), 61% (F = 23.80, df = 1,40, $P \leq 0.001$), and 55% (F = 20.51, df = 1,40, $P \leq 0.001$) respectively, but not significantly different from sampling period 3 (F = 5.46, df = 1,40, P = 0.025).

Antibiotic Cocktail. Viable gut bacteria (Figure 2F) and total gut microbes (Figure 2G) in both test and control treatments of this feeding study decreased over time, although this trend was only significant for viable counts (F = 8.98, df = 1,29, $P \leq 0.01$). Viable counts decreased by 56% from sampling period 1 to sampling period 4.

Gut Cellulase Levels

Although ingested antibiotics often significantly reduced the firebrat's viable gut bacteria and total gut flora, two-way ANOVA showed that the antibiotics ingested singly (nystatin, F = 0.45, df = 1,24, $P > 0.50$; metronidazole, F

$= 0.024$, $df = 1{,}40$, $P > 0.80$; streptomycin, $F = 0.80$, $df = 1{,}35$, $P > 0.30$; tetracycline, $F = 2.02$, $df = 1{,}38$, $P > 0.10$) or in combination ($F = 2.09$, $df = 1{,}32$, $P > 0.10$) did not significantly reduce the firebrat gut cellulase levels (Table 1). Additionally, although the firebrat's gut microflora often decreased significantly over time, no corresponding decreases over time were observed in the firebrat's gut cellulase levels in either test or control samples in any of the feeding studies (nystatin: $F = 0.28$, $df = 2{,}24$, $P > 0.70$; metronidazole: $F = 2.13$, $df = 4{,}40$, $P > 0.05$; streptomycin: $F = 0.88$, $df = 4{,}35$, $P > 0.40$; tetracycline: $F = 2.38$, $df = 4{,}38$, $P > 0.05$; antibiotic cocktail: $F = 1.73$, $df = 1{,}32$, $P > 0.10$) (Table 1). No significant treatment × sampling period interactions were observed (Table 2).

DISCUSSION

In this study firebrats were fed diets containing four antibiotics (nystatin, metronidazole, streptomycin, and tetracycline), singly and combined in a cocktail containing all four. The presence of these antibiotics in the food had no effect on consumption or survival. Each of the antibiotics singly and the combination of all four caused significant reductions in the number of microorganisms present in the firebrat's gut but had no effect on levels of activity of gut fluid toward microcrystalline cellulose. These findings argue against a microbial origin for the soluble cellulases present in the gut fluids of firebrats.

In several of our experiments, microbial populations in the gut declined over time, even in firebrats fed an antibiotic-free diet. Apparently proliferation of microbes in the gut is not always rapid enough to maintain high steady-state populations, and the maintenance of microbial populations at the levels observed in wild-caught individuals requires a constant ingestion of bacteria to replace losses due to digestion and excretion. Decreases in the size of the populations of gut microbes associated with maintenance in a laboratory culture were never accompanied by a corresponding reduction in gut cellulase levels. This observation also argues against a microbial origin for the soluble cellulases present in the gut fluids of this species.

Our findings are, therefore, in agreement with earlier studies that suggest that silverfish (Lasker and Giese, 1956) and firebrats (Zinkler and Götze, 1987) digest cellulose without the aid of symbiotic gut microbes. However, two important caveats must be attached to this conclusion. First, in no case did the antibiotic treatment remove all of the firebrat's gut microflora. Even in the most effective treatment, the gut still contained a 10^7 microbes. Even the removal of 90% of an insect's gut flora still leaves a large population of active microbes in the gut, and the possibility cannot be completely ruled out that cellulose

TABLE 1. GUT CELLULASE ACTIVITY[a] MEASURED IN FIREBRATS FEEDING ON ANTIBIOTIC-FREE AND ANTIBIOTIC-CONTAINING DIETS DURING COURSE OF EACH ANTIBIOTIC (Ab) FEEDING STUDY.

Sampling period[b]	Treatment	Antibiotic feeding study				
		Nystatin	Metronidazole	Streptomycin	Tetracycline	Ab cocktail
1	Ab-free	29.2 ± 7.6(5)	51.6 ± 11.8(5)	21.6 ± 8.5(5)	21.6 ± 8.5(5)	36.3 ± 5.8(10)
	Ab-containing	22.9 ± 4.2(5)	46.5 ± 7.1(5)	23.1 ± 9.4(5)	20.6 ± 5.9(5)	34.2 ± 5.1(10)
2	Ab-free		75.4 ± 12.3(5)	35.4 ± 22.6(5)	35.4 ± 22.6(5)	
	Ab-containing		55.7 ± 2.8(5)	33.5 ± 6.8(4)	30.4 ± 2.9(5)	
3	Ab-free	29.9 ± 6.3(5)	52.2 ± 9.6(5)	41.1 ± 13.4(5)	41.1 ± 13.4(5)	
	Ab-containing	29.9 ± 5.2(5)	59.4 ± 3.4(5)	39.6 ± 6.7(5)	55.4 ± 5.7(5)	
4	Ab-free		51.5 ± 9.0(5)	30.2 ± 11.7(5)	30.2 ± 11.7(5)	35.0 ± 4.0(8)
	Ab-containing		74.0 ± 3.2(5)	56.8 ± 15.2(5)	51.8 ± 5.7(5)	51.3 ± 6.0(8)
5	Ab-free	32.4 ± 6.2(5)	52.5 ± 4.3(5)	38.8 ± 6.4(3)	38.8 ± 6.4(3)	
	Ab-containing	28.2 ± 7.8(5)	44.0 ± 3.3(5)	51.6 ± 9.9(5)	50.4 ± 15.9(5)	

[a] Measured as micromoles glucose liberated per gram gut per hour for the conditions of the assay (45°C, pH 5.0, incubation volume 1.0 ml). Each value is the mean ± SEM for the number of replicates in parentheses.

[b] Sampling periods 1, 3, and 5 for the nystatin feeding study equal 7, 14, and 21 days, respectively. Sampling periods 1, 2, 3, 4, and 5 for the metronidazole, streptomycin, tetracycline, and Ab-cocktail feeding studies equal 3, 6, 9, 12, and 15 days, respectively.

TABLE 2. *F* STATISTICS FROM TWO-WAY ANOVAS ON VIABLE GUT BACTERIA, TOTAL GUT BACTERIA AND GUT CELLULASE LEVELS[a]

	Antibiotic feeding study				
	Nystatin	Metronidazole	Streptomycin	Tetracycline	Ab-cocktail
Viable gut bacteria					
Treatment	0.037		2.18	17.70***	13.06***
	(1, 23)		(1, 36)	(1, 32)	(1, 29)
Sampling period	1.84		0.46	1.54	8.98**
	(2, 23)		(4, 36)	(4, 32)	(1, 29)
Interaction	1.18		0.40	0.69	0.96
	(2, 23)		(4, 36)	(4, 32)	(1, 29)
Total gut bacteria					
Treatment	0.069	13.84***[b]	15.52***	30.85***	19.43***
	(1, 24)	(1, 40)	(1, 40)	(1, 40)	(1, 30)
Sampling period	0.49	15.89***	4.66**	8.88***	3.29
	(2, 24)	(4, 40)	(4, 40)	(4, 40)	(1, 30)
Interaction	1.82	2.61*	2.92*	1.63	1.24
	(2, 24)	(4, 40)	(4, 40)	(4, 40)	(1, 30)
Gut cellulase levels					
Treatment	0.45	0.024	0.80	2.02	2.09
	(1, 24)	(1, 40)	(1, 35)	(1, 38)	(1, 32)
Sampling period	0.28	2.13	0.88	2.38	1.73
	(2, 24)	(4, 40)	(4, 35)	(4, 38)	(1, 32)
Interaction	0.13	2.26	0.47	0.41	2.85
	(2, 24)	(4, 40)	(4, 35)	(4, 38)	(1, 32)

[a] Degrees of freedom are in parentheses.
[b] *$0.05 > P \geq 0.01$, **$0.01 > P \geq 0.001$, ***$P < 0.001$.

digestion is mediated by microbial populations not affected by the antibiotics employed. Second, it is possible that the antibiotics did remove a significant portion of the firebrat's cellulolytic flora but also selected for resistant strains, and that following the initial reduction the resistance strains recovered to initial levels and continued to bring about cellulose digestion.

Our experiments were designed to address these two possible complications. First, we used four antibiotics with different activity profiles: nystatin, a naturally occurring fungicide active against yeasts and filamentous fungi (Greenwood, 1989); metronidazole, a nitroimidazole antibiotic active against anaerobic bacteria, trichomonads, and amoeba (Conte and Barriere, 1988); streptomycin, a broad spectrum aminoglycoside antibiotic active against aerobic gram-negative bacteria and some gram-positive species (Kurylowicz, 1976); and tetracycline, a broad spectrum antibiotic active against aerobic and anaerobic bacteria, mycoplasmas, chlamydiae, and rickettsiae (Conte and Barriere, 1988). If cellulose

digestion were brought about by bacteria in firebrats, it seems unlikely that the subgroup of crop microflora responsible would be unaffected by any of the antimicrobial agents used. Second, we performed a series of closely spaced measurements of microbial numbers and cellulase activities in each feeding trial, an experimental design that would have detected an initial decline followed by a rebound of microbial numbers. No such pattern was observed in any of our experiments. Thus, we believe that this study provides strong support for the hypothesis that cellulose digestion in firebrats is not dependent upon the participation of symbiotic gut microbes.

Three mechanisms have been proposed to explain the ability of some insects to digest cellulose (reviewed in Martin, 1987, 1991): (1) cellulolysis mediated by microbial symbionts residing in or associated with the gut; (2) cellulolysis resulting from the presence of microbial cellulases originally present in the food that remain active in the gut following ingestion; and (3) symbiont-independent digestion brought about by enzymes exclusively of insect origin.

Evidence for symbiont-independent cellulose digestion mediated by enzymes exclusively of insect origin has been slow in coming but is mounting. The strongest cases for symbiont-independent cellulose digestion have been made for the nasute termites *Nasutitermes walkeri* and *N. exitosus* (Schulz et al., 1986; Hogan et al., 1988a) and the Australian wood-eating roach *Panesthia cribrata* (Scrivener et al., 1989). Slaytor (1992) has gone so far as to propose that cellulose digestion in all roaches and termites can be accomplished entirely by the action of endocellulases of insect origin. According to Slaytor (1992), symbiont-independent cellulose digestion in insects is possible, even in the absence of exocellulases, if endocellulases with low activity toward crystalline cellulose are produced in sufficient quantities, or if the food contains a significant component of noncrystalline cellulose that can be degraded by endocellulases alone. Endocellulases with low activity toward crystalline cellulose have been reported to be produced by eight species of termite (Potts and Hewitt, 1973, 1974a,b; Veivers et al., 1981, 1991; Schultz et al., 1986; Hogan et al., 1988a,b; Rouland et al., 1988a,b) and three species of roach (Scrivener et al., 1988; Zhang et al., 1993).

If cellulose digestion in the common firebrat and other primitive hexapods is mediated by insect-derived cellulases, then it is reasonable to conclude that symbiont-independent cellulolytic capacity is a primitive trait in insects. This conclusion is entirely compatible with Slaytor's suggestion (Slaytor, 1992) that cellulose digestion in termites and roaches can be accomplished effectively by endogenous enzymes, without the obligatory participation of microbial symbionts, and implies that symbiont-mediated cellulose digestion is a derived condition in insects.

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RED MAPLE (*Acer rubrum*) INHIBITS FEEDING BY BEAVER (*Castor canadensis*)

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Abstract—At many beaver (*Castor canadensis*) sites at Allegany State Park in New York State, red maple (*Acer rubrum*) is the only or one of the few tree species left standing at the ponds' edges. The relative palatability of red maple (RM) was studied in three ways. (1) At seven beaver sites, the available and utilized trees were recorded and an electivity index (E) computed. Of 15 tree species, RM ranked second or fourth lowest. (2) In experiment I, RM, sugar maple (*A. saccharum*, SM), and quaking aspen (*Populus tremuloides*) logs were presented cafeteria style at 10 colonies. RM was the least preferred. (3) Bark of RM was extracted with solvents. Aspen logs were painted (experiment II) or soaked (experiment III) with this RM extract and presented to beaver cafeteria-style, along with aspen and RM controls. This treatment rendered aspen logs less palatable, indicating that a chemical factor had been transferred.

Key Words—*Acer rubrum*, Adirondacks, aspen, beaver, *Castor canadensis*, feeding inhibition, *Populus tremuloides*, red maple.

INTRODUCTION

Beaver (*Castor canadensis*) at Allegany State Park (ASP) in New York State have clear-cut the areas around many of their ponds. The last remaining single mature deciduous trees are often red maple (*Acer rubrum*), even though its wood classifies it as a "soft maple" in contrast to sugar maple (*A. saccharum*), a

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"hard maple." Beaver prefer trees with soft wood. It is possible that red maple (RM) repels herbivores chemically. If so, the antifeedant compound(s) would be candidates for an effective beaver repellent. Ever-increasing beaver populations and damage complaints call for a potent chemical repellent, among other control measures. For these reasons, we undertook a study of food preferences by beaver at ASP. This included a survey of available and selected trees at several beaver sites, cafeteria-style palatability experiments at beaver ponds, chemical extraction of RM, and palatability experiments with RM extracts transferred to logs of the most preferred tree, quaking aspen (*Populus tremuloides*).

METHODS AND MATERIALS

This study consisted of three steps: (1) Tree preferences by beaver were determined by recording the number of trees available and those utilized. (2) Relative palatability was determined by cafeteria-style choice experiments at beaver ponds. (3) RM bark was extracted with solvents, and the extract transferred to aspen logs. These RM-extract-treated aspen logs were presented to free-living beaver at their ponds in cafeteria-style choice experiments.

Relative Utilization of Red Maple by Beaver. The number of intact trees and stumps left by beaver were recorded for each tree species at three sites on December 29 and 30, 1984, and at four different sites on February 19 and 20, 1985. We sampled around the ponds up to 60 m from the water. This is how far from the pond there were signs of beaver foraging. The number of felled trees of each species was related to the number of originally available trees (intact trees plus stumps) by computing the electivity index, E (Jacobs, 1974), for each species j:

$$Ej = \ln \frac{(rj)\,(1 - pj)}{(pj)\,(1 - rj)}$$

where r = number of utilized trees of a given species, and p = number of available trees of that species. An E larger than zero represents preference, smaller than zero avoidance. Values near zero mean a species is taken in proportion to its abundance. The significance of E is determined with a chi-square test (Jenkins, 1979).

Choice Experiments, Untreated Logs. For experiment I, branches from quaking aspen (*Populus tremuloides*, QA), sugar maple (*Acer saccharum*) (SM), and RM were cut into 20-cm-long (average diameter 2.71 ± 0.72 cm) sections. These logs were placed at 10 beaver ponds in the evening, 12 at a time (four replications of each species). Preferences for untreated logs were tested twice: at ASP on March 12–15, 1985, and at Cranberry Lake Biological Station (CLBS) in the Adirondacks on July 3–6, 1985. At CLBS, yellow birch (*Betula alle-*

gheniensis, YB) was added, because it is frequently utilized by beaver there. Equal numbers of logs were used; four for each of the three tree species at ASP, and three for each of the four species at CLBS. The logs were placed at the water's edge near the lodge and arranged 20 cm apart, parallel, in a row in predetermined random sequence. On the following day, the logs were examined for beaver activity. They were classified as removed, fully or partially peeled, sampled, or left intact. Most logs were still at the feeding site, while some were floating nearby or had drifted to the dam. Some were found one or several days later, after beaver had first taken them to the lodge and then discarded the peeled logs.

Solvent Extraction of Red Maple. Winter-dormant RM twigs were harvested in ASP from the areas within foraging range of beaver. They were collected and transported to the laboratory in freezing temperatures, and then kept at −10°C in a walk-in freezer. Still frozen, they were cut into short (20-cm) pieces and shredded in a Wiley mill. The resulting fine chips were placed in 2-liter amber glass jars and soaked in solvent. In the first experiment, five solvents of different polarities were used. These were methanol, ethyl acetate, acetone, ethyl ether, and hexane. The solvent was removed from the chips in a Buechner funnel. For every gram of wood chips, approximately one gram of extract was obtained. In the second experiment, only methanol was used as a solvent. An average log had about 15 g of bark. The original extract was concentrated so that 5 ml contained material from 15 g of bark, the amount to be transferred to each experimental log, as described below.

Choice Experiments, Treated Logs. For experiment II, aspen logs painted with RM extract were bioassayed three times at ASP (April 22–26, May 24–25, and November 27–30, 1985) and once at CLBS (July 8–9, 1985). As controls, untreated RM and aspen logs and solvent-treated aspen logs were used. Twelve logs per night were placed at each pond.

For experiment III, the three phases took place on April 6–10, 1991, April 7–12, 1992, and April 17–21, 1993. Based on our experience, experiment III differed from experiment II in three ways: (1) The logs were punctured by rolling them over a nailboard to increase the amount of extract they absorbed. These perforated logs were dried for 2 hr, soaked with concentrated extract or solvent (controls) for 2–3 hr in flat trays, and periodically turned over. Five milliliters of extract was used per log, representing the extract from 15 g bark. (2) After drying for 2–3 hr at ambient temperature, 10 logs each were nailed, evenly spaced 20 cm apart, to a 2-m long pole, so that the beavers removed only logs they were motivated to feed on. In 1991, two such 10-log arrays were placed, tied to two pegs, at each of four ponds as seen in Figure 1. In 1992 and 1993, one array each was placed at 10 sites. (3) Extracts were made from different types and parts of RM trees: In 1991 we used 10 different treatments. These included five different RM extracts: from RM trunk base (beaver area), twigs

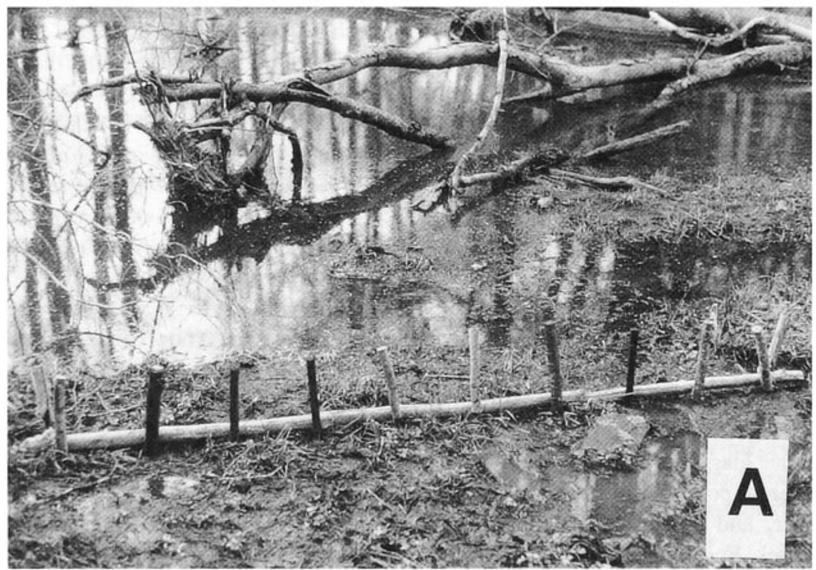

FIG. 1. (A) An array of the 10 treated samples, placed at the edge of an active beaver pond. (B) An array of samples on the day following its placing, after the beaver have made their selections.

TABLE 1. SAMPLES TESTED DURING 1991, 1992, AND 1993[a]

	1991	1992	1993
Controls			
Aspen, intact (Ai)	x	x	x
Aspen, punctured (Ap)	x	x	x
Aspen, EtOH soaked (Ae)	x	x	x
Red maple intact (Ri)	x	x	x
Red maple punctured (Rp)	x	x	x
Red maple, EtOH soaked (Re)		x	x
Scots pine, intact (Si)	x		
Extracts tested			
Extract of RM (juvenile regrowth) on aspen (Arj)	x	x	x
Extract of RM base on aspen (Arb)	x	x	
Extract of RM mature twigs on aspen (Art)	x	x	
Extract of aspen twigs on Aspen (Aat)		x	x
Extract of RM juvenile regrowth on RM (Rrj)			x
Extract of aspen on RM (Ra)			x

[a]Carrier logs: A = aspen; R = red maple; S = Scots pine. Extract treatments: a = aspen; b = tree base; e = EtOH; i = intact; j = juvenile regrowth (after cutting by beaver); p = perforated; r = red maple; t = twigs from mature tree.

of mature trees from two beaver colonies, and one upland site beyond the foraging range of beaver, and juvenile regrowth after beaver cutting. The remaining five samples were controls, including intact aspen, bark-punctured aspen, ethanol-soaked aspen, intact RM, and punctured RM. In 1992 we extracted RM tree base bark, juvenile regrowth after cutting by beaver, and twigs from mature trees at beaver sites. RM logs treated with aspen twigs extract were added as a new sample to test whether palatability of an undesirable species can be increased by a chemical factor from a preferred species. Because the different RM extracts did not have different effects in 1991 and 1992, only one type of RM extract (juvenile regrowth, beaver site) was used in 1993 and applied to both aspen and RM. Aspen extract was also applied to RM and aspen. The remaining six samples were controls: aspen and RM, each intact, perforated, or EtOH-soaked. The aspen logs for 1991 averaged 2.71 $\pm$ 0.72 cm in diameter (N = 80) and for 1993, 2.58 cm (162 cm^2 surface, N = 30) and the RM logs in 1993, 2.73 cm (171.4 cm^2, N = 30). For comparison, on one aspen stand, saplings not used by beaver averaged 0.89 $\pm$ 2.58 (SE) cm (range 0.4–1.8 cm, N = 12) in diameter, while those that were beaver-cut averaged 1.95 $\pm$ 1.88 cm (range 0.9–3.1 cm; N = 12). In another study, beaver first cut experimental saplings ranging between 3.1 and 4 cm diameter, depending on distance (Fryxell and Doucet, 1993). Beaver that had moved into a new area preferred aspen of 1.5–7.5 cm diameter (Basey et al., 1988).

Statistically, each 10-log array constituted a block. Because samples were either eaten or avoided (including untouched and sampled), and rarely halfway peeled, nonparametric tests were used. Lost logs were not included in the analysis, even though almost all that were found later had been peeled. Multiple samples were compared by Cochran *Q* test, and samples were compared pairwise by the McNemar's test.

RESULTS

Relative Utilization of Red Maple. The results of two surveys of available and utilized trees are presented as electivity indices in Figures 2 and 3. In the first survey (three beaver sites) RM ranks fourth lowest in preference. Only hawthorn (*Crataegus* sp.), white pine (*Pinus strobus*), and Scots pine (*P. sylvestris*) rank still lower (Figure 2). In the second survey, RM ranks second lowest, between Norway spruce (*Picea abies*) and Scots pine (Figure 3). In general, the rankings agree with food preferences known for beaver from other studies (e.g., Fryxell and Doucet, 1993). Small sample sizes—sometimes only

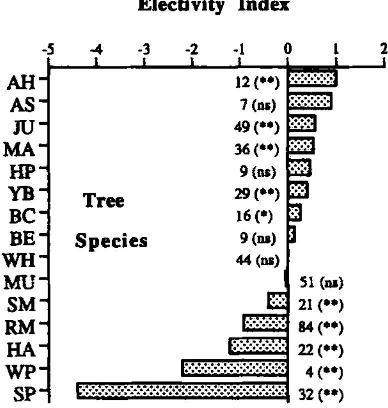

FIG. 2. Utilization of 15 tree species by three beaver colonies at Allegany State Park, New York, fall 1984, expressed by the electivity index *E*. Bars on right: preferred trees; bars on left: trees selected against. Significance levels: * = 0.05; ** = 0.01 (χ^2 test). Species abbreviations: AH: ash (*Fraxinus* sp.); AS: aspen (*Populus tremuloides*); JU: Juneberry (*Amelanchier* sp.); MA: maple (*Acer* sp.); HP: hop hornbeam (*Ostrya virginiana*); YB: yellow brich (*Betula lutea*); BC: black cherry (*Prunus serotina*); BE: beech (*Fagus grandifolia*); WH: witchhazel (*Hamamelis virginiana*); MU: musclewood (*Carpinus caroliniana*); SM: sugar maple (*Acer saccharum*); RM: red maple (*Acer rubrum*); HA: hawthorne (*Crataegus* sp.); WP: white pine (*Pinus strobus*); SP: Scots pine (*Pinus sylvestris*).

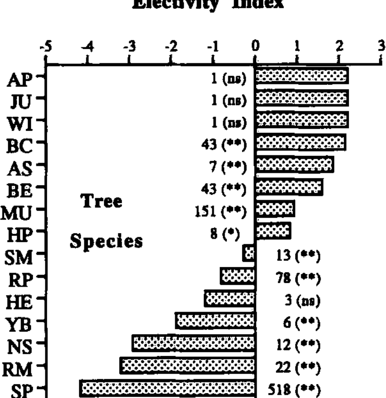

FIG. 3. Utilization of 15 tree species by four beaver colonies at Allegany State Park, New York, winter 1984/85. Species Abbreviations same as Figure 2 with these new species: AP: apple (*Pyrus malus*); WI: willow (*Salix* sp.); RP: red pine (*Pinus resinosa*); HE: eastern hemlock (*Tsuga canadensis*); NS: Norway spruce (*Picea abies*).

one tree or stump of one species—result in nonsignificant rankings. In Figure 3, for instance, the significant preferences series starts with black cherry (fourth bar).

Experiment I: Choice Experiments, Untreated Logs. In the first choice test at ASP, beaver consumed 94.7% of QA, 76.3% of SM, and 46.3% of RM. The preference is significant ($\chi^2 = 45.9$; $df = 6$; $P < 0.001$). The second choice test, at CLBS, took place in July when beaver take little woody vegetation. Of the four tree species offered, QA was taken most often, but only 38.9% of the logs were utilized (YB: 13.9%; SM: 11.1%). RM was never taken. The preference is significant ($\chi^2 = 21.68$; $df = 6$; $P < 0.01$).

Experiment II: Pilot Tests with Extract Painted Logs. First, three pilot tests were performed: In the first test, in April 1985, the painting of aspen logs with RM had no effect on consumption. Aspen, solvent-painted aspen, and RM-painted aspen were eaten equally often (80.6%, 86.8%, and 82.6%, respectively). Only intact RM was consumed less (58.3%). In the second test, in May 1985, aspen logs painted with RM extract were consumed as often (55%) as control logs painted with solvent (57.5%) or intact aspen (60%). Intact RM, however, was taken significantly less than the other three samples ($\chi^2 = 9.269$; $df = 2$; $P < 0.01$). Thus, the painting had no effect. For the third test, in November 1985, the percentages eaten were 42.5%, 40%, 36.6%, and 22.5% respectively, for aspen, solvent control, RM-treated aspen, and intact RM. Still, no significant differences were found. Likewise, in the test at CLBS in July 1985, painting logs did not reduce their palatability.

Experiment III. Choice Tests with Extract-Soaked Logs. The second series with treated logs at ASP covered the three spring seasons 1991–1993. In 1991, a total of 320 logs were placed. Eight arrays of 10 logs each were never touched and therefore not counted. Of the remaining 240 logs, 193 were recovered. Another 47 had been carried away and remained missing. (Most missing logs found later were peeled.)

Beaver consumed 80.0%, 60.0%, and 32.0%, respectively, of the three key samples ($N = 25$ for each)—intact aspen (Ai), aspen treated with extract from juvenile regrowth of red maple at a beaver site (Arj), and intact red maple (Ri). These differences were significant (Cochran Q test: $Q = 14.53$; $P < 0.001$, one-tailed). Samples Ai and Arj, compared by the McNemar test, were not significantly different, due to a small sample size. The three extracts of red maple (r) twig and tree base did not differ in their effects. Therefore, they were combined into the Ar category (aspen logs treated with red maple extract).

For a larger sample size, all 1991 RM extract samples (Ar; $N = 104$) were compared first to all three types of control aspen (Ac; $N = 75$) including intact, punctured, and soaked in EtOH, then to all red maple controls (Rc; $N = 48$) including intact and punctured (Rp), independent of the original blocks. Ac were consumed most often (73.6%), and Rc least often (37.5%). Ar were intermediate (57.7%). They were significantly more avoided than Ac ($\chi^2 = 4.02$; $P < 0.05$, two-tailed), and eaten significantly more often than Rc ($\chi^2 = 4.58$; $P < 0.05$; two-tailed).

In 1992, the same treatments were repeated, and an aspen extract (Aa) added to test whether a "super-aspen" could be created. First, the four treatments with expected extreme results were compared by Cochran Q test. These were Aa, Ap, Ar, and Rp ($N = 35$ for each treatment). The differences in this multiple comparison were highly significant ($\chi^2 = 13.47$; $df = 3$; $P < 0.001$, two-tailed). In a matched two-sample comparison, Ar was avoided more than Ap (McNemar test, $\chi^2 = 3.273$; $P < 0.05$; one-tailed). Ar was eaten less often than Ai, but not significantly so. The beaver did not treat Aa as super-aspen; they consumed Aa and Ai equally often ($\chi^2 = 0.8$; NS).

In 1993, the only type of RM extract tested on aspen was from juvenile regrowth (stump sprouts) at a beaver site, henceforth labeled Ar. As expected, Ri, Ar, and Ai differed significantly (Cochran Q test, $Q = 31.4$; $P < 0.001$; $N = 33$ for each treatment). The beavers' responses to the three key samples Aa, Ai, and Ar also differed significantly (Cochran Q test: $Q = 13.76$; $df = 2$; $P < 0.001$, two-tailed). Aspen extract was used on aspen (Aa) and RM (Ra). Treating RM with aspen extract (Ra) raised acceptance of RM significantly ($Q = 14.6$; $P < 0.0005$), compared with Ri and Rr. Pairwise comparisons by McNemar's test showed that beaver avoided Ar more than Aa ($\chi^2 = 2.77$; $P < 0.05$), while Ar was consumed slightly less than Ai ($\chi^2 = 2.5$; $P = 0.06$). They accepted Ra more often than Rr ($\chi^2 = 7.11$; $P < 0.005$). In other words,

treatment by RM or aspen extract resulted in clear palatability differences for both aspen and RM logs. Ri and Rr were never eaten (except for one Ri log), so that treatment with RM extract (i.e., changing Ri into Rr) could not make RM any more unpalatable than Ri already was. The results for 1993 are summarized in Figure 4.

Trends over Time. In 1991, the total number of logs utilized increased from 36.8% on the first day to 77.6% on the fourth, and in 1992 from 46% on the first, to 77.8% on the last day. Neither trend was significant (Spearman rank correlation coefficient). In 1993, no such trend was observed.

Colony Differences. The beaver colonies differed significantly in their responses. Some colonies never touched the experimental logs, others only on certain days. Eight of 40 arrays (20%) were not touched in 1991, 15 of 50 (30%) in 1992, and 12 of 50 (24%) in 1993. These were all excluded from the analysis on the assumption that beaver may not have encountered the samples or were not even present at the site when the samples were presented. For those colonies that consumed at least some of the logs, multiple range analysis revealed three significantly different groups. Colonies that consumed more logs were also less selective. In 1992 the three colonies (sites 50, 61, and 58) that consumed the least number of logs consumed the red maple controls Ri and Rp near zero levels (0.55 and 0.01%, respectively). By contrast, the three colonies that consumed the most (sites 49, 62, and 81B), used the two RM controls Ri and Rp at averages of 32.3 and 30.9%, respectively. In 1993, the three colonies that

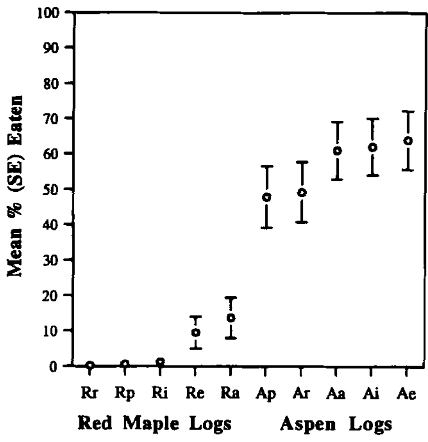

FIG. 4. Consumption of 10 differently treated samples during April 1993. For abbreviations see text.

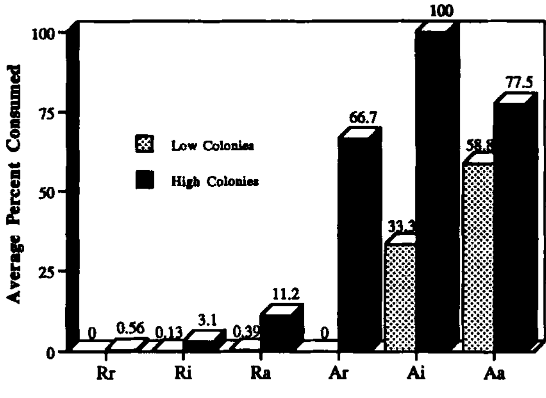

FIG. 5. Average percent of bark area removed from treated logs at the three least consuming (stippled bars) and three most consuming (black bars) colonies in 1993.

consumed the least (sites 42, 82, and 150L) only took aspen controls (Figure 5). Of these, super-aspen (Aa) was utilized most (59%). However, intact aspen (Ai) averaged only 33.3%. The three colonies that consumed the most (sites 53, 61, and 150U), increased their consumption of aspen controls and super-aspen, Ai, even to a 100% average, but their choices also extended to other samples: they accepted Ar (66.7%), and even some RM samples, such as Ra (11.2%) and Re (4.7%). Both probably were more palatable because of their specific modification by aspen extract or EtOH, respectively. The latter may have extracted some deterring compounds.

DISCUSSION

Red maple was clearly avoided by beaver in our study area. It may rank differently in other areas. For instance, we noted that beaver utilized red maple to a greater degree in the surroundings of Cranberry Lake Biological Station in the Adirondacks in New York State. The mature red maple trees still standing at otherwise clear-cut beaver sites at ASP most likely grew up during the beaverless time between the near-extinction of beaver in the 19th century and their reappearance at ASP in 1937 (Shadle and Austin, 1939). Logging further favored the RM by eliminating competition from other trees.

In other studies, beaver have utilized red maple least of all tree species (Shadle and Austin, 1939; Henry and Bookhout, 1970) or consumed it according to its local abundance (Belovsky, 1984; Johnston and Naiman, 1990). Beaver

have not been reported to prefer red maple. In some studies, the different species of *Acer* have not been distinguished (Shadle et al., 1943; Jenkins, 1975, 1978, 1979, 1980, 1981). The work by Shadle and Austin (1939) is particularly pertinent. They counted trees cut by the first beaver that moved into Allegany State Park. From their arrival in the summer of 1937 to November 1938, the beaver cut 226 trees. Only one was a red maple, leaving open how much of it the beaver actually consumed. The authors state ". . . the maples and birches, although present, were not much used . . . ." The abundance of the various tree species were not reported in that study. Silver maple (*Acer saccharinum*), a soft maple like RM, was selected against by a beaver population in Wisconsin to the point that it remained the dominant tree at lower elevations near beaver ponds (Barnes and Dibble, 1988).

It is well known to foresters that red maple is less afflicted by herbivorous insects and less browsed by deer than is sugar maple (N. Richards, personal communication). We assume that phenolics render the red maple less palatable to beaver. Most *Acer* species contain considerable amounts of condensed and hydrolyzable tannins, usually in a reciprocal relationship (Bate-Smith, 1977). In the field, a simple test with $FeCl_3$ solution placed on a cross section of a green branch shows a difference between red maple and sugar maple. Red maple turns a deep purple, sugar maple a pale green. Different growth forms are expected to contain different levels of secondary plant compounds. However, the relationships are not always clear. For instance, quaking aspen juvenile-type regrowth after cutting by beaver is consumed less by beaver, and yet it contains lower levels of salicin and tremuloidin than adult-form sprouts (Basey et al., 1990). A marsupial, the brush-tailed possum, *Trichosurus vulpecula*, fed more on different clones of poplars (*Populus* sp.) having lower levels of salicin and its derivatives (Edwards, 1978).

Colonies differed considerably in their feeding levels and selectivity. Some never touched the samples, others fed on some samples, while still others fed on most samples with little discrimination (Figure 5). Two colonies even preferred Scots pine (*Pinus silvestris*) to aspen samples. They had been feeding on Scots pine before and during the time of the experiment. Other authors (e.g., Jenkins, 1975, 1981) have also noted that tree preferences by beaver can vary between colonies and even within colonies, both spatially and over time.

It appears that the aspen logs remained attractive to the beaver to varying degrees despite the modification by the extracts. Therefore, for future experiments, we recommend not only higher concentrations of extracts, but also carrier logs from trees of medium preference, such as black cherry (*Prunus serotina*), sugar maple, or musclewood (*Ostrya* sp.) in areas similar to ASP. These neutral trees could be rendered more palatable by aspen extract and less palatable by RM extract. Eventually, of course, repellents will have to be tested on the

specific tree species that are to be protected. This includes ornamental, fruit, and forest trees.

Beaver take logs into the water and peel them there. They also pulled whole arrays of our experimental logs attached to a pole into the water. RM extract may leach out when the logs are thus immersed. We also observed occasionally that beaver felled a red maple into their pond, but did not consume the branches at all or only after a few days' soaking in the water. The winter food caches in the water may also permit leaching out of compounds that inhibit feeding. Could such "food processing" be a regular strategy of beaver? If so, in future experiments, presoaked logs should be more acceptable to beaver.

Beaver use smell (Doucet et al., 1994) and possibly taste and other senses to select food. The frequent sampling, i.e., biting into experimental logs and naturally growing trees, suggests that the sense of taste is important. Learning by feedback from ill effects of unpalatable plants is also likely. A taste experience may condition an odor aversion so that subsequently an odor suffices as a signal. In black-tailed deer (*Odocoileus hemionus columbianus*), compounds derived from red pepper failed to inhibit feeding, suggesting that the trigeminal nerve may not play a role (Sullivan et al., 1985).

It is possible that red maple has developed chemical defenses in response to herbivory by beaver. Beaver and red maple have coexisted since the Tertiary, ample time for coevolution to have occurred. Furthermore, red maple is insect-pollinated. This facilitates faster selection (than wind pollination) in the limited areas along streams and around lakes where beaver dwell. To test this hypothesis, the levels of secondary plant compounds in upland populations of red maple far removed from beaver should be compared with those of red maples along streams. More extensive experiments than our 1991 test are needed to establish palatability differences between upland red maples and those in beaver-populated stream valleys.

Red maple logs can also be made more attractive to beaver by treating them with extract of aspen, a preferred food. This fact, together with its opposite, the feeding inhibition by red maple extracts, shows that chemical factors, and not merely texture or other properties of bark or wood, play a role.

Our findings can be applied in beaver management to reduce wildlife damage. We propose that extracts and compounds from less palatable tree species should be potent beaver repellents that can be applied to individual specimen trees. To keep herbivores out of entire areas, "fear" repellents instead of negative odors or taste compounds are recommended (Sullivan et al., 1985). Recent field tests showed that predator odors applied to aspen sticks reduced feeding by beaver (Engelhart and Müller-Schwarze, 1994). A repellent has to prevent cutting bark and felling trees, not just feeding, as in our experiments. As beaver-proof tree cover in larger areas and for longer time-frames, we suggest planting the less vulnerable red maple near beaver sites, along with conifers such as pine

and spruce species. Conversely, where desirable, one could possibly render some tree species more palatable to captive or free-ranging beaver by treating them with extracts from aspen.

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ANALYSIS, ISOLATION AND INSECTICIDAL ACTIVITY OF LINEAR FURANOCOUMARINS AND OTHER COUMARIN DERIVATIVES FROM *Peucedanum* (APIACEAE: APIOIDEAE)

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Abstract—*Peucedanum arenarium* Waldst. & Kit., *P. austriacum* (Jacq.) Koch, *P. coriaceum* Reichenb., *P. longifolium* Waldst. & Kit, *P. officinale* L., *P. oreoselinum* (L.) Moench, *P. ostruthium* L., and *P. palustre* (L.) Moench accumulate different structural types of coumarins including simple coumarins, linear furanocoumarins, linear dihydropyranocoumarins, angular dihydrofuranocoumarins and angular dihydropyranocoumarins. Linear furanocoumarins, known for various biological activities, include some well-known antifeedants, such as bergapten, isopimpinellin, and xanthotoxin. The aim of this investigation was to screen the diverse coumarins from *Peucedanum* for insecticidal activity. LC was used to analyze and isolate coumarins for the bioassays. A growth inhibition bioassay with 17 derivatives, comprising all structural types from *Peucedanum*, carried out with *Spodoptera littoralis* (Boisduval) (Lepidoptera: Noctuidae) as test organism, indicated the majority of the linear furanocoumarins and the angular dihydrofuranocoumarin athamantin as active compounds. Oxygenation of the prenyl residue of linear furanocoumarins decreased activity. Further formation of an ester with angelic acid even resulted in complete inactivity. Five active linear furanocoumarins,

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bergapten, isopimpinellin, xanthotoxin, isoimperatorin, and imperatorin, and two linear furanocoumarins with a substituted furan ring, peucedanin and 8-methoxypeucedanin, were compared in a dietary utilization bioassay. Relative growth rate (RGR) and relative consumption rate (RCR) divided the tested coumarins in three groups of similar activity. Isopimpinellin and peucedanin slightly decreased RGR and RCR of the treated larvae, and xanthotoxin, isoimperatorin, and 8-methoxypeucedanin heavily decreased RGR and RCR. Bergapten and imperatorin differed by the lowest RGR values and rather high RCR values. The effects caused by these two coumarins indicate specific postingestive toxicity. The results obtained in this study add to the reputation of coumarins to be an effective chemical defense, postulating that chemical diversity is a necessary trait for well-defended plants.

Key Words—Apiaceae, *Peucedanum*, Lepidoptera, Noctuidae, *Spodoptera littoralis*, HPLC, preparative isolation, furocoumarins, furanocoumarins, pyranocoumarins, growth inhibition, dietary utilization, plant chemical diversity.

INTRODUCTION

The most recent review on coumarins (Murray, 1991) summarizes about 1250 monomeric derivatives isolated from plant sources. Within higher plants, simple coumarins occur in about 70 plant families. Coumarins with an additional ring system, furano- or pyranocoumarins, however, only occur in 15 plant families, the majority of them in two families, the Apiaceae and Rutaceae.

Berenbaum (1992) reviewed the various biological activities of this highly diversified class of secondary plant compounds including antifungal, antiviral, antibacterial, allelopathic, and insecticidal properties of some derivatives. Moreover, coumarins have been identified as phytoalexins in *Apium graveolens* L. and *Pastinaca sativa* L. within Apiaceae (Beier and Oertli, 1983; Heath-Pagliuso et al., 1992; Johnson et al., 1973) and in *Citrus* L. species within Rutaceae (Ben-Yehoshua et al., 1992; Gottstein et al. 1990). Furthermore, Berenbaum (for detailed references see Berenbaum, 1990) outlined a coevolutionary scenario between coumarin-containing Apiaceae and caterpillars specializing on them. According to investigations of Zangerl and Berenbaum (1993), the accumulation pattern of linear and angular furanocoumarins even explains to a large extent the distribution of *Depressaria pastinacella* (de Geer) larvae feeding on a population of *Pastinaca sativa*. The majority of heavily attacked individuals of *Pastinaca sativa* displayed comparatively low concentrations of furanocoumarins in contrast to the unattacked ones. These reports unequivocally demonstrate the biological relevance of certain coumarin derivatives within the defense system of plants accumulating them.

Within Apiaceae, *Peucedanum* L. is a genus classically defined by winged, dorsally flat fruits, widely distributed in Europe, Asia, and Africa (Drude, 1898). In North America fruits of this type characterize the genus *Lomatium* Rafin.

(Coulter and Rose, 1900). A phytochemical comparison of the underground parts of 13 *Peucedanum* species, indigenous to Central Europe, revealed 29 coumarins, including furano- and pyranocoumarins of the linear and angular type, which could be identified along the polyacetylenes, alkylbutenolides, and 2-methylchromones (Hadaček, 1989; Hegnauer 1990). With respect to the high degree of convergent and mosaic evolution observed in this rather heterogeneous genus (Solov'eva et al., 1985), the currently available data on the distribution of coumarins and other classes of compounds are still too few to allow insights into the chemical evolution within the genus. Thus, any taxonomic conclusions are preliminary, and hence chemotaxonomic implications of coumarin distribution with *Peucedanum* will not be discussed here.

Mothes (1976) describes the diversity of plant chemistry as a play of nature in which compounds only secondarily acquire a particular significance. Since they are often highly toxic to the organism that forms them, either a rapid excretion to the plant surface—Zobel and Brown (1991) report significant amounts of coumarins on fruit surfaces in coumarin-accumulating Apiaceae, Fabaceae and Rutaceae—or good internal protection in special compartments—like the characteristic oil ducts present in the fruits of Apiaceae—is necessary. Jones and Firn (1991) argue that this play of nature leads to a chemical diversity, the maintenance of which is the major requirement for sufficient chemical defense. Consequently, plants should display a high variety of inactive compounds besides a few active ones. Against recently adapted consumer organisms, mutations should generate new active molecules from the pool of inactive compounds and thus create new well-defended plants that can survive in a rapidly changing consumer environment. Screening procedures for lead structures displaying significant biological activity have revealed only few compounds so far, a fact which favors the theory outlined above. Jones and Firn (1991) explain this low hit rate with the high receptor specificity required of a highly active molecule. However, experimental data are still needed to test the hypothesis in detail.

Numerous reports reveal the antifeedant and toxic potential of xanthotoxin **(6)**, isopimpinellin **(9)**, and bergapten **(1)**, all linear furanocoumarins, against polyphagous herbivores (Berenbaum, 1978; Escoubas et al., 1992; Gebreyesus and Chapya, 1983; Klocke et al., 1989; Luthria et al., 1989; Muckensturm et al., 1981; Yajima et al., 1977; Yajima and Munakata, 1979). The chemical diversity of coumarins present in *Peucedanum* offers itself as an ideal basis to compare activity and diversity within a class of plant compounds already renown for insecticidal properties. As a test organism we chose *Spodoptera littoralis* (Boisduval), a polyphagous noctuid and well-known cotton pest, because it is easy to rearing in the laboratory. Preliminary experiments with this species revealed a similar susceptibility against bergapten **(1)** as observed in related *Spodoptera* species used in previous studies.

Little is known about herbivores associated with *Peucedanum*. Available studies focus on pests on umbelliferous crop plant (Ellis and Hardman, 1992). However, Carter and Hargreaves (1986) list *P. palustre* (L.) Moench as a food plant of *Papilio machaon* L., a butterfly who has adapted to host plants within Apiaceae and Asteraceae (Wiklund, 1975). *P. oreoselinum* (l.) Moench can also be a host to *P. machaon* (Hadaček, personal observation).

If a study is focused on chemical diversity in plants, all compounds included in the investigation have to be isolated from the plants directly. We used high-performance liquid chromatography (HPLC) in combination with UV-diode array detection to analyze crude extracts and to determine which of the different *Peucedanum* species and their particular organs were suitable sources for the isolation of coumarins needed for the bioassays. In order to present a low-cost solution—both to afford and to maintain—for a preparative LC system, we will illustrate separation techniques for complex coumarin mixtures employed in our laboratory.

METHODS AND MATERIALS

Plant Material. The following *Peucedanum* species are included in this investigation: *P. arenarium* Waldst. & Kit. (Hungary, Comitat Veszprém, SE of the village Bakonyszentlászlo, 17°40′ 47°07′ N, sandy grassland, 300 m.s.m.), *P. austriacum* (Jacq.) Koch (Austria, Lower Austria, Schneeberg near the village of Puchberg, 15°52′ 47°47′ N, forest clearings, 700 m.s.m.), *P. coriaceum* Reichenb. (Croatia (former Yugoslavia), Istria, Učka SW of the town Opatija, 14°05′ 45°10′ N, dry grassland, 1100 m.s.m.), *P. longifolium* Waldst. & Kit. (Turkey, NW Anatolia, ca. 20 km SW of the town Mudurnu, 31°02′ 40°24′ N, dry rocky grassland, 1700 m.s.m.), *P. officinale* L. (Austria, Lower Austria, NW of the town Marchegg, 16°49′ 48°17′ N, seasonally wet meadows, 170 m.s.m.), *P. oreoselinum* (L.) Moench (Austria, Lower Austria, N of the village Baumgarten, 16°53′ 48°19′ N, dry grassland, 150 m.s.m.), *P. ostruthium* L. (Austria, Carinthia, Plöckenpass, 12°56′ 45°37′ N, moist limestone ravines, 1500 m.s.m.), *P. palustre* (L.) Moench (Austria, Lower Austria, W of the village Amaliendorf, 15°05′ 45°46′ N, swamp, 580 m.s.m.). Herbarium specimens are deposited in the herbarium of the Botanical Institute, University of Vienna (WU). However, it was not always possible to obtain sufficient amounts for the bioassays from these particular *Peucedanum* species. The major amount of xanthotoxin (**6**) and isopimpinellin (**9**) was obtained from a fraction of a leaf extract of *Clausena indica* (Dalz.) Oliver (Rutaceae), collected near Anuradhapura in Sri Lanka (80°26′ 8°21′ N), and supplemental amounts of bergapten (**1**) were bought from Carl Roth GesmbH (Karlsruhe,

Germany). Umbelliferone (7-hydroxycoumarin) and aesculetin (6,7-dihydroxycoumarin) did not occur in the species investigated and were obtained from Fluka AG (Buchs, Switzerland).

Extraction. Fruits or underground organs (roots and/or stolons, 50–200 g) were dried, ground, and extracted with either petroleum ether (62–82°C)–diethyl ether (2 : 1), or hexane–diethyl ether–methanol (1 : 1 : 1) for three days, at room temperature.

Analysis of Crude Extracts. A Hewlett Packard 1090 II LC with UV diode array detection was used with 230 nm as the signal wavelength. The column (290 × 4 mm, Spherisorb ODS, 5 μm) was thermostatted at 40°C. MeOH (gradient 60–100%) in aqueous buffer (*o*-phosphoric acid 0.015 mol, tetrabutylammonium hydroxide 0.0015 mol, pH 3) served as mobile phase at a flow rate of 1 ml/min. The crude extract was concentrated to a final concentration of 10 mg/ml and filtered over RP-8 silicagel (0.040–0.060 mm). Ten microliters of this solution were injected.

Preparative Isolation. The crude extract was partitioned by open column chromatography into 50-ml fractions using silica gel (35–70 mesh). Hexane with increasing portions of diethyl ether and methanol was used as eluent. Thin-layer chromatography (TLC, Merck Silica Gel 60 F_{254} 0.25 mm) identified fractions containing similar compounds. These were united and subjected to preparative LC (column 400 × 40 mm, Merck Silicagel 60 Lichroprep 0.025–0.040 mm, UV detection, signal wavelength 254 mm, mobile phase ethyl acetate–hexane step gradients, flow rate: 20–25 ml/min). All fractions obtained were monitored by TLC. In most cases minor impurities could be removed by crystallization. Mixtures inseparable by these procedures were additionally subjected to cyclic LC. In contrast to a single run, this method allows exploitation of the separation capacity of the stationary phase. Additional valves enable the cyclic mode (see Figure 1A).

Structure Elucidation. ^{1}H NMR (Bruker 250 or 400 MHz, $CDCl_3$) was used to identify all compounds isolated (**1–20**). Commercially obtained umbelliferone and aesculetin were not included in spectroscopic analysis. The $[\alpha]_D^{20}$ was measured for all optically active coumarins with a Perkin Elmer 241 polarimeter in EtOH.

Insect Rearing. Larvae of *Spodoptera littoralis* (Lepidoptera: Noctuidae) were taken from a laboratory colony reared on artificial diet under controlled conditions as described previously (Srivastava and Proksch, 1991).

Incorporation of Coumarins into Artificial Diet. An adequate amount of test coumarin was dissolved in 96% ethanol and homogenized with 735 mg freeze-dried diet powder. The diet powder was made of 150 g ground beans, 3 g *p*-hydroxybenzoic acid ethyl ester, 3 g vitamin C, 30 g wheat germ, and 1 ml formaldehyde solution. After evaporation of the solvent, gentamicin sulfate (0.75 g in 2.12 ml water) and an agar solution (78.6 mg in 1.5 ml water) were

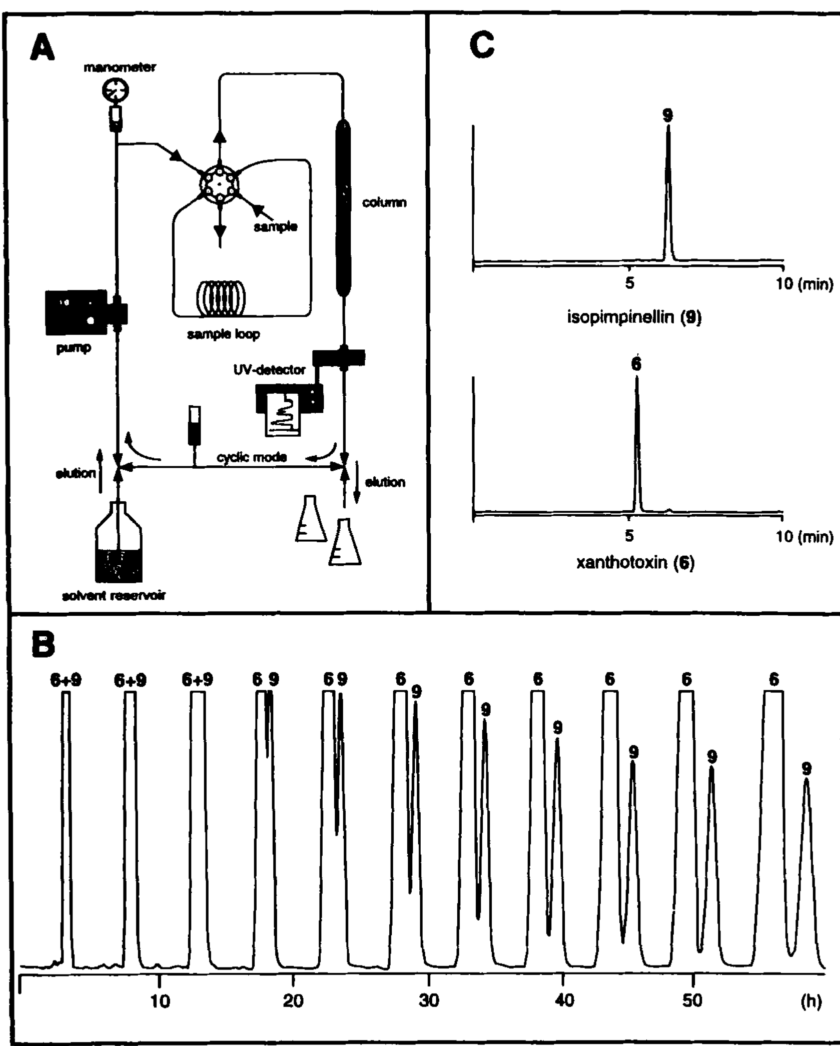

FIG. 1. (A) Low-cost preparative LC system with valve system for cyclic mode. (B) Cyclic separation of xanthotoxin (**6**, 28 mg) and isopimpinellin (**9**, 8 mg), mobile phase ethyl acetate–hexane (3:7). (C) Analytical reverse-phase HPLC of purified compounds **6** and **9**.

carefully added. After cooling, the diets were offered to *S. littoralis*. Controls were provided with diets treated with 96% ethanol only. All feeding experiments were conducted in the dark.

Growth Inhibition. Twenty neonate larvae were kept on 3.5 g diet spiked with different concentrations (1.0–5.0 μmol/g fresh wt) of the compounds studied. After six days, the combined weight of all surviving larvae was measured. The average weight of a single larva was determined by dividing the combined weight by the number of surviving larvae. The high number of tested individuals helps to obtain a reliable average value for the weight of a single larva. The growth of the treated larvae was expressed as a percentage of the average weight of the larvae kept on control diet (= 0 μmol coumarin, 100% growth). However, the procedure of this bioassay does not permit the calculation of a standard error.

Dietary Utilization. Seven linear furanocoumarins (**1, 2, 6, 7, 9, 10,** and **11**) were incorporated into the artificial diet at a concentration of 3 μmol/g fresh wt. Bergapten (**1**), xanthotoxin (**6**), and isopimpinellin (**9**), already well-known insecticidal compounds, had originally been excluded from the test series. To obtain information about structure–activity relationships, these compounds, together with 8-methoxypeucedanin (**11**), were included in the dietary utilization bioassay without previous testing for growth inhibition. Second-instar larvae of *S. littoralis* were weighed individually and transferred to treated diet cubes of known weight. Control larvae were weighed and transferred to the control diet. After three days, larvae and the remaining diet were weighed again to determine the net larval weight gain and the amount of diet consumed. Relative growth rates (RGR) and relative consumption rates (RCR) were calculated according to previously described methods (Kogan, 1986). A *t* test was performed according to standard procedures (Sachs, 1992). However, the limited amounts of the coumarins available excluded more specific tests for phototoxic and phytojuvenile hormone activity, both of which have to be carried through the whole life cycle of the insect for realistic evaluation.

RESULTS

HPLC-UV Analysis. Figure 2 gives an example of the diversity of coumarin accumulation in the fruits and underground organs of different *Peucedanum* species with predominant coumarin accumulation. *P. officinale* inhabits seasonally wet, but mostly dry meadows and grows a long woody tap root. Here, the coumarin pattern is dominated by isoimperatorin (**2**) and peucedanin (**10**). In contrast, the fruits display a variety of different linear furanocoumarins (**1, 2, 6, 7, 9, 10**). Similar patterns of coumarin diversity reflect the close relationship

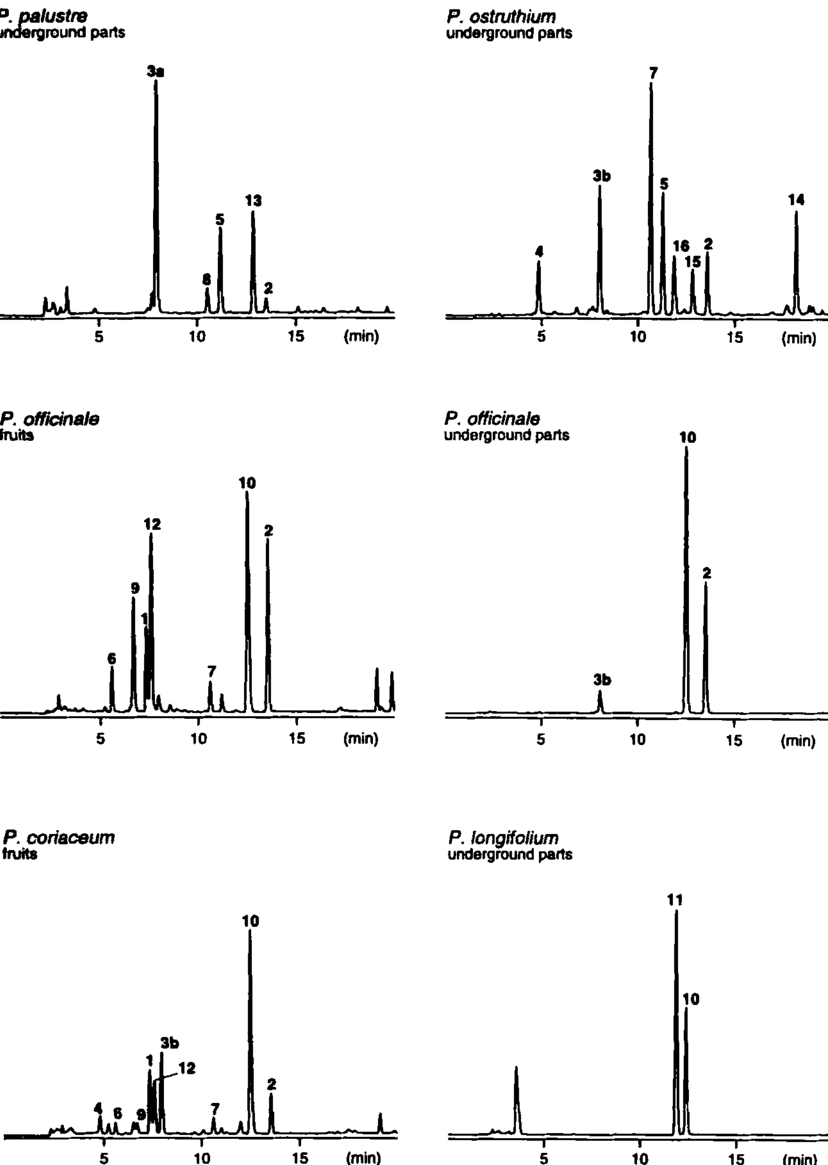

FIG. 2. HPLC analysis (RP18, MeOH, gradient 60–100% in aqueous buffer) of underground parts and fruits of different *Peucedanum* species. Identified coumarins: bergapten (**1**), isoimperatorin (**2**), (+)-oxypeucedanin (**3a**), (−)-oxypeucedanin (**3b**), (+)-oxypeucedanin hydrate (**4**), (+)-ostruthol (**5**), xanthotoxin (**6**), imperatorin (**7**), isobyakangelicin angelate (**8**), isopimpinellin (**9**), peucedanin (**10**), 8-methoxypeucedanin (**11**), ostruthin (**14**), osthol (**15**); identified 2-methylchromone: peucenin (**16**).

of *P. longifolium* and *P. coriaceum*. Besides (+)-oxypeucedanin (**3a**), *P. palustre* accumulates columbianadin (**13**), an angular dihydrofuranocoumarin. Simple coumarins, ostruthin (**14**) and osthol (**15**), can be found in *P. ostruthium* besides the linear furanocoumarins.

UV spectra of the single coumarins allow the assignment of the oxygenation pattern of the coumarin ring system (Figure 3). Especially within linear furanocoumarins 5- (**1–5**), 8-oxygenated (**6, 7**), or 5,8-oxygenated (**8, 9**) derivatives are easily distinguished. However, simple coumarins and coumarins with an additional saturated ring do not show such characteristic differences (see **13–15** in Figure 4). Only residues with a strong influence on the π-electron system of the 7-oxygenated coumarin ring cause significant changes in absorbance (e.g., **12**). UV spectra also help to differentiate between coumarins and 2-methylchromones (see **16**).

Isolation and Structure Elucidation. Sufficient amounts of coumarin derivatives for insecticidal bioassay studies were isolated from the following *Peucedanum* species: the fruits of *P. officinale* yielded bergapten (**1**), xanthotoxin (**6**), isopimpinellin (**9**), peucedanin (**10**), and isoimperatorin (**2**), the roots peucedanin (**10**) and isoimperatorin (**2**). 8-Methoxypeucedanin (**11**) was isolated from *P. longifolium* roots. The underground parts of *P. ostruthium* produced isoimperatorin (**2**), (±)-oxypeucedanin (**3b**), (+)-oxypeucedanin hydrate (**4**), (+)-ostruthol (**5**), ostruthin (**14**), and osthol (**15**). (+)-Oxypeucedanin (**3a**), isobyakangelicin angelate (**8**), and columbianadin (**13**) were obtained from the roots of *P. palustre*. Athamantin (**17**) is the major compound of the root extract of *P. oreoselinum*. (−)-Anomalin (**18**) was isolated from the underground organs of *P. austriacum*, peuarenarin (**19**) and xanthalin (**20**) from the roots of *P. arenarium*. Complete separation of mixtures of **6** and **9** and mixtures of **10** and **11** was only achieved by cyclic LC. Figure 1B illustrates the separation of **6** and **9**. Subsequent HPLC-analysis revealed the high purity of the fractions obtained (Figure 1C). ^{1}H NMR data of all compounds included in this investigation have already been published elsewhere (for **1**, **6**, and **9** see Steck and Mazurek, 1972; for **2–5**, **7**, **8**, **10**, **12–15**, and **17–20** see Hadaček, 1989; for **11** see Kozovska and Zheleva, 1980; for **16** see González et al., 1976).

Growth Inhibition. Table 1 lists the growth inhibitory effects of diet spiked with 1.0–5.0 μmol/g fresh wt. test coumarin on neonate larvae. The results revealed that all linear furanocoumarins, with the exception of ostruthol (**5**) and isobyakangelicin angelate (**8**), and one angular dihydrofuranocoumarin athamantin (**17**) reduced the growth of the treated larvae below 50% of the control larvae. However, no treated larvae died during the bioassay. A comparison of the growth inhibitory effects of isoimperatorin (**1**) and its derivatives shows that additional oxygenation on the prenyl side chain decreases the activity of (+)-oxypeucedanin (**3a**) and (+)-oxypeucedanin hydrate (**4**). The formation of an ester with angelic acid (**5**) even rendered the compound inactive (Figure 5).

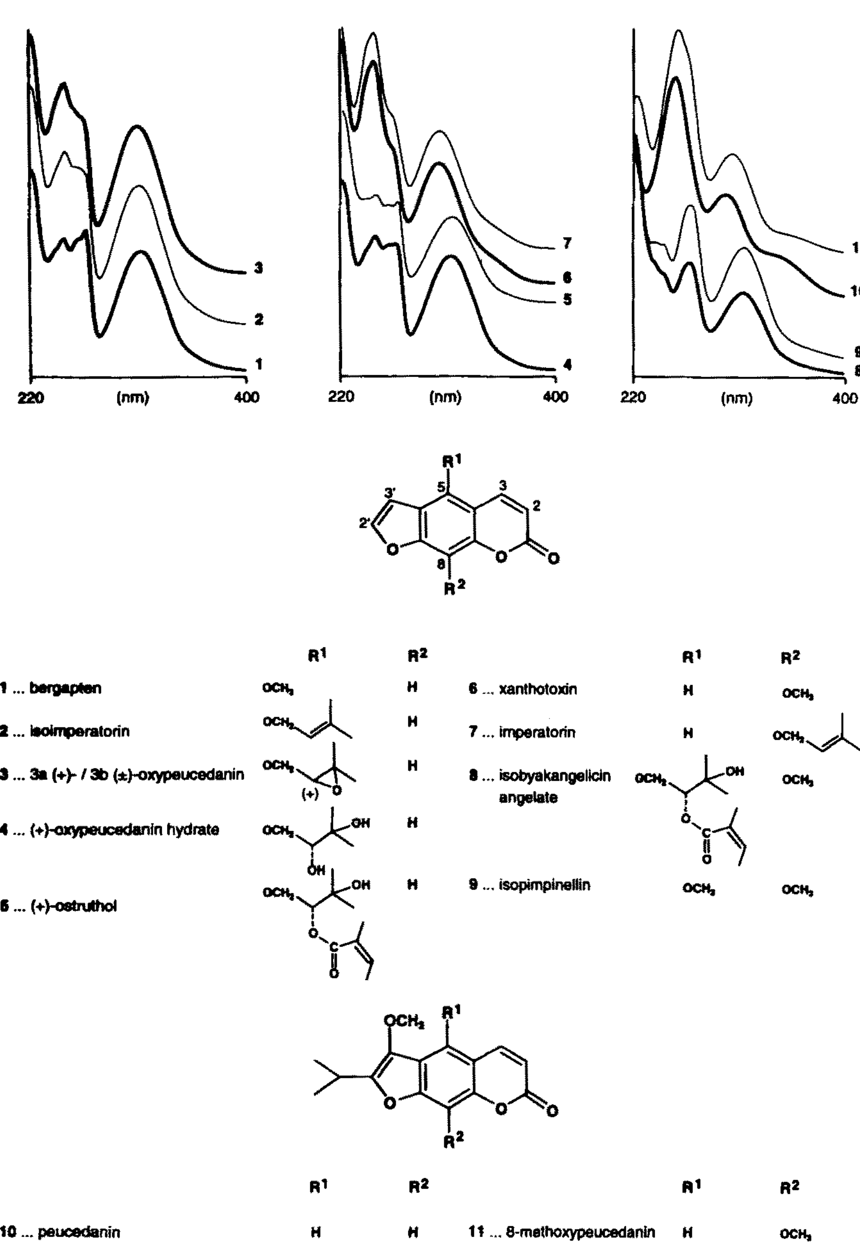

FIG. 3. UV spectra of linear furocoumarins from *Peucedanum* generated by the UV-diode array detector.

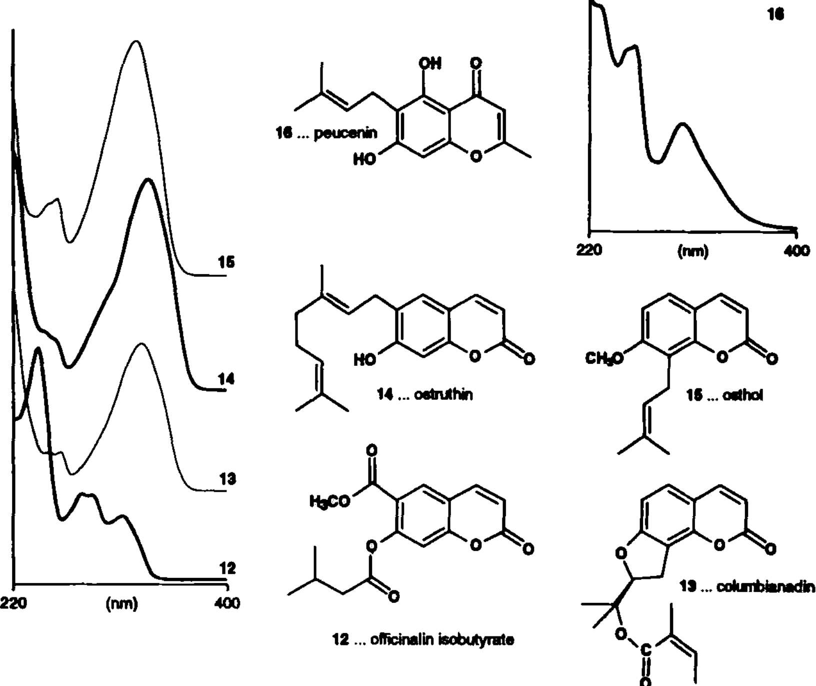

FIG. 4. UV spectra of simple coumarins, angular dihydrofuranocoumarins, and 2-methylchromones from *Peucedanum* generated by the UV-diode array detector.

Yajima and Munakata (1979) presented similar results for isopimpinellin and its prenylated derivatives, byakangelicol (epoxide at prenyl residue at C-8) and byakangelicin (diol at prenyl residue at C-8). Ostruthin (**14**) and osthol (**15**), both simple coumarin derivatives, did not inhibit the growth of the treated larvae at all. Other simple coumarins without bulky side chains, such as umbelliferone and aesculetin, were also inactive. This absence of any preingestive effects of umbelliferone and other simple coumarins is well documented (Berenbaum, 1978; Luthria et al., 1989). Only ostruthin (**14**) was reported to be a phytojuvenile hormone (Slama et al., 1971). However, during this bioassay, the larvae treated with ostruthin (**14**) did not gain less weight than the control larvae. The available amounts of ostruthin (**14**) did not allow monitoring of the development of the treated larvae until pupation, which would have been necessary in order to detect phytojuvenile activity.

Along with linear furanocoumarins, one angular dihydrofurano- (**17**), one angular dihydropyrano- (**18**) and two linear dihydropyranocoumarins (**19, 20**)

TABLE 1. GROWTH INHIBITION OF NEONATE LARVAE OF *Spodoptera littoralis* ON ARTIFICIAL DIET SPIKED WITH INCREASING CONCENTRATIONS OF VARIOUS COUMARINS ISOLATED FROM *Peucedanum*[a]

	Inhibition (%)				
μmol/g fresh wt.	1	2	3	4	5
Linear furanocoumarins					
Isoimperatorin (**2**)	63	**35**	**16**	**18**	**10**
(+)-Oxypeucedanin (**3a**)	83	**50**	**22**	**20**	**13**
(±)-Oxypeucedanin (**3b**)	n.t.	n.t.	**16**	n.t.	n.t.
(+)-Oxypeucedanin hydrate (**4**)	100	**43**	**54**	**29**	**36**
(+)-Ostruthol (**5**)	100	100	100	100	100
Imperatorin (**7**)	86	**29**	**14**	**11**	**11**
Isobyakangelicin angelate (**8**)	n.t.	n.t.	100	n.t.	n.t.
Peucedanin (**10**)	68	**23**	**22**	**18**	**8**
Simple coumarins					
Ostruthin (**14**)	100	100	100	100	100
Osthol (**15**)	91	84	91	81	94
Umbelliferone	100	100	100	100	94
Aesculetin	97	100	100	100	100
Angular dihydrofuranocoumarins					
Columbianadin (**13**)	n.t.	n.t.	100	n.t.	n.t.
Athamantin (**17**)	86	59	25	10	14
Linear dihydropyranocoumarins					
Xanthalin (**20**)	100	97	83	97	66
Peuarenarin (**19**)	97	72	55	76	69
Angular dihydropyranocoumarins					
(−)-Anomalin (**18**)	100	93	100	100	95

[a]Larval weight was monitored after six days of feeding (growth of controls was set at 100%). Growth inhibition values below 50% of the control are marked in bold letters.

were tested. Only athamantin (**17**) displayed growth inhibitory effects similar to the active furanocoumarins (Figure 6). This is the first report for an antifeedant angular dihydrofuranocoumarin so far.

Dietary Utilization. In this bioassay, the insecticidal activities of bergapten (**1**), isoimperatorin (**2**), xanthotoxin (**6**), isoimperatorin (**7**), and isopimpinellin (**9**) were compared with peucedanin (**10**) and 8-methoxypeucedanin (**11**), linear furanocoumarins with a substituted furan ring. Relative growth rate (RGR) and relative consumption rate were determined. All seven coumarins reduced the weight gain and the amounts of diet consumed compared to the control larvae. The RGR and RCR values of the coumarin-treated larvae, calculated from these data, differed significantly from the values of the control larvae (Table 2, $P <$

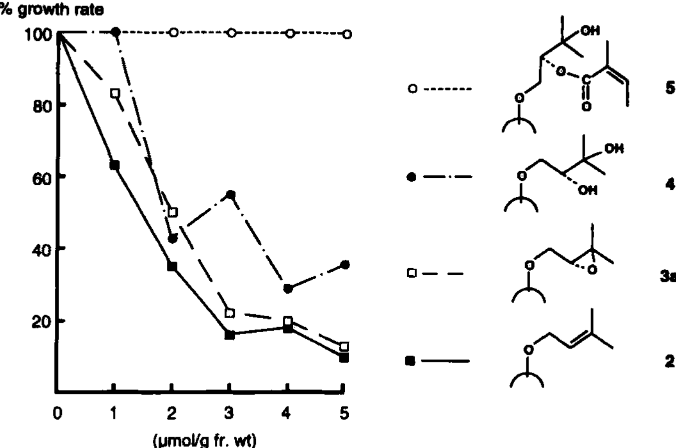

FIG. 5. Growth inhibition of *Spodoptera littoralis* neonate larvae reared on artificial diet with increasing concentrations of the linear furocoumarins isoimperatorin (**2**), (+)-oxypeucedanin (**3a**), (+)-ostruthol (**4**), and (+)-oxypeucedanin hydrate (**5**). Larval weight was monitored after six days of feeding (growth of controls was set at 100%).

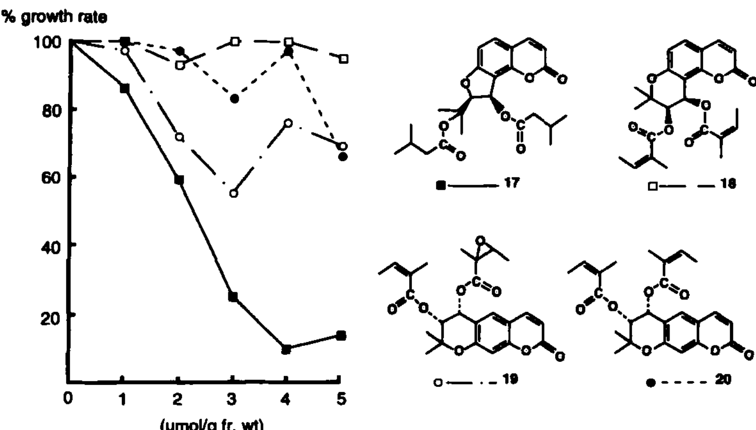

FIG. 6. Growth inhibition of *Spodoptera littoralis* neonate larvae on artificial diet with increasing concentrations of athamantin (**17**), (−)-anomalin (**18**), peuarenin (**19**), and xanthalin (**20**). Larval weight was monitored after 6 d of feeding (growth of controls was set at 100%).

TABLE 2. NET WEIGHT GAIN, DIET CONSUMED, RELATIVE GROWTH RATE (RGR), AND RELATIVE CONSUMPTION RATE (RCR) FOR *Spodoptera littoralis*[a]

	Weight gain (mg dry wt) $\bar{X} \pm$ SD	Diet consumed (mg dry wt) $\bar{X} \pm$ SD	RGR (mg/mg/day) $\bar{X} \pm$ SD	RCR (mg/mg/day) $\bar{X} \pm$ SD
Control	16.33 ± 4.10	91.34 ± 29.11	0.80 ± 0.07	4.44 ± 0.85
Peucedanin (**10**)	10.81 ± 2.61	42.46 ± 19.55	0.66 ± 0.06	2.56 ± 0.91
Isopimpinellin (**9**)	8.65 ± 3.87	41.15 ± 13.14	0.61 ± 0.12	2.83 ± 0.82
Bergapten (**1**)	**5.27 ± 1.15**	35.62 ± 10.33	**0.46 ± 0.06**	3.21 ± 0.91
Imperatorin (**7**)	**2.81 ± 1.33**	30.32 ± 15.17	**0.33 ± 0.11**	3.79 ± 2.28
Isoimperatorin (**2**)	6.63 ± 0.67	**23.33 ± 7.38**	0.53 ± 0.02	**1.49 ± 0.59**
Xanthotoxin (**6**)	6.78 ± 2.33	**23.15 ± 8.12**	0.49 ± 0.06	**1.67 ± 0.47**
8-Methoxypeucedanin (**11**)	5.89 ± 1.13	**17.06 ± 7.96**	0.51 ± 0.04	**1.67 ± 0.63**

[a] 3 μmol/g fresh wt. test furocoumarin ($P < 0.05$ for all tested coumarins, $N = 12$) characterizing three groups of similar insecticidal activity. The most deleterious effects within each category are marked in bold letters.

0.05, $N = 12$). All tested compounds decreased RCR and RGR of the larvae reared on treated diet.

DISCUSSION

HPLC is recognized as a reliable method for the analysis of coumarins (Berenbaum, 1992). On-line UV detection with a diode array system additionally provides UV spectra of the compounds separated. This feature, together with characteristic retention times, is a great help in identifying individual coumarin derivatives using spectra library software during screening procedures as well as during the isolation process. This system is especially useful with mixtures that are difficult to separate. For example, with xanthotoxin (**6**) and isopimpinellin (**9**), this technique not only provides analytical information, but also helps with the isolation of pure compounds. Due to the easy recycling of the used solvents, and the employment of unmodified silica gel as stationary phase, step-gradient LC is a rather cheap preparative chromatography system. The separation capacity can be greatly enhanced by the cyclic mode as illustrated in Figure 1. Pure compounds are essential for bioassay studies. Impurities can introduce activity where there is none and thus lead to contradictory results. Recently, this was demonstrated for the reported photobiological activity of isopimpinellin (**9**), which was actually caused by contamination with bergapten (**1**) (Ashwood-Smith et al., 1992).

Within coumarins, prenylation produces a variety of different ring systems.

The formation of esters with various acids, frequently with acetic, angelic, isovaleric, senecionic, or tiglic acid, additionally contributes to the structural diversity of coumarins found in plants. The wide range of biological and pharmacological activities attributed to coumarins indicates the wide range of receptor specificity of different derivatives of this class of compounds. This has to be expected from a class of compounds that contributes to the defense system of the accumulating plant. In particular, linear furanocoumarins, tricyclic planar molecules, have attracted attention because of their ability to intercalate in DNA. On subsequent irradiation, the react with pyrimidine bases to form mono- or biadducts, depending on the type of the molecule (Towers, 1984). Similarly, other planar tricyclic phytochemicals, furanochromones and furanoquinolines, derived by different biosynthetic routes, are also well-known photogenotoxins. Photoexcitable furanocoumarins are able to react efficiently with proteins (see Cadet et al., 1990) and thus postingestive toxic effects of UV treated phototoxic furanocoumarins, such as xanthotoxin **(6)**, are well documented (Arnason et al., 1992). However, insect species adapted to furanocoumarin-containing plants have developed various strategies, both physiological and behavioral, to cope with photoactivated phytochemicals (Arnason et al., 1992). Apart from the light-activated toxicity, linear furanocoumarins are known as potent antifeedants (reviewed by Berenbaum, 1992).

Since the majority of coumarins were not available in large enough amounts for detailed studies, a bioassay comparing the growth inhibition of neonate larvae of *S. littoralis* enabled a comparison of different structural types of coumarins. The results identified isoimperatorin **(2)**, the isomer imperatorin **(7)**, and the derivatives oxypeucedanin **(3a, 3b)** and oxypeucedanin hydrate **(4)** as active. Only the ester with angelic acid, ostruthol **(5)**, turned out to be inactive (see Table 1 and Figure 5). A possible explanation for this effect would be that the addition of angelic acid to the molecule dramatically reduces the affinity of this coumarin type to the involved receptor or receptors. Consequently, regarding the high number of active compounds, these results confirm the classification of linear furanocoumarins, as they can be found in *P. ostruthium* and *P. palustre* as potent allomones against herbivores in a broad sense.

The growth inhibition induced by athamantin **(17)**, an angular dihydrofuranocoumarin, decisively a nonplanar molecule, however, reveals an alternative to linear furanocoumarins, probably involving a different mode of action on a different receptor site. Considering the high degree of diversification found in coumarins, and classifying coumarin containing plants as well defended, the existence of different structural types confirms coumarins as efficient in chemical defense in the sense of Jones and Firn (1991). Comparing athamantin **(17)** with the other inactive dihydropyranocoumarins in the test series (Figure 6), only athamantin **(17)** shows isovaleric acid as an ester component. The second angular dihydrofuranocoumarin, columbianadin **(13)**, with angelic acid as an ester

component, however, is inactive. More detailed studies, involving more dihydrofurano- and dihydropyranocoumarins with different ester components, should be done in order to determine the relevance of the ester type for the activity of the molecule.

Peucedanin (**10**) is a furanocoumarin that has a substituted furan ring, in contrast to the previously discussed derivatives, but equally inhibits the growth for neonate larvae of *S. littoralis*. Thus, a more detailed study of the insecticidal activity of peucedanin (**10**) and 8-methoxypeucedanin together with various methoxylated linear furanocoumarins was carried out in order to compare these two structural types of linear furanocoumarins. A closer characterization of insecticidal activity of a plant compound demands a distinction between deterrent (preingestive) and toxic (postingestive) effects (Berenbaum, 1986). Toxicity depends on complex additive processes such as absorption, binding to proteins, and transport by proteins to the target tissue in the hemolymph (Shapiro, 1991). However, complex interactions between nutrients and allelochemicals frequently prevent a clear distinction between these two types of effects (Slansky, 1992). Thus, the possibilities of interpretation remain limited, especially when the involved receptor or receptors are unknown (Jones and Firn, 1991).

A plot of the mean RGR against the mean RCR from data obtained in the dietary utilization bioassay, however, still allows one to draw some careful conclusions. The plot characterizes three groups within the tested compounds [bergapten (**1**), xanthotoxin (**6**), isopimpinellin (**9**), isoimperatorin (**2**), imperatorin (**7**), peucedanin (**10**), and 8-methoxypeucedanin (**11**)] on the basis of their similarity concerning the effects on RGR and RCR of *S. littoralis* (Figure 7). Isopimpinellin (**9**) and peucedanin (**10**) show slightly reduced RGR and RCR values compared to the control. Isoimperatorin (**2**), xanthotoxin (**6**), and 8-methoxypeucedanin (**11**), however, distinctly decrease RGR and RCR. Both neural antifeedant and toxic properties of these compounds may be responsible. Bergapten (**2**) and imperatorin (**7**) clearly deviate with RCR values that are slightly reduced compared to the control, and very low RGR values. Thus, larvae feeding on a diet spiked with bergapten (**1**) and imperatorin (**7**) have gained less weight than the amount of diet eaten would suggest. These observed effects could be caused by postingestive effects.

Peucedanin (**10**) and 8-methoxypeucedanin (**11**) are both linear furanocoumarins of a rare structural type with a restricted occurrence within *P. officinale* and closely allied species (Murray et al., 1982; see HPLC of *P. coriaceum*, *P. officinale*, and *P. longifolium* in Figure 2). Moreover, a biosynthetic pathway discrete from that of the majority of furanocoumarins has been outlined (González et al., 1977). Hence, this coumarin type can be regarded as a useful chemotaxonomic characteristic for this group of closely allied species. Our experiment clearly demonstrates that additional residues on the furan ring do not diminish the insecticidal activity of these coumarins. Xanthotoxin (**6**) and

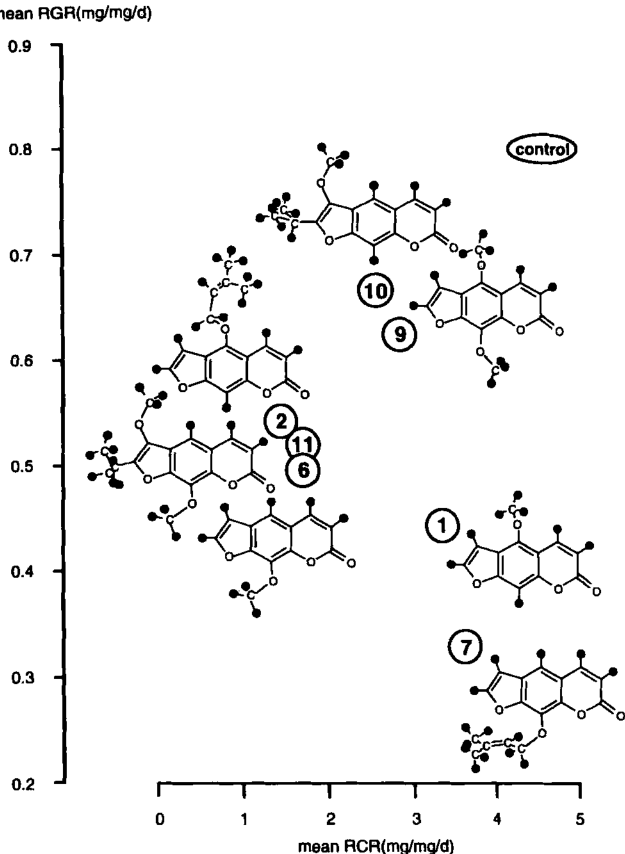

FIG. 7. Relative growth rate (RGR, $\overline{X}$) versus relative consumption rate (RCR, $\overline{X}$) for *Spodoptera littoralis* reared on diet spiked with 3 μmol/g fresh wt diet bergapten (**1**), isopimpinellin (**9**), xanthotoxin (**6**), isoimperatorin (**2**), imperatorin (**7**), peucedanin (**10**) and 8-methoxypeucedanin (**11**). The drawing of the chemical formulae is based on models calculated by the molecular modeling software PCMODEL (Serena Software, Bloomington, Indiana).

8-methoxypeucedanin (**11**) are characterized by very similar RGR and RCR values. Thus, peucedanin (**10**) and 8-methoxypeucedanin (**11**) should be regarded as potent allomones as are isopimpinellin (**9**) and xanthotoxin (**6**). However, to what extent these chemical traits contribute to the adaptive radiation that can be observed within the *P. officinale* group (Frey, 1989; Hadaček and Samuel, 1994), is not easy to estimate because only the gross effects of morphological,

physiological, and biochemical traits determine the fitness of a species in a certain plant community associated with specific pathogens and herbivores. Nevertheless, the results obtained in this study certainly add to the reputation of coumarins as effective chemical defensive agents.

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SEASONAL VARIATION IN VOLATILE SECONDARY COMPOUNDS OF *Chrysothamnus nauseosus* (PALLAS) BRITT.; ASTERACEAE SSP. *hololeucus* (GRAY) HALL. & CLEM. INFLUENCES HERBIVORY

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Abstract—*Chrysothamnus nauseosus* (rubber rabbitbrush) is used by browsing animals, especially mule deer (*Odocoileus hemionus*), as a forage in the winter months. It is used only slightly, if at all in the summer. This dietary difference may result from changes in the secondary chemical composition of the leaves. Solvent extracts from summer and winter rabbitbrush leaves were analyzed by gas chromatography and mass spectrometry, and the volatile compounds were quantified and identified. Hexane and chloroform extracts from winter leaves exhibit a marked concentration decrease in most chemicals when compared to summer extracts. The methanol extracts revealed the presence of several chemicals in the summer leaves that were absent in winter leaves. Rubber rabbitbrush has fewer secondary volatile chemicals in the winter than in the summer. These chemical differences may influence the seasonal dietary difference observed in mule deer and other browsing animals.

Key Words—Rabbit brush, *Chrysothamnus nauseosus*, secondary chemicals, essential oils, herbivory, hydrocarbons, natural products, mule deer, *Odocoileus hemionus*.

INTRODUCTION

Rubber rabbitbrush (*Chrysothamnus nauseosus* [Pallas] Britt.; Asteraceae) is adapted to grow in semiarid lands. It is a shrub that grows in a wide range of environmental conditions from sea level to 3000 m (Weber et al., 1985). Its range of distribution encompasses most of the Intermountain West in the United States and Canada, from New Mexico to California in the south and from

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southern British Columbia to Alberta in the north (Anderson, 1986). The shrub is winter dormant, but the leaves hang on the plant during most of the winter. It flowers in late August to early September.

Rabbitbrush could be a significant source of nutrients for foraging animals. Plant material analysis has shown that crude protein levels in plant leaves range from 9% in the winter to 11.8% when the new leaves appear in the spring (Weber et al., 1987). Bhat et al. (1990b) found that rabbitbrush contains an average of 0.16% phosphorus and has an *in vitro* digestibility of 44.4%. Ward (1971) found 40–50% *in vitro* digestibility for rabbitbrush treated with elk inoculum. These values are comparable to most other forages. These data indicate that rubber rabbitbrush could be a significant source of nutrients for foraging animals such as deer. In fact, rabbitbrush was found in the rumens of 48% of wintering mule deer (*Odocoileus hemionus*) in California (Sampson and Jesperson, 1963).

Although rabbitbrush is browsed heavily in the winter, wildlife and livestock browse the plant only lightly in the summer (Hanks et al., 1975; McArthur et al., 1979; Sankhla et al., 1987). The cause of this seasonal difference in eating habits has not been determined. A lack of forage in the winter may result in rabbitbrush browsing. Another explanation could be a difference in chemical factors.

Rabbitbrush produces considerable quantities of secondary chemicals (Bhat et al., 1990a), especially in the form of rubber, resins, and terpenoid compounds, which are important elements of many plant defense mechanisms. Hegerhorst et al. (1988) found that the levels of rubber and resin in the plant vary by season. Their research evaluated the hydrocarbon content of the woody tissue for the months from April to November. Rubber content was highest in the summer. Resin content was highest in the spring and fall. Many of the essential oils present in the *Chrysothamnus* genus have been characterized by Hegerhorst et al. (1987), who evaluated leaves and new stems collected in the month of November. No data are currently available to indicate whether or not terpene composition and content in rubber rabbitbrush leaves varies with season. If such variation is found, it might be linked to the observed seasonal foraging by mule deer.

The purpose of our research was to determine if a correlation exists between secondary chemical composition of *C. nauseosus* and seasonal foraging by browsing animals.

METHODS AND MATERIALS

Samples of *Chrysothamnus nauseosus* ssp. *hololeucus* were collected from a site in Provo, Utah. Samples were collected in July 1992, a month before flowering, and in January 1993. Sixteen samples were collected at each harvest.

All samples were stored at −20°C until mid-February. A second summer sampling of 12 plants from the same accession, in July 1993, was analyzed to confirm the results of the previous year. Leaves were removed from the stems and ground under liquid nitrogen. The powder (1 g) was then placed in a filter bag. Three 5-ml aliquots of the solvents hexane, chloroform, and methanol (HPLC grade) were successively used in extraction. The solvent extracts were grouped by sample and solvent and then concentrated under a nitrogen atmosphere to a 1.5-ml volume and transferred to sampling vials.

Quantitative analysis was performed by gas chromatography on an HP 5890, with FID, cross-linked methyl silicone column (25 m × 0.2 mm × 0.5 μm) and a temperature program of 40°C for 1 min followed by 6°C min ramp to 280°C with a 10-min hold. Standards of *d*-limonene, docosane, and a C_{36} wax ester were used to estimate the concentrations of the extracted compounds. A dry weight correction was made for the difference in water content between summer and winter leaves.

Analysis of large hydrocarbons was performed on summer leaves by gas chromatography on an HP 5890, with FID, aluminum-coated cross-linked methyl silicone column SGE 12AQ3/HT5 (25 m × 0.2 mm × 0.5 μm) and a temperature program of 60°C for 1 min followed by 10°C/min ramp to 400°C with a 25-min hold.

Compounds were identified using chromatographic and spectral comparisons from an HP GC-MS 5995, cross-linked methyl silicone column (25 m × 0.2 mm × 0.5 μm), with published standards and computer search library of 90,000 spectra. In many cases initial extraction concentrations were insufficient to obtain spectral data. An 80-g sampling of July 1993 leaves from the same accession location was extracted with 3 × 400 ml chloroform and methanol. These extractions were concentrated to about 20 ml in a rotovap. The concentrates were fractionated on a silica gel (60–200 mesh) column using hexanes, dichloromethane, chloroform, ethanol, methanol, water, and acetic acid as eluting solvents. The fractions were reconcentrated under nitrogen gas to 1.5 ml and analyzed by GC-MS as described above.

The Behrens-Fisher test (Dowdy and Wearden, 1983) was used to determine if significant differences existed between summer and winter concentrations of extracted compounds.

RESULTS

Many compounds from both the summer and winter extracts of *Chrysothamnus nauseosus* were quantified and identified or characterized. The data from the hexane and chloroform extracts were combined because a similar chromatogram was obtained for both extractions. Table 1 illustrates differences

TABLE 1. DIFFERENCES BETWEEN SUMMER AND WINTER HYDROCARBONS EXTRACTED WITH HEXANES AND CHLOROFORM

Compound[a]	CAS number	Kovat's index	Summer leaf content (μg/g dry wt.)	Winter leaf content (μg/g dry wt.)	Confidence interval (%)
(*E*)-Sabinene hydrate	17699-16-0	1021	3.1	2.0	99
Camphor	76-22-2	1098	2.0	1.1	99
Geraniol	106-24-1	1250	1.2	0.6	99
β-Cubenene	13744-15-5	1400	0.3	0.17	99
β-Elemene	515-13-9	1407	2.4	0.8	99
β-Caryophyllene	87-44-5	1444	0.5	0.6	
β-Humulene	116-04-1	1481	3.9	2.0	99
γ-Muurolene	30021-74-0	1521	9.8	0.9	99
α-Muurolene	10208-80-7	1548	7.1	1.2	99
α-Farnesene	502-61-4	1591	0.16	[b]	
(*E*)-β-Farnesene	39029-41-9	1598	23.7	3.4	99
δ-Cadinene	29873-99-2	1615	1.3	[b]	
γ-Elemene	5273-84-7	1630	0.19	0.11	98
(*Z*)-Isoelemicin	481-34-5	1655	0.16	[b]	
β-Bisabolol	15352-77-9	1704	0.3	[b]	
(Sesquiterpene)		1721	0.18	[b]	
(Sesquiterpene)		1737	0.22	[b]	
(Sesquiterpene)		1765	0.3	[b]	
(Sesquiterpene)		1791	0.25	[b]	
Unknown		1874	0.9	0.13	99
Unknown		1845	0.8	0.12	99
Unknown		1850	1.0	0.5	95
Nonadecane	629-92-5	1900	1.1	0.4	99
Methyl palmitate	112-39-0	1955	1.3	0.14	99
Phytol	150-86-7	1963	4.3	0.9	99
(Labdane)		1975	0.5	0.12	98
Eicosane	3452-07-1	2004	1.1	0.5	99
(Diterpene)		2010	0.21	[b]	
(Diterpene)		2036	0.6	0.13	99
(Diterpene)		2050	0.7	0.3	99
(Diterpene)		2066	0.7	0.3	99
Octadecanol	112-92-5	2080	1.7	[b]	
Heneicosane	629-94-7	2096	0.7	0.8	
(Diterpene)		2113	0.8	[b]	
(Diterpene)		2146	0.4	[b]	
(Diterpene)		2175	[b]	0.22	99
Spiro[furan-2(3H),1(4H) napthlene]-acetic acid, 4,4'a,5',6',7',8',8'a-octahydro-2',5,5',8'a-pentamethyl-,[1'*R*[1'.alpha.(*S**),4'aQ]]	1438-57-9	2191	1.0	0.01	99

TABLE 1. (Continued)

Compound[a]	CAS number	Kovat's index	Summer leaf content (μg/g dry wt.)	Winter leaf content (μg/g dry wt.)	Confidence interval (%)
Docosane	629-97-0	2206	3.0	0.2	99
Tricosane	638-67-5	2297	2.0	[b]	
Unknown		2320	1.0	[b]	
Tetracosane	646-31-1	2400	0.3	[b]	
Unknown		2413	0.18	0.10	99

[a] Tentative assignments.
[b] Compound not detected.

between summer and winter hydrocarbons extracted with hexanes and chloroform. The majority of the compounds identified were monoterpenes, diterpenes, and *n*-alkanes. The most abundant compounds identified were (*E*)-β-farnesene, α-muurolene, γ-muurolene, and β-humulene. Almost all the compounds had a significantly greater concentration in the summer leaves than in the winter leaves.

Table 2 illustrates differences between summer and winter hydrocarbons extracted with methanol. The major compounds are chrysolane and caryophyllene oxide. Many of the compounds found in the summer extracts are absent in the winter leaf extracts. Farnesol isomers and 8,13-cedrane-diol are present in the winter but not in the summer extracts.

Large wax esters and long-chain hydrocarbons were found in the summer 1993 extracts (Table 3). C_{28} and C_{38} (C=C) waxy esters and a C_{29} hydrocarbon were most prevalent. Even numbered chain waxy esters were found as large as C_{56}. Two odd-numbered chain, C_{31} and C_{41}, waxy esters were present.

Figure 1 illustrates the concentrations of total volatile compounds found in the summer and winter leaves of rubber rabbitbrush.

DISCUSSION

(*E*)-β-Farnesene, the major volatile compound extracted from rabbitbrush leaves, has an aphid alarm pheromone component (Pickett, 1991). β-Caryophyllene and caryophyllene oxide appear to have antimicrobial (Harborne, 1991) and antifungal (Hubbell et al., 1983) activities. β-Humulene has antimicrobial activities (Deans and Svoboda, 1989).

Other work has shown that rabbitbrush produces compounds that have nematocidal and insecticidal activities (Bohlmann et al., 1979; Maugh, 1982). The compounds listed in these works were myrcene, α-pinene, δ-3-carene, and

TABLE 2. DIFFERENCES BETWEEN SUMMER AND WINTER HYDROCARBONS EXTRACTED WITH METHANOL

Compound[a]	CAS number	Kovat's index	Summer leaf content (μg/g dry wt.)	Winter leaf content (μg/g dry wt.)	Confidence interval (%)
(Z)-Nerolidol	142-50-7	1556	0.3	[b]	
Caryophyllene oxide	1139-30-6	1605	0.9	[b]	
(Z)-,(E)-Farnesol	3790-71-4	1731	[b]	0.14	
(Z)-,(Z)-Farnesol	16106-95	1744	[b]	0.08	
(Sesquiterpenol)		1878	0.13	[b]	
(Sesquiterpenol)		1886	0.13	[b]	
8S,13-Cedrane-diol	62600-04-8	1930	[b]	0.11	
Phytol	150-86-7	1963	0.15	0.3	
Hexadecyl acetate	629-70-9	2026	0.01	0.12	
Unknown		2034	0.15	0.14	
Unknown		2045	0.01	0.12	
Manool	596-85-0	2062	0.15	[b]	
Octadecanol	112-92-5	2085	0.15	[b]	
(Diterpenol)		2097	0.4	0.18	98
Unknown		2110	0.25	0.20	
Unknown		2120	0.19	[b]	
Chrysolane	82731-92-8	2200	1.4	[b]	
(Diterpenol)		2218	0.3	0.07	99
Unknown		2256	0.14	[b]	
(Diterpenol)		2266	0.6	[b]	
Unknown		2283	0.16	[b]	
Unknown		2292	0.16	[b]	
Unknown		2329	0.11	[b]	

[a] Tentative assignments.
[b] Compound not detected.

α-phellandrene. Previous work has identified these compounds in *Chrysothamnus nauseosus* ssp. *hololeucus* (Hegerhorst et al., 1987, 1988), although we were unable to measure these compounds. Our work does, however, show that most of the essential oils present in *C. nauseosus* ssp. *holoeucus* are present in significantly lower concentrations in the winter than in the summer.

The presence of the farnesol isomers in the winter methanol extracts can be seen as oxidation products of α-farnesene and other terpenoids present in the summer. Phytol is present in both winter and summer as a degradation product of chlorophyll.

Ward (1971) found that rubber rabbitbrush was comparable to other forages in digestibility for both elk and cattle. When rabbitbrush was tested for digestibility by Ward (1971), essential oils had no harmful effect. Although no diges-

TABLE 3. LONG CHAIN HYDROCARBONS AND WAXES IN SUMMER LEAVES OF *Chrysothamnus nauseosus* ssp. *hololeucus*.

Compound[a]	Concentration (μg/g dry wt.)
C_{26} hydrocarbon	0.3
C_{28} hydrocarbon	0.4
C_{29} hydrocarbon	1.9
C_{30} hydrocarbon	0.4
C_{28} waxy ester	3.0
C_{32} hydrocarbon	0.13
C_{31} waxy (C=C) ester	0.16
C_{36} waxy ester	0.4
C_{34} waxy (C=C) ester	0.09
C_{38} waxy (C=C) ester	2.2
C_{40} waxy ester	1.0
C_{41} waxy ester	0.16
C_{42} waxy (C=C) ester	0.3
C_{44} waxy ester	0.14
C_{46} waxy ester	0.09
C_{48} waxy ester	0.15
C_{50} waxy ester	0.05
C_{52} waxy ester	0.08
C_{54} waxy ester	0.09
C_{56} waxy ester	0.08

[a] Compounds listed in order of elution.

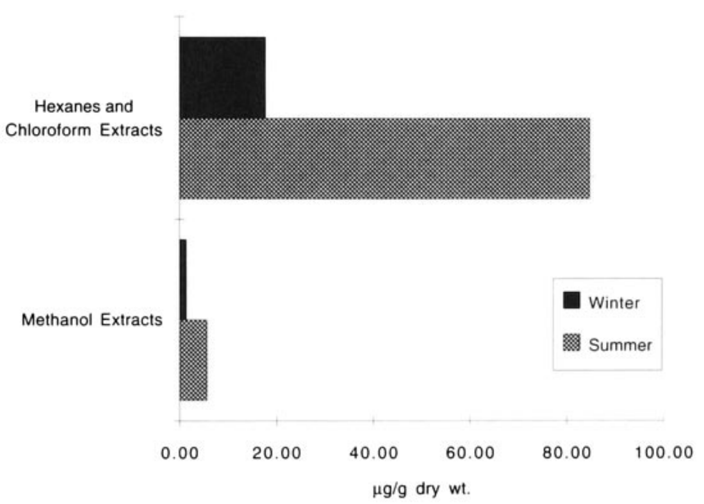

FIG 1. Summer and winter concentrations of total volatile compounds in the leaves of *Chrysothamnus nauseosus* ssp. *hololeucus*.

tibility effects were seen, the high concentrations of the terpenoids in the summer could act as a feeding deterrent to deer and other browsers. Although the leaves remain on the plant during the winter months, *C. nauseosus* ssp. *hololeucus* is dormant, leading to a reduction of the levels of defensive chemicals. Because the defensive chemicals are reduced in the winter and alternative forage is scarce, *C. nauseosus* ssp. *hololeucus* becomes a more attractive forage for wintering animals such as mule deer.

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PROXIMITY OF RELEASE POINTS OF PHEROMONE COMPONENTS AS A FACTOR CONFUSING MALES OF THE SPOTTED STEM BORER, *Chilo partellus*, APPROACHING THE TRAP

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Abstract—The effect of proximity of the release points of the two pheromone components (Z)-11-hexadecenal and (Z)-11-hexadecen-1-ol of the spotted stem borer, *Chilo partellus* (Lepidoptera: Pyralidae) on behavior of the males and on trapping efficiency was investigated. Separating the dispensers of the two components in the trap by a mere 3 cm resulted in a threefold decrease in trap performance, compared to very close release of the components. The result is attributed to possible distortion of the pheromone signal, resulting in confused behavior of *C. partellus* males in the vicinity of the trap. The ethological and practical implications of the phenomenon are discussed.

Key Words—Lepidoptera, Pyralidae, *Chilo partellus*, spotted stem borer, female sex pheromone, (Z)-11-hexadecenal, (Z)-11-hexadecen-1-ol, multicomponent pheromones, signal integrity, dispensing technique, trap efficiency, proximity effect.

INTRODUCTION

Effective trapping of insects depends on the continuous attractivity of the lure being used over a fairly large distance ranging from several meters to a few

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centimeters from the bait. To achieve this, the signal emitted from the trap should bear sufficient resemblance to the natural one and must be perceivable by the receptive insect all the way to the source without significant interruption. Although these requirements are fairly obvious, in practice they are often difficult to fulfill. Where a multicomponent blend is to be dispensed, the way in which the signal is perceived needs to be carefully considered. Components of such a blend known to react chemically to form products that are antagonistic to the pheromone, or that appreciably reduce the duration of its performance, present a special problem. For such cases, separate dispensing of the components has been recommended (Nesbitt et al., 1979). However, not enough attention has been given to possible effects of the degree of proximity of the release points of the pheromone components on trap efficiency, although the possibility of compositional heterogeneity of a signal that could impair its perception in the vicinity of the trap is obvious.

The female sex pheromone of *Chilo partellus* is composed of two major components, (*Z*)-11-hexadecenal (*Z*11-16:Ald) and (*Z*)-11-hexadecen-1-ol (*Z*11-16:OH), which were reported to react together even in dilute hexane solution at −20°C (Nesbitt et al., 1979); therefore, separate dispensing of the two components was recommended. Although *Z*11-16:Ald alone was reported to be highly attractive to male *C. partellus* in field studies conducted in India (Nesbitt et al. 1979), this compound, and its blend with *Z*11-16:OH were later found to be ineffective in inducing substantial catches of male *C. partellus* in Kenya (Unnithan and Sexena, 1990a, b), Malawi (D.R. Hall, ODNRI, cited by Beevor et al., 1990), and South Africa (Kfir and Van Rensburg, personal communication 1993), as well as in India (K.V. Seshu Reddy, ICIPE, personal communication).

Recently, we demonstrated that the two components are essential for optimal trap efficiency (unpublished results). However, we surmised that the erratic catches obtained by different workers in the past may have been due to possible distortion of the pheromone signal resulting from "careless" placing of the dispensers of the two components in the trap. Accordingly, we studied the effect of the relative proximity of the release points of the two components on the behavior of males and on trapping efficiency, and in view of the broader implication of our results for situations where separate dispensing of pheromone components is necessary, we report our findings in this paper.

METHODS AND MATERIALS

Insect Material. Insects used for laboratory and screenhouse experiments were of African origin and obtained from the ICIPE mass-reared colonies. The insects were reared on an artificial diet (Ochieng et al., 1985) and were kept under natural illumination (12L:12D photoperiod).

Synthetic Pheromone Components. Synthetic (Z)-11-hexadecenal (Z11-16:Ald) and (Z)-11-hexadecen-1-ol (Z11-16:OH) (referred to hereafter as the aldehyde and the alcohol or ALD and ALC, respectively) were obtained from commercial sources and their purity confirmed to be greater than 99% by GC analysis. Previous studies had indicated that (Z)-11-hexadecenoic acid (Z11-16:COOH), the oxidative by-product of Z11-16:Ald, inhibited hovering and attempted mating behavior of male *C. partellus* moths (Ogwayo et al., 1990). Hence, the synthetic Z11-16:Ald was purified further before use by extraction with sodium hydrogen carbonate to remove any acid present.

Construction of Filter Paper Dispenser. The dispenser (referred to hereafter as the "paper dispenser") was constructed from two filter papers (Whatman No. 40) and a piece of polyethylene foil. One square filter paper (2.5 cm^2) was loaded with 100 μl *n*-hexane solution containing 100 μg of the aldehyde and the other paper with 100 μl hexane containing 10 μg of the alcohol. For close release, filter papers loaded with the two components were separated only by a piece of polyethylene foil and held together with a paper clip, which was prevented from touching the filter papers by additional small pieces of foil. For release from spatially distinct points, the two pieces of filter paper were placed 3 cm apart. The dispensers were loaded with synthetic pheromone and "aged" for 12–14 hr before use. For the screenhouse or field experiments, the dispensers were loaded around 1400–1500 hr and placed in screenhouses or field around 1600–1800 hr the same day (the maximum catches were expected around 0300–0500 hr the following day).

Ethological Observations. Pupae were separated by sex and held in separate cages to prevent mating after moth emergence. The observations were conducted in the laboratory under photoperiod 12L:12D, 8–11 hr into the scotophase. For convenience, the photoperiod was shifted by 6 hr, so that the observations were made at 0800–1100 hr. The pupae were allowed to adjust to the shifted photoperiod for a few days. To assess the extent of the possible effect of the photophase shift, the results obtained were further confirmed by several observations of insects kept under natural illumination, which were also observed during 8–11 hr into the scotophase (0200–0500 hr). The experiments were conducted in a chamber (60 × 60 × 100 cm) made of glass and aluminum and a wire mesh on the two opposite sides. An electric fan was used to generate air movement across the chamber. The conditions were as follows: 20–22°C, 50–60% relative humidity, 0.2–0.8 m/sec wind speed, red light of about 1.0 lux intensity. For each experiment, a single virgin 1-day-old male was released and its behavior towards the source of the pheromone observed for 1 hr. For each observation, a new individual was used.

The following parameters of male behavior towards the pheromone source were quantified: (1) time spent resting before upwind flight; (2) time taken walking while wing fanning; (3) rate of antennal beats per minute; (4) total time

spent resting; (5) total time spent flying (random flight); (6) general vigor (graded arbitrarily from 0 to 10); (7) nearest distance reached to the source; (8) time taken to locate the source; (9) time spent flying upwind up to 5 cm from the source (oriented flight); and (10) time taken moving 5 cm or closer around the source.

Three types of pheromone sources were used: (1) a virgin female confirmed in a small screen cage; (2) a paper dispenser baited with the two synthetic pheromone components, which were released separately but close to each other as described above; and (3) a similar dispenser but with the two components placed 3 cm apart. Each type of the pheromone source was tested independently. Every day, three observations were conducted in a single session, one observation for each pheromone source. The order of the pheromone sources used during the successive sessions (days) was systematically rotated. The observation of male behavior towards each pheromone source was repeated 21 times.

Screenhouse Experiments. Screenhouse experiments were conducted at the ICIPE Mbita Point Field Station (MPFS) located on the shores of Lake Victoria, in western Kenya. In a screenhouse (7 × 12 m), sticky Delta traps placed 50 cm above the ground were placed and arranged according to a randomized block design (3 treatments × 4 replications). To facilitate more uniform distribution of insects flying inside the screenhouse, potted sorghum plants were set between the traps. For each experiment, 50–100 *C. partellus* males were released per screenhouse. Recapture rate ranged between 3 and 28% (mean about 15%).

Field Experiments. The experiments were carried out in two locations: in artificially infested maize and sorghum fields at MPFS and in naturally infested fields at the Kenya coast (Kilifi, Mtwapa, Kwale, Kaloleni). At MPFS, plants were artificially infested with eggs or with first-instar larvae obtained from the ICIPE mass-rearing laboratory and the adults that emerged were trapped. In all field experiments, sticky Delta traps were used, arranged according to the randomized block design (3 treatments × 4 replications) or the Latin-square design (4 × 4). The traps were spaced 4 m apart and placed 50 cm above the ground. The distance between traps was deliberately kept relatively short, to allow competition between traps equipped with different baits and to enhance any differences in their attractiveness. However, trap density (1 trap/16 m^2) was still kept much lower than the expected density of females in the field.

Comparison of Attractiveness of Virgin Females versus Two Pheromone Components Dispensed at Different Levels of Proximity. The experiment was conducted in screenhouses and on artificially infested fields at MPFS. The following three types of baits were used: (1) two virgin females; (2) paper dispenser with pheromone components released very close to each other as described previously; and (3) paper dispenser with pheromone components released 3 cm apart. Each experiment lasted three days. Virgin females were replaced daily but dispensers with the synthetic pheromone were changed after three days.

Screenhouse experiments were repeated more than 40 times and field experiments 20 times.

Comparison of Proximity Effect between Two Dispensing Media: Filter Paper and Rubber Septa. The experiment was conducted on naturally infested fields at the Kenya coast, using four types of baits: (1) paper dispenser pairs with pheromone components released close to each other; (2) paper dispenser pairs with components separated by 3 cm; (3) dispenser pairs made from rubber septa, separated by less than 0.5 cm; and (4) dispenser pairs made from rubber septa separated by 3 cm. The dispensers were changed daily. The experiment was conducted on three fields concurrently and was repeated 20 times.

Statistical Analysis. For data from screenhouse and field experiments at MPFS, analysis of variance (ANOVA) on log-transformed data, followed by Tukey's test, was employed for comparisons between averages of treatments. Since the total number of insects caught during the field experiments at the Kenya coast was very small due to the very low population density of *C. partellus* at the time of the experiments, only the chi-square test was used to detect differences between the summations of catches.

RESULTS

During observations in the chamber, only relatively small differences were noticed in the male behavior in response to virgin females as compared to paper dispensers with the pheromone components dispensed close to each other. However, males approaching the virgin females were slightly more vigorous, spent more time fanning wings, moved their antennae at a higher rate, and spent more time moving around the pheromone source, showing that the dispenser does not wholly simulate the signals emitted by the calling female. Interestingly, the female behavior changed dramatically at the time of male arrival, which could contribute to more complex, short-range communication between sexes (Lux, unpublished data). In both cases, however, all males were able to locate the pheromone source (came closer than 5 cm) (Table 1).

In contrast, separation of the release points of the pheromone components resulted in a notable confusion of males at a distance of about 30–40 cm from the source. On average, the nearest distance to the source reached was about 25 cm. The males were much less vigorous, spent much less time fanning wings, moved their antennae at a lower-rate, and spent less time flying and more time resting. Remarkably, only three of 20 males came closer than 5 cm to the source, but even then they spent only a very short time moving around the source (40 and 100 times, respectively, less than in the cases where dispensers with components close to each other and virgin females were used) (Table 1). This seems to suggest that the three males located the source fortuitously.

TABLE 1. BEHAVIOR OF *Chilo partellus* MALES TOWARDS VIRGIN FEMALE VERSUS FEMALE PHEROMONE COMPONENTS DISPENSED AT DIFFERENT LEVELS OF PROXIMITY (LABORATORY OBSERVATION)[a]

	Type of pheromone source		
		Paper dispenser	
Male behavior	A, virgin female	B, components close	C, components separated
Time spent resting before upwind flight	5.6 a	35.1 a	55.4 a
Time taken walking while wing fanning	750.5 a	332.1 b	64.6 c
Rate of antennal beats per minute	148.1 a	120.4 b	50.8 c
Total time spent resting	2018.8 b	2256.6 b	3103.3 a
Total time spent flying (random flight)	538.3 a	319.2 a	135.2 b
General vigor (graded arbitrally from 0 to 10)	7.8 a	6.1 b	2.5 c
Nearest distance reached relative to the source	0.0 b	0.6 b[b]	26.6 a
Time taken to locate the source	185.1 a	313.5 a	588.3 a[c]
Time spent flying upwind up to 5 cm from the source (oriented flight)	14.7 a	15.7 a	11.3[c]
Time taken moving 5 cm or closer around the source	948.8 a	373.0 b	9.3 c[c]

[a] Within rows, differences between averages followed by the same letter are not significant ($P > 0.05$: Tukey's test on log-transformed data).
[b] All males came closer than 5 cm to the source and only 4 males did not land directly on the source.
[c] Only three males of 20 got closer than 5 cm to the source and the numbers represent means for those three males only.

Screenhouse as well as field experiments also indicated that separation of the release points of the pheromone components by a mere 3 cm resulted in a threefold decrease in catches (Tables 2 and 3). This effect was found to be very consistent during the following two nights despite the fast evaporation of the pheromone components and consequent decrease in the attractiveness of the bait (Tables 2 and 3). A similar decrease in bait performance due to spaced release of the pheromone components was also shown in results of the experiment in naturally infested fields, in spite of very low catches because of low population density of the pest at the time of the experiment. The effect of spacing of the pheromone components was equally pronounced in the case of dispensers made of filter paper as in those made of rubber septa. Numbers of insects caught in traps baited with dispensers with the pheromone components placed together vs

TABLE 2. COMPARISON OF ATTRACTIVENESS OF VIRGIN *Chilo partellus* FEMALES VERSUS TWO FEMALE PHEROMONE COMPONENTS DISPENSED AT DIFFERENT LEVELS OF PROXIMITY (SCREENHOUSE EXPERIMENT)

	Number of males caught	Catches as percentage of virgin female catch	Average ± SE[a]
1st night			
A, 2 virgin females	85	100	0.47 ± 0.065 b
B, paper disp. (components close)	76	89	0.42 ± 0.075 b
C, paper disp. (components separated)	26	31	0.14 ± 0.033 c
2nd night			
A, 2 virgin females	119	100	0.71 ± 0.078 a
B, paper disp. (components close)	47	39	0.28 ± 0.044 bc
C, paper disp. (components separated)	15	13	0.09 ± 0.022 c
3rd night			
A, 2 virgin females	149	100	0.89 ± 0.103 a
B, paper disp. (components close)	45	30	0.27 ± 0.038 bc
C, paper disp. (components separated)	19	13	0.11 ± 0.025 c

[a] Average denotes mean number of males caught per trap per night; differences between averages in this column followed by the same letter are not significant ($P > 0.05$; Tukey's test on log-transformed data).

those with spaced components was 17 vs 5 for filter paper and 10 vs 3 for rubber septa, respectively ($\chi^2 = 13.34^{**}$, $df = 3$).

During the first night, traps baited with filter paper dispensers with the pheromone components released very close together were as effective as traps baited with two virgin females (Tables 2 and 3). During the next two days, the effectiveness of the paper dispensers was reduced considerably, but the dispensers still retained some attractiveness during the second and third night, catching about 35% and 28% males, respectively, as compared to virgin females (Tables 2 and 3). Although results from the screenhouse and field are consistent, it is clear that deterioration of the pheromone in the open field was faster, probably due to more harsh atmospheric conditions.

DISCUSSION

The results of our experiments provide strong evidence that even a small separation between the release points of the female *C. partellus* pheromone components substantially decreases trap efficiency. Direct observations of male flight behavior suggest that this decrease is associated with confused behavior

TABLE 3. COMPARISON OF ATTRACTIVENESS OF VIRGIN *Chilo partellus* FEMALES VERSUS TWO FEMALE PHEROMONE COMPONENTS DISPENSED AT DIFFERENT LEVELS OF PROXIMITY (FIELD EXPERIMENT)

	Number of males caught	Catches as percentage of virgin female catch	Average ± SE[a]
1st night			
A, 2 virgin females	96	100	1.20 ± 0.38 a
B, paper disp. (components close)	111	116	1.39 ± 0.39 a
C, paper disp. (components separated)	44	46	0.55 ± 0.26 b
2nd night			
A, 2 virgin females	94	100	1.18 ± 0.19 a
B, paper disp. (components close)	31	33	0.39 ± 0.09 b
C, paper disp. (components separated)	4	4	0.05 ± 0.02 c
3rd night			
A, 2 virgin females	64	100	0.80 ± 0.13 ab
B, paper disp. (components close)	16	25	0.20 ± 0.05 bc
C, paper disp. (components separated)	1	2	0.01 ± 0.01 c

[a] Average denotes mean number of males caught per trap per night; differences between averages in this column followed by the same letter are not significant ($P > 0.05$: Tukey's test on log-transformed data).

of the male approaching the trap. Although these findings are not unexpected, paucity of definitive documentation of the phenomenon in the literature suggests that the ethological consequence of separate release of pheromone components may have been unduly neglected in the past. A comment on this is, therefore, in order.

It is generally known that a flying insect approaching a pheromone trap follows a very complicated, filamentous and/or plumelike structure of the odor carried by an airstream. It is assumed that the pheromone components, if dispensed together from a pointlike source, do not become separated within the airstream carrying them, thus maintaining the integrity of the signal over a distance of many meters (Baker and Haynes, 1989; Liu and Haynes, 1992). This was elegantly demonstrated long ago in experiments with pheromone inhibitors. Remarkably, in these experiments, pheromone traps remained effective even when located in an atmosphere permeated with the corresponding inhibitors, as reported for *Pectinophora gossypiella* (McLaughlin et al., 1972), *Lymantria dispar* (Cardé et al., 1975), *Rhyacionia buoliana* (Daterman et al., 1975), and *Trichoplusia ni* (Kaae et al., 1974; McLaughlin et al., 1974; Mitchell, 1976). In such situations, enhancement of the trap performance was sometimes even noticed, e.g., for *Grapholita molesta* (Rothschild, 1974) and

Spodoptera frugiperda (Mitchell et al., 1974). Only close dispensing of the inhibitor along with the corresponding pheromone from the same trap could disrupt the signal and decrease catches (McLaughlin et al., 1974; Mitchell, 1976).

In the case of separate emission of two pheromone components, each would be expected to be carried independently by a windstream and thus to develop its own pattern of filaments and plumes. As a consequence, the presence of two distinct zones would be anticipated. Within the first zone closer to the source, the components would remain separated since their filament/plume patterns are unlikely to overlap. The range of this zone would depend on the proximity of the odor sources, their orientation toward the wind direction, wind speed, turbulence, etc. In the second zone, a variation of the composition of pheromone components would be expected as the two plumes begin to overlap: in certain regions, one component would predominate, and in the others a blend of the two would dominate but in a variable ratio depending on the degree of plume overlap. The range of this zone may be quite considerable, covering several meters, since it is known that the filament/plume structure of odors is surprisingly durable (Elkinton et al., 1987; Murlis and Jones, 1981), especially at low wind speeds when flying moths (including *C. partellus* males) are most active.

It is also recognized that although at the sensory level, stimulation due to each pheromone component is received independently, successive steps of a behavioral sequence are evoked by relevant biological signals perceived as an integral whole rather than as component stimuli. The view that an animal in its environment deals with integral biological messages rather than with isolated stimuli is widely accepted as a fundamental rule of ethology. Usually, the activity of a bicomponent pheromone is a result of the synergistic action of its constituents. In such cases, a critical time interval has been postulated, within which the stimuli due to the two components must be received to ensure the ability of the insect's nervous system to integrate the sensory information into a biologically meaningful signal (Liu and Haynes, 1992).

Therefore, it would be anticipated that males arriving within a distance of a few meters from the trap baited with a blend of separately dispensed components would encounter a succession of pulses mostly made up of single components, but some containing both and thus with a potential for providing a meaningful message. In the case of *C. partellus*, the males seem rather tolerant to large fluctuations in the ratio of the pheromone components (Lux et. al., unpublished results), so some orientation toward the trap would be expected in response to pulses containing both components. However, the situation changes dramatically as the insect enters the zone closer to the trap. Here it encounters only individual pheromone components and receives discrete stimuli elicited by each one with a time lapse probably exceeding the critical interval essential for

meaningful integration; in this zone the signal disappears, and the insect is left in a confused state. The results of our study conform to such a scenario.

A very similar decrease in trap catches resulting from separate release of the pheromone components relative to joint release of the blend has been reported for the cabbage looper moth, *Trichoplusia ni* (Linn and Gaston, 1981). Analogous effects were also reported during a study involving simultaneous release of the pheromone of this insect and the corresponding pheromone inhibitor. In this study, a cross wind separation of only a few centimeters between the release points of the compounds decreased considerably the negative effect of the inhibitor on trap catches (Liu and Haynes, 1992; Witzgall and Priesner, 1991). Thus, the phenomenon is not without precedent in the pheromone literature.

In conclusion, where components of a pheromone or other semiochemical blends need to be dispensed separately, special attention may need to be given to proximity effects resulting from the spacing of component dispensers. This may be particularly important where the components act together synergistically and would be expected to be of less consequence where they act additively. It would also be anticipated that the effect may be minimized through appropriate design of the trap. However, the development of a dispensing device that allows separate but close release of the components and that can simulate release of the blend from a calling female would appear to be the best solution.

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FECAL VOLATILES AS PART OF THE AGGREGATION PHEROMONE COMPLEX OF THE DESERT LOCUST, *Schistocerca gregaria* (FORSKAL) (ORTHOPTERA: ACRIDIDAE)

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Abstract—Olfactometric bioassays showed that nymphs of crowded desert locusts, *Schistocerca gregaria*, aggregated in response to volatiles derived from their feces and to volatiles emitted from the feces of young adults, but were indifferent to volatiles emitted by older adult feces. On the other hand, young and older adults were not only responsive to their own fecal volatiles but also cross-responsive to each other's and that of the nymphs. Charcoal-trapped volatiles from the feces and synthetic blends of the fecal volatiles also elicited similar responses. Young adults responded moderately to a blend of nymphal volatiles and those derived from nymphal feces. GC-EAD and GC-MS analysis of the trapped volatiles revealed the presence of guaiacol and phenol as predominant electrophysiologically active components of nymphal and young adult feces. Fecal volatiles of older adult contained phenylacetonitrile in addition to guaiacol and phenol, which were present in relatively lower proportion. These results suggest that fecal volatiles are part of the aggregation pheromone complex of the desert locust, which includes the pheromone blends produced by nymphs and older adults, respectively.

Key Words—Airborne volatiles, fecal volatiles, aggregation pheromone, bioassay, gregarious locusts, olfactometer, Orthoptera, Acrididae, *Schistocerca gregaria*, guaiacol, phenol, phenylacetonitrile.

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INTRODUCTION

Recently, we demonstrated the existence of two distinct sets of releaser pheromones that appeared to modulate the aggregation behavior of the two stages of the gregarious desert locust: a juvenile aggregation pheromone produced by nymphs and specific to nymphal stages and an adult pheromone produced by older adults and specific to the adult stages (Obeng-Ofori et al., 1993). The responses of the insects, whether tested individually or in groups, were not significantly different, indicating that visual and tactile stimuli are not prerequisites for the pheromone modulated-aggregation behavior, contrary to previous speculations (Gillett et al., 1976; Fuzeau-Braesch et al., 1988). We showed further that the production of the pheromone in the adult is confined predominantly to the older males, while no such differentiation occurs in the nymphs (Obeng-Ofori et al., 1994). More recently, we found that the aggregation pheromone system of adults consists of phenylacetonitrile (benzyl cyanide), which is the dominant component, present in as much as 75–85% in the volatile emission of older males along with benzaldehyde, guaiacol, and phenol (Torto et al., 1994). Significantly, the nymphs demonstrated total indifference to this pheromone blend, thus confirming our earlier conclusion on the occurrence of stage differentiation in pheromone-mediated aggregation of the desert locust (Obeng-Ofori et al., 1993). Characterization of the nymphal pheromone system is not yet complete.

The young adults represent an interesting stage because they do not emit any significant amount of pheromone themselves and are thus not induced to aggregate significantly by their own volatile emission (Obeng-Ofori et al. 1993, 1994; Torto et al., 1994). Since our bioassays have consistently shown that they respond only to the pheromone produced by older males, the question that needed to be addressed is how this stage of the insect maintained its cohesiveness in the absence of older pheromone-producing males. Field observations have shown a close association between hoppers and fledglings (Uvarov, 1977), which cannot be accounted for by the latter's indifference to the pheromone emission of the former in our bioassays. On the other hand, the presence of a large amount of fecal droppings on the ground where hoppers and fledglings cooccur suggests a possible role for fecal volatiles in the aggregation of the young stages of the insect. Interestingly, previous studies by Nolte et al. (1970, 1973) had implicated nymphal feces as the source of locust gregarization pheromone. Indeed, 5-ethylguaiacol (locustol) proposed by Nolte et al. (1973) and Nolte (1976) as the principal component of the gregarization pheromone of *S. gregaria* and *Locusta migratoria*, was isolated from the feces of nymphal *L. migratoria*. However, subsequent studies by Fuzeau-Braesch et al. (1988) failed to detect this compound in the volatile collections of either *L. migratoria* or *S. gregaria*.

Similarly, our own analysis of airborne volatiles of different stages of *S. gregaria* have not revealed the presence of this compound.

The present study was undertaken to help throw some light on the role of fecal volatiles on the aggregation behavior of different stages of the desert locust, and in particular, that of young adults to volatiles probably encountered on the ground when in close association with nymphs. Herein we report the results of these studies as well as the aggregation responses of the insect to blends of compounds identified in these volatiles.

METHODS AND MATERIALS

Insects. Crowded desert locust, *Schistocerca gregaria* (Forskal) (Orthoptera: Acrididae) from the ICIPE colony originating from a stock obtained for the Desert Locust Control Organization for Eastern Africa (DLCO-EA) in Addis Ababa, Ethiopia, was used for the study. Insects (300–400) of both sexes were bred under crowded conditions in aluminum cages (50 × 50 × 50 cm). They were reared in a special room (4.5 × 4.5 m) that was well aerated by a duct system that maintained a negative pressure with a nycthemeral temperature 30–35°C and light–dark cycle of 12:12 hr. Fresh wheat shoots (Nyangumi variety) and wheat bran were provided daily.

Collection of Fecal Volatiles. Aerations of feces obtained from fifth instars and young and older adults were carried out as previously described (Torto et al., 1994) in 12-cm-long × 2.5-cm-ID glass tubes. Volatiles were trapped for 1 hr from ca. 6.0 g of fresh feces, which were obtained after feeding the three different stages of locusts each overnight on fresh wheat shoots. Thermally conditioned charcoal (80–100 mesh, Chrompack) packed between two glass wool plugs in 6-cm-long × 5-mm-ID glass tubes were used as traps. Trapped volatiles were eluted with HPLC grade dichloromethane (Aldrich Ltd., 5 ml) and concentrated under a stream of nitrogen at 0°C. All the volatile concentrates were stored in the freezer at −15°C until used. For chromatographic analyses, samples were concentrated to 100 μl, and 500 ng of *o*-methylacetophenone were added as internal standard.

Analyses of Volatiles. Volatiles were analyzed employing methods previously described by Torto et al. (1994) by capillary gas chromatography (GC) and by gas chromatography–mass spectrometry (GC-MS). Aliquots (2 μl) of the extracts, were analyzed by GC performed on a Hewlett-Packard (HP) 5890 Series II gas chromatograph equipped with a flame ionization detector (FID) and a HP capillary column (Carbowax 20 M, 50 m × 0.2 mm ID × 0.2 μm) using nitrogen as the carrier gas at a flow rate of 0.35 ml/min. The oven temperature was initially isothermal at 60°C for 10 min, then temperature programmed at 5°C/min to 180°C and from 180°C to 220°C at 10°C/min. Chro-

matographic peaks were integrated using a HP 3396 integrator. GC-MS analysis were carried out on a VG Masslab 12-250 mass spectrometer (EI, 70 eV) coupled to a HP 5790 gas chromatograph.

Coupled gas chromatography–electroantennography (GC-EAD) was performed as previously described (Torto et al., 1994) employing the same column and GC conditions as for analyses of volatiles. The effluent from the column was split 1:1 and FID and EAD signals were monitored synchronously using a program on a GC-EAD interface card (Syntech) installed in a PC (Harvard Professional Computer, American Megatrends Inc.).

Behavioral Bioassays. Bioassays were conducted in a glass olfactometer previously described in detail by Obeng-Ofori et al. (1993). Feces from locusts, volatiles collected from the feces, and synthetic compounds of electrophysiologically active fecal volatile components were tested. Feces were transferred into muslin bags while solutions of the volatile were transferred into 2 ml of paraffin oil (Merck) in 3.7-ml vials as previously described in detail by Torto et al. (1994). The test stimuli was held in one flask and the control in the other flask of the olfactometer. All the synthetic compounds were obtained from Aldrich Ltd., and the purity of each compound was checked by GC.

Males and females and the mixed sexes of fifth-instar nymphs (3–5 days after molt), young adults (4–10 days after molt), and older adults (18–25 days after molt) were each bioassayed in the following situations: (1) control treatment with both sides free of fecal odor; (2) a choice of air column (empty muslin bag) and column treated separately with fifth-instar nymphal or young or older adult feces (10 g each) in a muslin bag; (3) a choice of control air column and a column treated with volatiles collected from the feces of the insect stages mentioned above at doses of 1 FH, 5 FH, and 10 FH, (1 FH = volatiles emitted by 1 g of fresh locust feces per hour); (4) a choice of control air column and a column treated with a 100:35:15 blend of synthetic phenylacetonitrile, phenol, and guaiacol, representing older adult male fecal volatile at pheromone blend (PB) loadings of 37.5, 75, 150, 300, and 600 ng; (5) a choice of air column and column treated with a 100:25 blend of synthetic guaiacol and phenol, representing fifth-instar nymphal or young adult fecal volatiles at pheromone blend (PB) loadings as in (4); (6) a choice of two air columns, one enriched with fifth-instar nymphal volatiles corresponding to a release rate of 100 LH (Obeng-Ofori et al., 1993) and another treated with a blend of 100 LH and 10 FH volatiles derived from fifth-instar nymphal feces; and (7) as in (6) with one column enriched with 10 FH of fifth-instar fecal volatile and the other enriched with a blend of 10 FH and 100 LH volatiles of the same insect stage.

Test insects were introduced into the bioassay arena of the olfactometer in groups of five, and after 15 min the number of insects in each section of the arena was counted. Uncommitted insects found in the middle part of the arena were treated as nonresponders. All tests were replicated five times and locusts

were used only once and then discarded. The aggregation index (AI) was calculated as 100 $(T - C)/N$ where T = number of locusts found in the treated compartment, C = number of locusts found in the control compartment, and N = total number of locusts tested. Means of aggregation responses over the dose range were transformed into arcsine and submitted to a two-way analysis of variance by insect stage (V1) and volatile (V2). Interaction S1 vs S2 would reveal aggregation responses of a particular stage to the volatiles. Mean aggregation responses over the dose range were compared using the LSD test ($\alpha = 0.05$).

RESULTS

Analysis of Volatiles. Compounds represented by peaks 2 and 4 were the dominant and electrophysiologically active components in the fecal volatiles of nymphs and young adults (Figure 1). These compounds were also present in

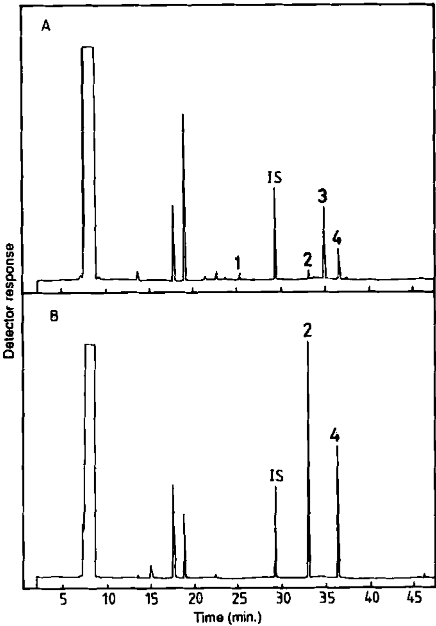

FIG. 1. Gas chromatograms of volatiles collected from feces of *S. gregaria* injected onto a 50-m Carbowax 20 M capillary column: (A) older adult male feces (B) fifth-instar or young adult feces. Labeled peaks are: 1, benzaldehyde; 2, guaiacol; 3, phenylacetonitrile; 4, phenol; IS, internal standard.

older adult fecal volatiles but in relatively lower proportions. The feces of older adults contained an additional electrophysiologically active component labeled 3. Comparison of the profiles of the volatiles from older adult males and females showed that compound 3 was specific to the fecal volatiles of the males. GC-MS analysis of 1, 2, 3, and 4 gave mass spectra characteristic of the following respectively: 1, benzaldehyde *m/z* (rel. intensity) at 77(100), 106(88), 105(84), 51(48), 50(27), 78(17), 76(17), 52(11) and 39(7); 2, guaiacol, *m/z* (rel. intensity) at 109(100), 81(68), 124(64), 53(12), 40(10), 39 and 51(8); 3, phenylacetonitrile, *m/z* (rel. intensity) at 117(100), 90(51), 116(42), 89(34), 51(17), 63(16), 39(14), 77(10), 65(8); 4, phenol, *m/z* (rel. intensity) at 94(100), 66(36), 39(29), 65(27), 40(18), 55 and 38(17). GC retention times of 1, 2, 3, and 4 and coinjection with authentic samples on Carbowax and methyl silicone columns confirmed the identities of the four compounds. Combined GC-EAD recordings using synthetic 2, 3, and 4 confirmed the retention times and EAG activities observed with the natural volatile collection. Benzaldehyde (1) did not elicit significant EAG activity at fecal volatile concentration.

Behavioral Response to Fecal Volatiles. Tables 1 and 2 show the aggregation responses of nymphs and adults to volatiles emanating directly from locust feces and charcoal-trapped fecal volatiles, respectively. The responses of locusts to the synthetic blend of nymphal and older male adult fecal volatiles are shown in Figure 2. In all the three sets of experiments, the nymphs showed aggregation responses to nymphal and young adult fecal volatiles but were indifferent to older adult fecal volatiles. Young and older adults were cross-responsive to each others volatiles. Both were also responsive to nymphal fecal volatiles. There was no sexual differentiation in the responses of nymphs and young and older

TABLE 1. AGGREGATION RESPONSES OF CROWDED *S. gregaria* TO VOLATILES EMANATING DIRECTLY FROM 10 g FIFTH-INSTAR NYMPHAL AND ADULT FECES IN THE OLFACTOMETER[a]

Odor source (10 g feces in muslin bags)	Aggregation index (%)[b]					
	5th-instar nymphs		Young adults		Older adults	
	Male	Female	Male	Female	Male	Female
5th-instar feces	68**	64**	64**	60**	60**	56**
Young adult feces	64**	68**	64**	64**	60**	64**
Older adult feces	4 NS	8 NS	60**	60**	64**	64**

[a]25 insects tested per replicate. Airflow = 120 ml/min.
[b]Difference from control (chi-square test) indicated by NS = not significant; $**P < 0.01$.

TABLE 2. AGGREGATION RESPONSES OF CROWDED *S. gregaria* TO FIFTH-INSTAR NYMPHAL AND ADULT CRUDE FECAL VOLATILES IN THE OLFACTOMETER[a]

Odor source (dose in FH)[b]	Aggregation index (%)[c]					
	5th-instar nymphs		Young adults		Older adults	
	Male	Female	Male	Female	Male	Female
5th-instar fecal volatiles						
1	68**	68**	60**	64**	60**	60**
5	76**	72**	72**	72**	68**	72**
10	76**	76**	76**	72**	72**	72**
Young adult fecal volatiles						
1	64**	68**	60**	64**	56**	60**
5	72**	68**	72**	72**	68**	72**
10	76**	76**	76**	72**	72**	72**
Older adult fecal volatiles						
1	4 NS	4 NS	60**	64**	64**	60**
5	4 NS	0 NS	72**	68**	72**	68**
10	0 NS	4 NS	72**	76**	72**	76**

[a]25 insects tested per replicate.
[b]Volatiles in 2 ml paraffin oil. Air flow = 120 ml/min.
[c]Difference from control (chi-square test) indicated by NS = not significant; $**P < 0.01$.

adults to the fecal volatiles (Table 2). The response of the different insect stages to the fecal volatiles is dose dependent (Figure 2).

The response of fifth-instar nymphs and young adults to simultaneous exposure to either fifth-instar nymphal volatiles or nymphal fecal volatiles and a blend of nymphal and fecal volatiles are shown in Table 3. The nymphs responded equally to the different volatiles. The young adults showed moderate response to the blend of nymphal and fecal volatiles and were indifferent to nymphal volatiles. They were, however, more responsive to the nymphal fecal volatiles than to the blend of nymphal and fecal volatiles.

DISCUSSION

Two patterns of aggregative responses from different stages of the desert locust have been observed with respect to their own volatiles and to those of their feces. In the case of volatiles emitted by live insects, a clear differentiation occurs between the nymphal and adult stages: nymphs of all stages responding only to volatiles emitted by nymphs and, likewise, both young and older adults responding to volatiles of the adult emitted by the older members (Obeng-Ofori et al., 1993; Torto et al., 1994). On the other hand, in the case of fecal volatiles,

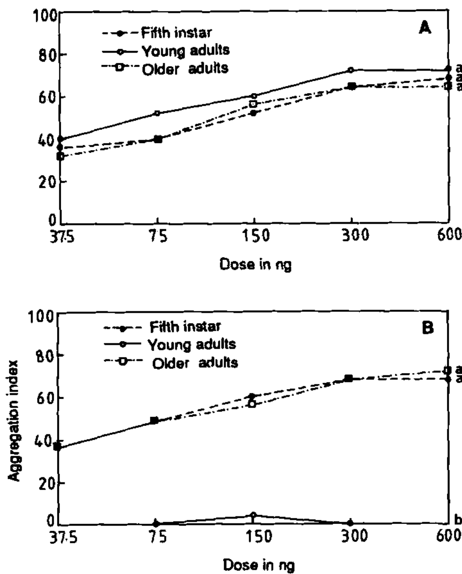

FIG. 2. Dose-response relationships for fifth-instar nymphs, young adults, and older adults to electrophysiologically active fecal volatile components of: (A) fifth-instar nymphs (guaiacol + phenol) and (B) older adult males (guaiacol + phenylacetonitrile + phenol) at different pheromone blend (PB) loadings. Overall means followed by the same letter are not significantly different, $P < 0.05$.

TABLE 3. PERCENT RESPONSES OF *S. gregaria* TO SIMULTANEOUS EXPOSURE TO EITHER NYMPHAL VOLATILES (NV) OR NYMPHAL FECAL VOLATILES (NFV) AND A BLEND OF NYMPHAL VOLATILES AND NYMPHAL FECAL VOLATILES IN THE OLFACTOMETER[a]

	Response (%)					
	5th-instar nymphs			Young adults		
Odor source	NV + NFV	NV	NFV	NV + NFV	NV	NFV
NV + NFV vs NV	52a	44a	NT	52a	20b	NT
NV + NFV vs NFV	56a	NT	44b	40b	NT	60a

[a] Means in each row followed by different letters are significantly different at $P < 0.05$; t test.

as revealed in the present study, differentiation between the two stages is less clear-cut. Thus, the nymphs aggregated in response not only to volatiles of their own feces but also to those of the young adults (Tables 1 and 2). The young and older adults showed even less specificity: they were responsive not only to their own fecal volatiles but also to each other's and that of the nymphs (Figure 1, Tables 1 and 2). Volatiles collected from the feces also elicited similar responses (Table 2). These results are consistent with GC-EAD and GC-MS analyses, which showed that fecal volatiles of nymphs and young adults contain guaiacol and phenol as the dominant electrophysiologically active components, whereas the fecal volatiles of older adults contain phenylacetonitrile in addition to guaiacol and phenol (Figure 2). Moreover, synthetic blends of these compounds elicited the same patterns of responses (Figure 1).

The exact role of phenylacetonitrile in the bioecology of the desert locust is not clear at present. For example, the adaptive significance of older adult locusts generating a volatile fecal blend that is not pheromonally active for the nymphs is unknown. It is also not known whether phenylacetonitrile is an olfactory inhibitor for the nymphs. Detailed single-cell electrophysiological studies could help throw more light on this. Furthermore, our studies on the primer effects of locust volatiles on accelerated maturation and phase dynamics of the desert locust currently in progress would provide some understanding on the possible significance of phenylacetonitrile in the biology of locusts.

Our results point toward specific functions for fecal volatiles in desert locust aggregation and a more complex pheromonal system of modulating social cohesion in *S. gregaria*. An obvious function of compositionally identical fecal volatiles of nymphs and young adults may not only be to hold the latter together but also to do so in close association with the former when these are fledgling. This would ensure the cohesiveness of the whole fledgling population that emerges, which would continue to rely on its fecal volatiles until it starts to produce the adult pheromone. Interestingly, the receptor cells of the young adults, unlike those of the hoppers, show the plasticity needed to respond to their own (and that of nymphal) fecal pheromone system and to that produced by the older adults.

Do fecal volatiles also play a role in the aggregation of nymphs and older males? In the case of the former, the close association of this stage of the insect with their feces during and after feeding, especially while roosting (Uvarov, 1966; Steedman, 1988), suggest that fecal volatiles may augment the nymphal pheromone system in modulating cohesion in this stage of the insect. On the other hand, the flight and feeding behavior of older adult locusts make them less likely to come into more than casual contact with their feces. However, a sustained contact is possible during roosting periods, during which fecal volatiles of older adults could similarly play an augmentative role in aggregating older adults. Significantly, the three major active components of the feces of older

adults constitute the same pheromone emission of these insects (Torto et al., 1994), although phenylacetonitrile is present in this emission in proportionately larger amounts than in feces (75–85% compared to 10–15% in fecal volatiles).

In summary, fecal volatiles appear to be part of the releaser pheromone complex of the desert locust with an augmentative role in aggregating the hoppers and older adults and with an important function of keeping young adults cohesive during the critical period when they transit from fledglings to full adults at which time they do not produce any other aggregating factors. It may be noted that previous studies by other workers had implicated feces as the primary sources of phase-modulating factors for the desert locust and migratory locust (*L. migratoria*). Thus, Nolte et al. (1970) traced the gregarization pheromone for the two species—assumed to be identical—to their hopper feces. Indeed, locustol (5-ethylguaiacol) and guaiacol were identified as the main constituents of the steam distillate of the hopper feces of *L. migratoria* and the former as primarily responsible for effects relating to pigmentation, morphometric changes, and increase in chiasma frequency reportedly associated with the gregarization pheromone of the two locust species (Nolte et al., 1973; Nolte, 1976). However, in our studies with the desert locust, we did not detect any 5-ethylguaiacol in any of our volatile collections either from the feces or the insects themselves. Likewise, Fuzeau-Braesch et al. (1988) were not able to detect this compound in their analyses of airborne collections from colonies of different stages of the two locust species although, interestingly, they found guaiacol and phenol, in addition to veratrole and an unidentified constituent. Adult feces have previously been evaluated by several authors. Gillett and Phillips (1977) concluded that adult feces had the effect of making nymphs less gregarious. This was confirmed by Gillett (1983), who found that the grouping behavior of nymphs exposed to adult feces was significantly less compared with those exposed to hopper feces and concluded that a solitarizing stimulus was produced by gregarious adults. These observations are accounted for by the results of our present work as well as our previous studies (Obeng-Ofori et al., 1993; Torto et al. 1994). We have established that hoppers are indifferent to blends that make up the feces of older adults and their volatile emissions. It would be interesting to establish whether older adult fecal volatiles constitute the solitarizing stimulus proposed by Gillett and Phillips (1977).

Our studies to date have focused on the chemical stimuli mediating the releaser effects in the gregarious behavior of the desert locust. In this regard, the pheromone system produced by the nymphal stages remains to be fully characterized. We have now initiated a detailed study of the primer effects of the different components of locust gregarization pheromone complex to allow us to establish their respective roles in phase transformation of different stages of the insect.

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ISOLATION OF 8-HYDROXYGERANIOL-8-*O*-β-D-GLUCOSIDE, A PROBABLE INTERMEDIATE IN BIOSYNTHESIS OF IRIDOID MONOTERPENES, FROM DEFENSIVE SECRETIONS OF *Plagiodera versicolora* AND *Gastrophysa viridula* (COLEOPTERA: CHRYSOMELIDAE)

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Abstract—8-Hydroxygeraniol and its 8-*O*-β-D-glucoside have been found as trace components in the defensive secretions of *Plagiodera versicolora* and *Gastrophysa viridula* larvae. This discovery supports the hypothesis that the evolution of the utilization of plant precursors by some chrysomelid species was favored by the plesiomorphic occurrence of a β-glucosidase and an oxidase in the defensive secretion of iridoid-producing species.

Key Words—Coleoptera, Chrysomelidae, defensive secretion, *Plagiodera versicolora*, *Gastrophysa viridula*, β-glucosidase, oxidase, 8-hydroxygeraniol, 8-hydroxygeraniol-8-*O*-β-D-glucoside.

INTRODUCTION

The larvae of leaf beetles belonging to the subtribe Chrysomelina and to the genus *Phratora* (Phyllodectina) secrete volatile compounds acting as irritants and quite active in repelling small predators (e.g., ants) (Pasteels et al., 1988). Although *Phratora* spp. are currently classified in a distinct subtribe (Seeno and Wilcox, 1982), their defensive glands are clearly homologous to those of the Chrysomelina, suggesting a monophyletic origin for all those taxa possessing

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these defensive glands (Pasteels and Rowell-Rahier, 1989; Pasteels, 1992). Recently published phylogenies based on the similarity of 12S and 16S mtDNA support this view (Hsiao, 1994).

In this group, most taxa synthesize de novo iridoid (cyclopentanoid) monoterpenes but others derive aromatic compounds from plant glucosidic precursors, e.g., salicylaldehyde in some *Chrysomela* and *Phratora* species and juglone in *Gastrolina depressa* (ref. in Pasteels et al., 1988). Circumstantial evidence based on the distribution of larval de novo and host-derived compounds among leaf beetle taxa suggests that the synthesis of iridoid monoterpenes is the plesiomorphic condition, whereas the secretion of host-derived compounds are apomorphic conditions that independently evolved at least three times (Pasteels and Rowell-Rahier, 1991). This suggestion is supported by mtDNA evidence (Hsiao, 1994).

The transformation of plant glucosides into volatile irritants only requires two enzymes located within the secretion stored in the glandular reservoirs. It was suggested (Pasteels et al., 1990) that the repeated evolution of such utilization of plant glucosides was favored by the plesiomorphic occurrence of a β-glucosidase and an oxidase in the defensive secretions of species producing iridoid monoterpenes. It was similarly hypothesized that the two enzymes are required in the final steps of the biosynthesis of the latter compounds and, thus, that small changes in the specificity of these preexisting enzymes would allow the shift from de novo synthesis to the utilization of plant precursors in the insect defensive chemistry (Pasteels et al., 1990). This suggestion was supported by the presence of glucose in the secretion of species producing iridoid monoterpenes and by the demonstration of a β-glucosidase activity in the secretion of at least three species: *Plagiodera versicolora* (Duffey and Pasteels, unpublished results), *Phratora tibialis* and *Ph. laticollis* (Soetens et al., 1993). The above hypothesis postulates that a monoterpene glucoside should be one of the biosynthetic precursors of the defensive iridoid monoterpenes and that this glucoside should be present in the glandular reservoir in which the final steps leading to the iridoid monoterpenes occur.

The purpose of this work was to search in the defensive secretions of *Plagiodera versicolora* and *Gastrophysa viridula* for this hypothetical monoterpene glucoside and its aglycone, and thus, to refine the biosynthetic scheme recently proposed by Lorenz et al. (1993). The larvae of *P. versicolora* used in this study secrete two iridoid monoterpenes, plagiodial and plagiolactone, and those of *G. viridula*, two related compounds, chrysomelidial and epichrysomelidial (Pasteels et al., 1982). Using deuterated nor-precursors, Lorenz et al. (1993) demonstrated that these compounds are biosynthesized by the larvae, through a pathway similar to that established for the biosynthesis of iridoids in plants (Inouye and Uesato, 1987). Geraniol formed from mevalonic acid is ω-hydroxylated into 8-hydroxygeraniol, which is further oxidized into

8-oxocitral. The latter is finally cyclized to afford plagiodial or chrysomelidial, depending on the species.

METHODS AND MATERIALS

Biological Material

Plagiodera versicolora and *Gastrophysa viridula* were collected near Brussels on various *Salix* species and on *Rumex obtusifolia*, respectively. They were raised in the laboratory in plastic, 500-ml containers with perforated transparent plastic lids. The bottom of the containers were covered with moist paper and the insect fed with fresh leaves of *Salix babylonica* and *Salix fragilis* × *alba* (*P. versicolora*) or *Rumex obtusifolia* (*G. viridula*), renewed every two days.

Collection of Secretions. To increase the probability of finding the biosynthetic precursors, secretions of larvae that were active in the process of synthesis were collected. Since the secretion of a larvae is renewed in about 24 hr, third-instar larvae were milked twice a day and on each consecutive day until pupation. These secretions were collected in glass capillaries half filled with methanol in order to inactivate as soon as possible the enzymes present in the secretion. No attempt was made to quantify the amount of secretion collected, but the secretions of several hundreds of larvae were collected in this way and stored in methanol.

Isolation of 8-Hydroxygeraniol [*2,6-dimethyl-2*(E)*,6*(E)*-octadiene-1,8-diol*] *(1), and its 8-*O-*β*-D-glucoside *(2) from Defensive Secretion of* P. versicolora *(Figure 1)*

The constituents of the defensive secretion of third-instar larvae of *P. versicolora* were partitioned between CH_3OH/H_2O/hexane (5:1:5), by stirring magnetically for 10 min. The phases were then allowed to separate and the

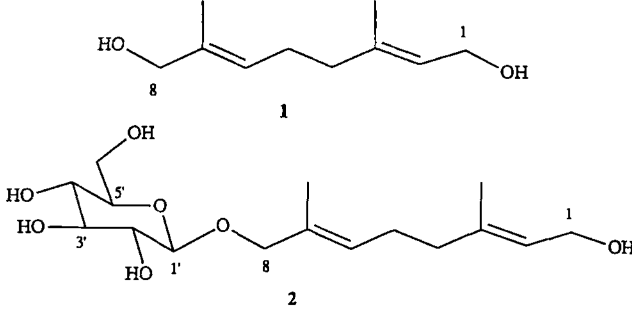

FIG. 1. Structures of the trace constituents found in the defensive secretions of *Plagiodera versicolora* and *Gastrophysa viridula*.

upper hexane layer removed with a Pasteur pipet. This procedure was repeated five times, after which most of the iridoid monoterpenes (plagiodial and plagiolactone) were concentrated in the hexane phase. The CH_3OH/H_2O phase was evaporated under reduced pressure, leaving 4.7 mg of material. A TLC analysis of the latter (SiO_2, $CHCl_3/CH_3OH$, 8:2, visualized by spraying with anisaldehyde or vanillin/conc. H_2SO_4) showed the presence of free glucose (major spot, *Rf* = 0.1), and a small amount of plagiodial (*Rf* = 0.8), accompanied by two faint spots (*Rf* = 0.4 and 0.7), exhibiting characteristic blue–green (anisaldehyde) or red colors (vanillin). This mixture was flash chromatographed on a silica gel column (5 mm diameter), eluted with $CHCl_3/CH_3OH$ (95:5, 90:10, and 70:30, 20 ml each). This separation afforded plagiodial and glucose, identified by direct comparison with authentic samples (GC or TLC, ^1H NMR), as well as two compounds corresponding to the aforementioned spots as *Rf* = 0.7 and 0.4. These were isolated in very small amounts and thus could not be weighed accurately. The analyses performed on these samples suggest that less than 50 μg of each compound was isolated.

*8-Hydroxygeraniol **(1)** (Figure 1)*

The compound at *Rf* = 0.7 was identified as 8-hydroxygeraniol by comparison with an authentic sample (Aldrich) in TLC ($CHCl_3/CH_3OH$, 95:5), by coinjection in GC on two different capillary columns (Rescom, 25 m, 0.32 mm diameter): OV-1701, 160°C isothermal and PEG 200°C isothermal, and by GC-MS (Finnigan, ITD 800, coupled to a Tracor gas chromatograph equipped with an OV-1701 column, isothermal at 180°C. CI-MS (isobutane): *m/z* 153 [10, $(M + H - H_2O)^+$]; 135 [100, $(M + H - 2H_2O)^+$]; 123 (10); 121 (15); 109 (12); 107 (40); 95 (38); 93 (25); 81 (22); 67(10).

*8-Hydroxygeraniol-8-O-β-*D-glucoside ***(2)** (Figure 1)*

The ^1H NMR spectrum (CD_3OD, 600 MHz, Varian Unity 600) of the compound at *Rf* = 0.4 showed that it was still accompanied by impurities. It was rechromatographied on a Pasteur pipet containing silica gel previously washed with redistilled $CHCl_3/CH_3OH$ (1:1) and subsequently heated at 140°C for 1 hr. The eluent was a 85:15 mixture of the same redistilled solvents. The purity of the sample thus obtained was now sufficient for spectroscopic analyses. CI-MS (NH_3): *m/z* 350 [100, $(M + NH_4)^+$]; 333 [58, $(M + H)^+$]; 332 (40, M^+); 315 [56, $(M + H - H_2O)^+$]; 304 (32); 256 (14); 212 (14); 180 (40); 169 (12); 162 (15); 153 (18); 135 (40); 118 (20); 110 (30); 108 (20). ^1H NMR (CD_3OD, 600 MHz, Varian Unity 600): 5.48 (H-2 or H-6, t, 7.0 Hz); 5.35 (H-6 or H-2, t, 7.0 Hz); 4.23 (H-1′, d, 8.0 Hz); 4.04 and 4.19 *H_2-8, AB system, J_{AB} = 11.0 Hz); 4.07 (H_2-1, d, 7.0 Hz); 3.85 (H-6′a, dd, 12.2, 2.5

Hz); 3.64 (H-6′b, dd, 12.2, 5.5 Hz); 3.20 (H-2′, dd, 9.0, 8.0 Hz); 2.18 (H_2-5, m); 2.08 (H_2-4, t, 7.0 Hz); 1.68 and 1.66 (2 × 3H, s, H_3-9 and H_3-10).

Acetylation of a few micrograms of **2** overnight with the mixture Ac_2O-pyridine afforded pentaacetate **3** (Figure 2) that was purified by chromatography on a Pasteur pipet filled with silica gel, with tridistilled hexane/AcOEt, 7:3, as eluent. ^{1}H NMR spectrum of **3** [$CDCl_3$, 600 MHz, 48,448 scans: 5.35 (2H, m, H-2 + H-6); 5.18 (H-3′, t, 9.0 Hz); 5.08 (H-4′, t, 9.0 Hz); 5.0 (H-2′, dd, 9.0, 7.8 Hz); 4.58 (H_2-1, d, 7.5 Hz); 4.47 (H-1′, d, 7.8 Hz); 4.25 (H-6′a, dd, 12.0, 5.0 Hz); 4.14 (H-6′b, dd, 12.0, 2.0 Hz); 4.12 and 3.95 (2H, AB system, J_{AB} = 11.5 Hz); 2.3 (4H, m, H_2-4 + H_2-5); 2.08, 2.04, 2.02, 2.0, 1.98 (5 × CH_3COO, s); 1.68 and 1.64 (2 × 3 H, s, H_3-9 and H_3-10)].

Compounds **1** and **2** were also detected in the defensive secretion of *Gastrophysa viridula* larvae by TLC comparison with the secretion of *P. versicolora* (*Rf* and characteristic color with anisaldehyde/conc. H_2SO_4 spray).

*Synthesis of 8-Hydroxygeraniol-8-O-β-D-glucoside **(2)** (Figure 3)*

Koenigs-Knorr Reaction. See Reyle et al. (1950). Typically, α-bromotetraacetylglucose (0.375 g, 0.91 mmol) and 8-hydroxygeraniol (0.210 g, 1.24 mmol) were dissolved in anhydrous toluene. Ag_2CO_3 (0.15 g, 0.54 mmol), prepared according to the procedure of Organic Synthesis (1955) and dried for three days in a desiccator containing P_2O_5, and anhydrous $CaSO_4$ (0.180 g, 1.32 mmol) were added and the mixture was stirred at room temperature in the dark. The reaction was followed by TLC and was stopped after 30 hr by dilution with CH_2Cl_2 and filtration on Celite. The two isomeric tetraacetyl glucosides **4** and **6** (Figure 3) were only resolved in TLC with toluene/AcOEt mixtures. Flash chromatography (eluent: toluene/AcOEt 6:4) of the reaction mixture afforded 20 mg of **4**, 40 mg of **6**, and 117 mg of a mixture of the two compounds.

Compound **4.** ^{1}H NMR ($CDCl_3$, 250 MHz, Bruker WM 250): 5.39 (H-2 and H-6, m); 5.22 (H-4′, dd, 9.5, 9.5 Hz); 5.08 (H-3′, dd, 9.5, 9.5 Hz); 5.00 (H-2′, dd, 9.5, 8.0 Hz); 4.49 (H-1′, d, 8.0 Hz); 4.25 (H-6′a, dd, 12.0, 5.0 Hz); 4.15 (H_2-1, d, 7.0 Hz); 4.13 (H-6′b, dd, 12.0, 2.7 Hz); 4.16 and 3.96 (H_2-8, AB system, J_{AB} = 12.0 Hz); 3.65 (H-5′, m); 2.15 and 2.10 (H_2-4 and

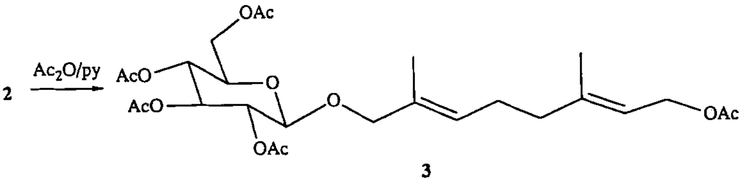

FIG. 2. Synthesis of pentaacetate **3** from 8-hydroxygeraniol-8-*O*-β-D-glucoside **(2)**.

FIG. 3. Synthesis of 8-hydroxygeraniol-8-*O*-β-D-glucoside (**2**) and 8-hydroxygeraniol-1-*O*-β-D-glucoside (**5**).

H_2-5, m); 2.09, 2.03, 2.01, 2.0 (4 × CH_3COO, s); 1.68 and 1.60 (H_3-9 and H_3-10, s).

Compound **6**. ^{1}H NMR ($CDCl_3$, 250 MHz, Bruker WM 250): 5.38 (H-2 or H-6, t, 6.5 Hz); 5.26 (H-6 or H-2, t, 6.5 Hz); 5.22 (H-4′, dd, 9.5, 9.5 Hz); 5.07 (H-3′, dd, 9.5, 9.5 Hz); 4.98 (H-2′, dd, 9.5, 8.0 Hz); 4.54 (H-1′, d, 8.0 Hz); 4.23 (H-6′a, H-6′b and H_2-1, m); 4.01 (H_2-8, bd, 6.0 Hz); 3.65 (H-5′, m); 2.10 (H_2-4 and H_2-5, m); 2.08, 2.04, 2.02, 2.0 (4 × CH_3COO, s); 1.66 (H_3-9 and H_3-10, s).

Hydrolysis of **4** *and* **6** *(Figure 3).* Compounds **4** and **6** were each treated for 48 hr with a saturated methanolic solution of K_2CO_3. The CH_3OH was evaporated under vacuum, water was added, and the aqueous solution extracted successively with $CHCl_3$ and *n*-BuOH. The *n*-BuOH extract containing the glucoside was dried under reduced pressure with toluene and chromatographied on a small silica gel column (eluent: CH_2Cl_2/CH_3OH, 85 : 15). This procedure afforded 7 mg of **2** from 20 mg of **4** and 12 mg of the isomer **5** from 40 mg of **6**.

*8-Hydroxygeraniol-8-*O-β-D-glucoside *(2).* Amorphous; CI-MS and ^{1}H NMR spectrum identical to those of the natural compound (see above).

8-Hydroxygeraniol-1-O-β-D-glucoside (5). Amorphous; CI-MS identical to that of **2**; ^1H NMR (CD_3OD, 250 MHz, Bruker WM 250): 5.38 (H-2 and H-6, t, 6.5 Hz); 4.26 (H_2-1, AB of ABX, J_{AB} = 12.0 Hz, J_{AX} = J_{BX} = 6.5 Hz); 3.90 (H_2-8, s); 3.86 (H-6'a, dd, 12.0, 2.2 Hz); 3.66 (H-6'b, dd, 12.0, 5.3 Hz); 3.36 to 3.15 (H-2', H-3', H-4'); 2.17 and 2.11 (H_2-4 and H_2-5, m); 1.69 and 1.64 (H_3-9 and H_3-10, s).

RESULTS

Examination of the crude defensive secretion of larvae of *Plagiodera versicolora* by TLC ($CHCl_3/CH_3OH$, 8:2, anisaldehyde conc. H_2SO_4 spray) allowed us to detect the presence of the familiar monoterpenes, plagiodial and plagiolactone, together with a much more polar compound identified as glucose by comparison with an authentic sample (Pasteels et al., 1982). However, after partitioning this secretion between the two phases of a CH_3OH/H_2O/hexane (5:1:5) system, most of the plagiodial and plagiolactone were transferred into the hexane layer. TLC analysis of the concentrated CH_3OH/H_2O phase now allowed detection of, besides glucose (*Rf* = 0.1), two more spots at *Rf* = 0.4 and 0.7.

The compounds corresponding to these spots were isolated by careful chromatography on small silica gel columns. The compound at *Rf* = 0.7 was easily identified as 8-hydroxygeraniol (**1**) (Figure 1) by TLC, GC, and GC/MS comparison with an authentic sample. The spectral properties of the more polar compound (*Rf* = 0.4) (see Methods and Materials) suggest that it is 8-hydroxygeraniol-8-*O*-β-D-glucoside (**2**) (Figure 1). In particular, the CI-MS shows a $[M + H]^+$ ion at *m/z* 333, together with fragment ions at *mz* 180 and 162, characteristic for an hexose sugar, and at *m/z* 169, 153, and 135, characteristic for an acyclic dihydroxylated monoterpene. The ^1H NMR spectrum at 600 MHz was in complete agreement with the proposed structure. The glucose was located at C-8 of the 8-hydroxygeraniol moiety since the CH_2-8 appears as an AB system at δ 4.04 and 4.19 (3.95 and 4.12 in pentaacetate **3**) and CH_2-1 as a doublet at δ 4.07 (4.58 in **3**). This identification was further confirmed by comparison of **2** with synthetic 8-hydroxygeraniol-8-*O*-β-D-glucoside and 8-hydroxygeraniol-1-*O*-β-D-glucoside (**5**), obtained by a Koenigs-Knorr reaction (Reyle et al., 1950) between α-bromotetraacetylglucose and 8-hydroxygeraniol, followed by separation of the two isomeric tetraacetyl glucosides **4** and **6**, and, finally, basic hydrolysis of the acetate groups (Figure 3). The identity of the natural and synthetic compounds also defines the absolute configuration of the glucose moiety as D.

The presence of 8-hydroxygeraniol and its 8-*O*-β-D-glucoside in the defensive secretion of *Gastrophysa viridula* was demonstrated by careful TLC comparisons with authentic material.

DISCUSSION

The presence in the defensive secretion of *Plagiodera versicolora* of traces of 8-hydroxygeraniol-8-*O*-β-D-glucoside (**2**), and of both 8-hydroxygeraniol (**1**) (Figure 1) and free glucose, together with the demonstration of a β-glucosidase activity in this secretion (Duffey and Pasteels, unpublished results support the view that 8-hydroxygeraniol 8-*O*-β-D-glucoside (**2**) is the first or immediate precursor of the glandular chemistry. Thus, it seems likely that the glucoside **2** is rapidly hydrolyzed in the reservoir, liberating 8-hydroxygeraniol, which is further transformed according to the scheme put forward by Lorenz et al. (1993). The final steps of the biosynthesis occur in the gland reservoirs, outside the glandular cell, and require several enzymes, among which are a β-glucosidase and at least one oxidase. Since both 8-hydroxygeraniol 8-*O*-β-D-glucoside (**2**) and 8-hydroxygeraniol (**1**) (Figure 1) were also found in the defensive secretion of *Gastrophysa viridula* producing other iridoid monoterpenes—chrysomelidial and epichrysomelidial (Pasteels et al., 1982)—the first steps in the biosynthesis of iridoids in these chrysomelid larvae must be similar and only differ after the formation of 8-hydroxygeraniol and its subsequent oxidation into 8-oxocitral (see also Lorenz et al., 1993).

Our results thus support the hypothesis that the evolution of the utilization of plant precursors by some species was facilitated by preexisting enzymes in the glandular reservoir. In the absence of information on the biosynthesis of iridoid monoterpenes in leaf beetles larvae, it was suggested that the precursor of iridoid aldehydes could be a glucoside of an already cyclized monoterpene (Pasteels et al., 1990). This obviously was an incorrect guess, since we have now shown that the glucoside is formed earlier in the biosynthetic pathway. In this context, it is interesting to point out that the classical scheme of iridoid glucoside biosynthesis in plants postulates that the glucosidation of the monoterpene aglycone is always effected after the cyclization of the latter into a cyclopentane ring (reference in Inouye and Uesato, 1987). However, this view has been questioned recently, following the isolation of several acyclic monoterpene glucosides in iridoid glucoside-producing plants (Gross, 1985; Gross et al., 1987; Gering-Ward and Junior, 1989). Thus, the presence of glucosylated precursors at an early stage of iridoid biosynthesis could be a common feature in both plants and insects.

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SQUASH LEAF GLANDULAR TRICHOME VOLATILES: IDENTIFICATION AND INFLUENCE ON BEHAVIOR OF FEMALE PICKLEWORM MOTH [*Diaphania nitidalis* (Stoll.)] (Lepidoptera: Pyralidae)[1]

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Abstract—Fourteen volatile compounds occurring in leaf trichomes of yellow squash (*Cucurbita pepo* L. cv. Early Prolific Straightneck) were identified. These compounds accounted for 83.5% of the volatile matrix. Ubiquitous constituents of the epidermis (myristic, palmitic, and stearic acids, *n*-tricosane, and *n*-pentacosane) accounted for 73.7%; these compounds were not bioassayed. The volatiles *o*-, *m*-, and *p*-xylene, toluene, 2-heptanone, (*R*)-(+)- and (*S*)-(−)-limonene, and germacrene D were tested for their influence on attraction and oviposition by the pickleworm moth (*Diaphania nitidalis* Stoll.). No single compound, except germacrene D, was attractive. (*R*)-(+)-Limonene and 2-heptanone were weakly repellent. Mixtures of the highly volatile fractions were as attractive as volatiles emanating from whole, intact leaves. Oviposition levels on treated artificial sites corresponded with levels of visitation. Oviposition was significantly stimulated by "whole-leaf" volatiles, and (*S*)-(−)-limonene caused a slight but significant reduction.

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Key Words—Cucurbitaceae, *Cucurbita pepo*, Insecta, Lepidoptera, Pyralidae, *Diaphania*, oviposition, *nitidalis*, pickleworm moth, egg laying, visitations, stimulation.

INTRODUCTION

The pickleworm *Diaphania nitidalis*, is a cucurbit pest of considerable economic importance in the southeastern United States, Central America, northern South America, and the Caribbean Islands (Elsey et al., 1984). The degree of susceptibility to infestations varies considerably among cucurbit species, cultivars, and varieties (Corley, 1973; Day et al., 1978; Pulliam, 1979; Quisumbing and Lower, 1975). Nonpreference to oviposition was shown in glabrous mutants of muskmelon and cucumber (Day et al., 1978; Elsey and Wann, 1982). Since nearly isogenic lines of glabrous and pubescent lines of cucumber were evaluated, it seemed possible that the lack of trichomes was the cause of nonpreference. Subsequently it was shown that small, highly polar nonvolatile compounds obtained from the leaves of yellow squash were responsible for prolific egg-laying on artificial sites, and addition of "whole-leaf" volatiles to the active water-soluble fraction increased oviposition considerably. Substitution of the whole-leaf volatile matrix by volatiles emanating from glandular trichomes resulted in the same levels of oviposition (Peterson and Elsey, 1994). It seemed likely that the glandular trichome volatiles served as attractants for the nocturnal gravid female in her search for oviposition host plants and in addition may stimulate oviposition.

METHODS AND MATERIALS

Insect Rearing. Diaphania nitidalis were raised in the laboratory according to methods described by Elsey et al. (1984), and a continuous supply of imagos was maintained.

Culture of Plants and Collection of Leaves and Volatiles. Seedlings of yellow squash (*Cucurbita pepo* L. var. Early Prolific Straightneck) were planted in the field and raised using standard cultural practices. Healthy leaves, of approximately one half of ultimate size (approximately 10–12 cm longest dimension) were collected from the field early in the morning and immediately processed.

For preliminary gas chromatography (GC), glandular exudates (membrane-bound droplets) were collected under a dissection microscope and dropped into *n*-pentane, and the solutions were concentrated at room temperature under a stream of nitrogen. These solutions were used to obtain a reference GLC profile, reflecting the chemical profile present during the early morning hours in the

field. Larger amounts, to be used for GC-mass spectrometry were obtained by dropping whole leaves, approximately one half of ultimate size, in *n*-pentane. These solutions were similarly concentrated under a stream of N_2 and stored at 4°C under N_2 until use.

Leaves for bioassays were collected early in the morning, immediately prior to their use. Leaf sizes were around 10 cm (longest diameter) and the tips were removed in order to obtain a leaf that fitted well within the borders of a 10-cm-diam. filter paper (see bioassays).

Gas Chromatography and GC-MS. The GC column was a 15-m × 0.25-mm-ID fused silica capillary coated with methylsilicone (DB-1, J & W Alltech, Inc., Deerfield, Illinois). The chromatograph was a Hewlett Packard (Avondale, Pennsylvania) model 5890, equipped with a split mode injector. Injector and detector temperatures were 225 and 280°C, respectively. The oven temperature was held at 60°C for 1 min, then programmed to 90°C at 3°C/min, held for 0.5 min, and finally programmed to 240°C at 8°C/min. The injection volume was 1.0 μl.

Mass spectral analyses were made with an Extrel model C 50/400 (Pittsburg, Pennsylvania) quadrupole mass spectrometer interfaced with a Perkin-Elmer (Norwalk, Connecticut) Sigma 300 gas chromatograph equipped with a cold on-column injector (Horvat et al., 1990). Chromatographic separations were made on a 50-m × 0.32-mm-ID fused silica capillary column coated with Se 54. Helium was used as a carrier gas at 50 kPa. The oven was programmed from 50 to 220°C at 3°C/min and held for 34 min.

The trichome volatile compounds were identified by comparison of mass spectra and GLC retention times with authentic standards when available. Mass spectra were also compared with spectra in the NIST/EPA/MSDC mass spectral data base (PC version 3.0) (Stein, 1990).

Bioassays. Assays involved observations of relative visitation frequencies and determination of the number of eggs deposited on glass fiber pads according to Elsey and McFadden (1981), with several modifications. The artificial visitation/oviposition sites were composed of the following layers: a circular pad of building insulation glass fiber (9 cm diam., 1.5 cm thickness), seven filter papers (Whatman 1, 9 cm diam.), one circle of aluminum foil, and finally one more filter paper. After application of test material, all layers were stapled together close to the periphery.

Volatiles were applied to the middle filter paper, small leaves (approx. 8 cm longest dimension) were placed immediately below the glass fiber with the underside of the leaf facing the pad; and leaf "sap" was brushed on top of the glass fiber. All applications were made immediately before testing. Volatiles were mixed with silicone oil: 1:9, v/v) and stored in vials under a nitrogen atmosphere in the dark. Single compounds were applied in amounts of 20 μl, mixtures 40 μl, and leaf sap 1.0 ml. Pads used as reference (blanks) received

silicone oil only. Leaf "sap" was prepared by grinding one small leaf with 3 ml water using a mortar and pestle. The resulting brei was subsequently centrifuged and 1.0 ml supernatant was brushed onto the surface of a glasswool pad. These pads were blown with N_2 until the surface became sticky.

Pads were suspended 20 cm below the top of a wire cage (120 × 90 × 90 cm) in a linear fashion and in random order with spacings of 20 cm. The cage was located in a humidified room held at 23°C, 16L:8D photoperiod. A population of approximately 400 imagos of mixed sex was maintained in the cages. The bioassays were conducted during the first 3 hr of the scotoperiod. The pads were placed in the cages for a 15-min waiting period; subsequently, the number of moths present on the pads was counted every 5 min (10 observations per pad) using a flashlight covered with four layers of red cellophane. At the end of the observation period, the pads were removed and the number of eggs was determined.

Individual compounds [ortho-, meta-, and paraxylene, toluene, (*R*)-(+)-limonene, (*S*)-(−)-limonene and 2-heptanone] were tested as follows: three pads received a randomly chosen but different compound, one pad had a leaf sandwiched in the middle and one pad received silicone oil only (blank). Tests were conducted until each compound was tested 10 times (100 visitation observations per compound). Germacrene D was tested separately: a test consisted of two pads with germacrene D, two pads with a leaf, and two pads without application (blanks). This test was repeated eight times (total: 160 visitation observations per treatment).

Two compound mixtures were tested: mix A and mix B. Mix A consisted of all compounds listed in Table 2B below. No germacrene D was present in this mixture. The relative concentrations were as found in the GC profile (see Table 1). A test consisted of two pads with the mixture, two pads with a leaf, and two pads without application. This assay was repeated six times (120 visitation observations per treatment).

Mix B was similar to mix A; however, mix B contained germacrene D in addition. Again the relative concentrations were as listed in Table 1. The tests were performed the same as for mix A. Leaf sap was tested using the same arrangement (two pads with sap, two with a leaf, and two blanks), and this test was performed three times.

Chemicals. *o*-Xylene (98%), *m*-xylene (99+%), *p*-xylene (99+%), toluene (99+%), (*R*)-(+)-limonene (97%), (*S*)-(−)-limonene (96%), 2-heptanone (98%), and silicone oil (n_4^{20} = 1.4040, *d* 0.963) were obtained from Aldrich Chemical Company (Milwaukee, Wisconsin). Germacrene D (97.9%) was a gift from Robert A. Flath (USDA, Albany, California). Germacrene D contained 1.5% caryophyllene and 0.6% miscellaneous compounds. The xylene isomers and germacrene D were stored and handled under a nitrogen atmosphere. *n*-Pentane was obtained from Fisher Scientific (Fair Lawn, New Jersey).

TABLE 1. VOLATILE CONSTITUENTS OF SQUASH LEAF TRICHOMES

Compound name[a]	Percent of total[b]
Toluene	4.85
2-Heptanone	0.94
Xylene isomer	0.21
Xylene isomer	0.23
Limonene isomers	tr[c]
Germacrene D	0.95
Germacrene B	1.89
β-Bourbonene	0.67
Myristic acid	12.33
Palmitic acid	4.35
Dibutyl phthalate[d]	12.07
n-Tricosane	2.22
Stearic acid	48.77
n-Pentacosane	6.06

[a] Compounds are listed in order of their elution from the GC capillary column.
[b] Amount in percent based on GC peak area. Values are averages of two samples.
[c] Tr (trace) represents less than 0.2% of the volatile fraction based on area of GC peaks. In bioassays 0.1% peak area was used for (+)- and (−)-limonene in testing mixtures.
[d] Artifact; not natural product.

Statistics One-way ANOVAs were used to analyze the effects of sap and leaf volatiles on moth visitations and oviposition (Table 2A–E). One-way ANOVA was also used to analyze effects of sap and leaf volatiles on the oviposition index, which we defined as (oviposition/blank) $(\text{visitation/blank})^{-1}$ (Table 3). When this index is significantly larger than 1, treatment causes stimulation of oviposition.

Mean separations were performed by the least significant difference (LSD) multiple comparison test ($\alpha = 0.05$). These analyses were performed with the PROC GLM module of SAS software (SAS Institute, version 6.07).

Regressions of visitations versus time (Figure 1A and B) were calculated and plotted using the model: $Y = a + b\ (\ln x)$, with Freelance Graphics for Windows 2.0 (Lotus Development Corp.).

RESULTS

Identification of Volatiles. Thirteen naturally occurring compounds were identified (Table 1). Most abundant were myristic and palmitic acid, *n*-tricosane, stearic acid, and *n*-pentacosane. These acids and hydrocarbons accounted for 73.7% of the volatile matrix. The low-molecular-weight compounds toluene,

TABLE 2. RELATIVE NUMBER OF VISITATIONS AND AMOUNT OF OVIPOSITION ON TREATED ARTIFICIAL SITES BY FEMALE PICKLEWORM MOTHS[a]

Treatment	Visitations	Oviposition
A		
Sap[b]	59.7 a[e]	74.7 a
Whole-leaf volatiles[c]	23.0 b	17.0 b
Blank[d]	17.2 b	8.3 c
B		
Whole-leaf volatiles	25.3 a	30.4 a
(−)-Limonene	20.7 b	16.7 b
Blank	20.7 b	19.1 b
m-Xylene	19.0 bc	16.6 b
o-Xylene	19.0 bc	18.7 b
p-Xylene	18.9 bc	18.9 b
Toluene	18.0 bc	16.8 b
(+)-Limonene	16.7 cd	15.0 b
2-Heptanone	14.5 d	14.7 b
C		
Whole-leaf volatiles	42.1 a	42.3 a
Germacrene D	34.6 b	32.6 b
Blank	23.5 c	25.1 c
D		
Whole-leaf volatiles	39.9 a	48.8 a
Mix A[f]	37.2 a	32.6 b
Blank	23.0 b	18.7 c
E		
Whole-leaf volatiles	39.8 a	43.8 a
Mix B[g]	36.4 a	36.7 a
Blank	23.8 b	19.5 b

[a] All data are presented as percent of total visitations or oviposition within the 5 sets of experiments.
[b] Sap: aqueous liquid derived from fresh leaves. Applied to outside of oviposition pad, both solids and volatiles are perceived by moths.
[c] Only whole-leaf volatiles can be perceived by moths.
[d] Blank: pad did not receive application.
[e] Numbers followed by the same letter within a column and within an experiment (e.g., 2A) are not significantly different ($P = 0.05$).
[f] Mix A: mixture of volatiles as listed in B, and in ratios as listed in Table 1.
[g] Mix B: mixture of volatiles as listed in B and in addition germacrene D. Relative amounts as listed in Table 1.

2-heptanone, and two xylene isomers accounted for 6.2%, and the sesquiterpenes germacrene B and D and β-bourbonene contributed 3.5%. Limonene was present in trace amounts. Approximately 4.5% of components were unidentified.

Bioassays: Visitations and Oviposition. Aqueous leaf extract, referred to as sap, was tested against whole-leaf volatiles and blanks (no application). The

TABLE 3. STIMULATION OF OVIPOSITION BY PICKLEWORM MOTH ON TREATED ARTIFICIAL OVIPOSITION SITES.

Treatment	OI[a]	Treatment	OI
Leaf sap	2.8 a[b]	(+)-Limonene	1.2 bc
Whole-leaf volatiles	1.5 b	Mix A	1.1 bc
Mix B	1.3 bc	*p*-Xylene	1.1 bc
o-Xylene	1.2 bc	Germacrene D	1.0 bc
Toluene	1.2 bc	*m*-Xylene	1.0 bc
2-Heptanone	1.2 bc	(−)-Limonene	0.8 c

[a]Oviposition index: (oviposition/blank)(visitation/blank)$^{-1}$. When index is significantly larger than 1, treatment causes stimulation of oviposition.
[b]Numbers followed by the same letter are not significantly different ($P = 0.05$).

sap was most attractive and strongly stimulated oviposition (Tables 2A and 3). Whole-leaf volatiles caused visitations higher than the blank, but these increases were not significant. Oviposition, however, was significantly higher.

Volatile compounds, as found in the trichome droplets, were tested individually (Table 2B). Pads emanating whole-leaf volatiles were most attractive, and those treated with a single compound were visited as much or less than the blanks. (*R*)-(+)-Limonene and 2-heptanone were visited significantly less than blanks. Oviposition on pads treated with a single compound was not significantly different from blanks and all were significantly lower than for whole-leaf volatiles.

Germacrene D received more visits than blanks, but was not as attractive as the whole-leaf volatiles and oviposition followed the same pattern (Table 2C).

Pads that received mix A (mixture of volatiles as listed in Table 2B and in ratios as listed in Table 1) were visited no less than pads that emanated whole-leaf volatiles. Oviposition, however, was lower but still significantly higher than for blanks (Table 2D).

When a mixture of compounds, including germacrene (mix B; i.e., mix A plus germacrene D, ratios as in Table 1), was tested, both visitations and oviposition were not significantly different from whole leaf volatiles (Table 2E).

Five treatments (sap, whole-leaf volatiles, germacrene D, mix A, and mix B) caused visitations and oviposition significantly higher than blanks. In order to indicate that increased oviposition may not be due solely to increased visitation, the data were processed in such a manner that an estimate of stimulation of oviposition was obtained (see legends of Table 3). Data for leaf sap and whole-leaf volatiles indicate significant stimulation of oviposition (Table 3).

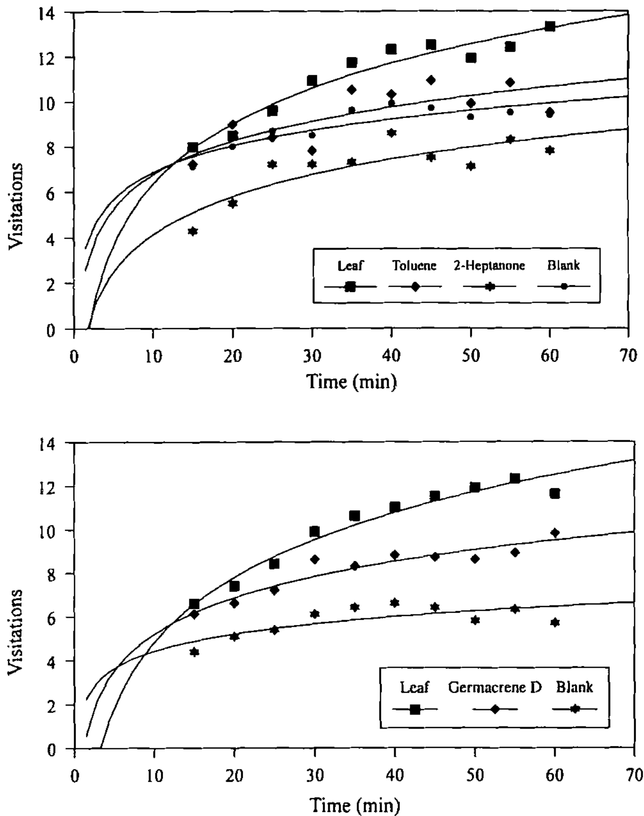

FIG. 1. Visitations of female pickleworm moths to artificial oviposition sites (treated as indicated) versus time. Visitations represent number of moths present on a site at a given time. Each datum point represents an average of 10 (A) or 16 (B) observations. (A) $y = a + b\,(\ln x)$—leaf: $a = -2.51$, $b = 3.85$, $R^2 = 0.94$; toluene: $a = 1.78$, $b = 2.16$, $R^2 = 0.58$; 2-heptanone: $a = 2.93$, $b = 1.71$, $R^2 = 0.77$. (B) $y = a + b\,(\ln x)$—leaf: $a = -5.03$, $b = 4.28$, $R^2 = 0.96$; germacrene D: $a = -0.31$, $b = 2.39$, $R^2 = 0.90$; blank: $a = 1.84$, $b = 1.12$, $R^2 = 0.55$.

DISCUSSION

The Cucurbitaceae have multicellular, uniseriate trichomes of varying length, and they may be glandular or nonglandular (Theobold et al., 1979). The leaves of yellow squash (*Cucurbita pepo* L. cv. Early Prolific Straightneck) have both large and small, thorn-shaped multicellular and uniseriate trichomes. The large trichomes occur on the petioles and large veins of the leaves. The

small trichomes, occurring on the small leaf veins, have the same shape as the large ones (personal observations). A large number of the small trichomes carry on the tip of their stalk, a globular membrane-bound droplet, which disengages upon slight touch. The trichomes, including the membrane-bound droplets, contain a colorless sticky liquid, which, upon drying and possible polymerization, becomes hard, white, and brittle.

In previous experiments it was shown that the contents of these trichomes increased pickleworm oviposition when applied to artificial sites treated with leaf extract from which all volatiles were removed (Peterson and Elsey, 1994).

Preliminary studies indicated that squash leaves were most attractive early in the morning and young leaves were more attractive than old ones. Gas chromatographic profiles showed that the low boiling compounds disappear during the course of a warm day (data not shown here). For these reasons, young leaves, approximately one-half of full size, were collected for GC-MS studies as well as for bioassays. In the field, most oviposition occurs during the early part of the night. In order to optimize these parameters, the day–night cycle was reversed for the insect colonies. Young leaves were collected in the field around 7:30 AM and used for bioassays starting at 8:00 AM, which coincided with the start of the scotoperiod of the insect colony. The colonies were frequently monitored for their oviposition activity, and bioassays were performed during peak periods. A flashlight covered with four layers of red cellophane was used for a brief time to count the number of female moths present on the pads. This interference did not visibly disturb the moths. Male moths rarely visited these pads, and even rarer was the presence of a mating couple; these were not counted. It has to be kept in mind that the visitation data were taken by counting the number of female moths present on a pad at a given time, and the data do not represent the actual number of landings. Since counts were made every 5 min, most ovipositing moths were counted several times during the observation period.

GC-MS. Of the 13 compounds identified, six were common components of epidermal waxes. These compounds were also found when isolated trichome droplets were extracted. This is to be expected since the epidermis is continuous across the trichomes (Esau, 1965; Uphof, 1962). Considering the ubiquitousness and very low volatility of these acids and hydrocarbons, no bioassays were performed with these compounds. The individual structural configurations of the isomers of xylene, and the absolute configuration of limonene were not determined; therefore, *o*-, *m*-, and *p*-xylene, (*R*)-(+)- and (*S*)-(−)-limonene were all bioassayed. Germacrene B and D, as well as β-bourbonene were identified by comparison of their mass spectra in the NIST MS data base and Kovat's indices on a DB-1 fused silica capillary column (Flath, 1993 personal communication). Germacrene D was also compared with authentic samples. Ger-

macrene B and β-bourbonene could not be obtained in adequate quantities for use in bioassays.

Bioassays. Single compounds were applied to the visitation/oviposition sites in amounts of 20 μl (compound–silicone oil, 1:9). Preliminary assays suggested that, within limits, the amounts applied did not affect the relative number of visitations. Large variations in concentrations are expected under natural conditions, where temperature, time of night, humidity, differential volatility of compounds, age of trichomes, and genetic (Dell and McComb, 1978) and intraspecific variations (Levin, 1973) are factors that influence absolute and relative concentrations. Due to the high volatility, the concentrations of volatiles around the oviposition pads decrease significantly during the observation period, a fact readily smelled by the experimenter. In order to evaluate these factors, visitations were plotted against exposure time (Figure 1A and 1B). In Figure 1A visitation data for whole-leaf volatiles, toluene, 2-heptanone, and blanks were used. All other compounds showed values between the whole-leaf volatiles and 2-heptanone and were not plotted to avoid cluttering of the graph. Similarly, the values for germacrene D were plotted (Figure 1B). In all cases, moth visitations increased, then leveled off, and there were no cases where visitations dropped off significantly toward the end of the observation period.

Since five treatments, i.e., sap, whole-leaf volatiles, germacrene D, mix A, and mix B, showed oviposition significantly higher than the blanks, it was relevant to know whether increased oviposition was due solely to increased visitation or if stimulation played a role as well. For all single experiments, relative visitation data (average visitation to treated pads/average visitation to nontreated pads) were compared with relative oviposition data (average oviposition on treated pads/average oviposition on non-treated pads). These numbers, presented in Table 3, were termed oviposition indices (OI). When these ratios (see legend of Table 3) are significantly larger than 1.0, stimulation of oviposition is indicated. The aqueous extract of squash leaves (sap), had the highest value (OI = 2.7). This was expected because this extract contains the nonvolatile oviposition stimulants (Peterson and Elsey, 1994). In addition, the index for whole-leaf volatiles (OI = 1.5) indicated that these treatments stimulated oviposition.

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POPULATION AND SEX DIFFERENCES IN ANTIPREDATOR RESPONSES OF BREEDING FATHEAD MINNOWS (*Pimephales promelas*) TO CHEMICAL STIMULI FROM GARTER SNAKES (*Thamnophis radix* and *T. sirtalis*)

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Abstract—We conducted a predator bite survey on a population of fathead minnows (*Pimephales promelas*) considered to be under substantial predation pressure by western plains garter snakes (*Thamnophis radix*). Scarring, due to failed predation attempts by garter snakes and crayfish (*Orconectes virilis*), was observed significantly more often in breeding males than in breeding females and nonbreeding minnows. Likely, territorial nest defense under the edges of rocks along the water's edge, a habitat occupied by crayfish and frequented by snakes, caused the breeding males to be differentially vulnerable to predation. Under controlled laboratory conditions, breeding males from this population exhibited an antipredator response to chemical stimuli from live snakes (*T. sirtalis* and *T. radix*) significantly more often than breeding female minnows from the same population and breeding minnows of both sexes from a population that was presumed to be under lower predation pressure from snakes.

Key Words—Differential vulnerability, differential predation, alarm substance, cost of reproduction, learned predator recognition, predator avoidance, fathead minnow, *Pimephales promelas*, garter snake, *Thamnophis radix*, *Thamnophis sirtalis*.

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INTRODUCTION

Differences in predation intensity frequently result in interpopulation differences in antipredator behavior. Generally, individuals from populations in which predators are common exhibit stronger antipredator responses than individuals from populations that experience lower levels of predation. Interpopulation differences in antipredator behavior have been documented in many taxa, including spiders (Riechert and Hedrick, 1990), salamanders (Ducey and Brodie, 1991), snakes (Herzog and Schwartz, 1990), deermice (Hirsch and Bolles, 1980), and fishes (Giles and Huntingford, 1984; Huntingford, 1982; Magurran and Pitcher, 1987; Magurran and Seghers, 1990; Seghers 1973, 1974; Mathis et al., 1993). In Trinidadian guppy (*Poecilia reticulata*) populations, for example, there are differences in attack cone avoidance (Magurran and Seghers, 1990) and school cohesiveness (Seghers, 1973, 1974). European minnows (*Phoxinus phoxinus*) from pike-sympatric populations increase shoaling (Magurran and Pitcher, 1987) and numbers of inspection visits, following exposure to live or model pike (*Esox lucius*), compared to pike-allopatric populations (Magurran and Pitcher, 1987). Similarly, pike-sympatric fathead minnows (*Pimephales promelas*) show a greater increase in cover use and greater decrease in overall activity levels than pike-allopatric populations following exposure to visual or chemical stimuli from pike (Mathis et al., 1993). Huntingford (1982) and Giles and Huntingford (1984) documented that populations of three-spined stickleback (*Gasterosteus aculeatus*) with high predation risk showed more extreme fright responses than populations from low-risk sites.

Differences in predation pressure have also resulted in intrapopulation sex differences in antipredator behavior and predation vulnerability. For example, Huntingford (1982) showed sex differences in antipredator responses in three-spined stickleback. Males were found to be bolder than females when encountering predators. Sex-specific predation due to differential vulnerability has been documented in various taxa, including birds (Sargeant, 1972), mammals (Bergerud, 1971), and fishes (Britton and Moser, 1982). Sargeant (1972) demonstrated that differential vulnerability associated with nesting caused female dabbling ducks to be selectively preyed upon by red foxes (*Vulpes vulpes*). Male caribou (*Rangifer tarandus*) calves, due to their greater tendency to wander away from the protection of the herd than females, are more often captured by lynx (*Lynx canadensis*) (Bergerud, 1971). Likewise, Britton and Moser (1982) demonstrated that female mosquito fish (*Gambusia affinis*) are more vulnerable to capture by grey herons (*Ardea cinerea*) because they are larger and more conspicuous than males.

Breeding male fathead minnows associate with defined nesting areas, which they defend for 10–30 days (McMillan, 1972; McMillan and Smith, 1974). At a pond located on the University of Saskatchewan campus, breeding male min-

nows defend territories under the edges of rocks in shallow water. This territoriality in shallow water likely results in an increase in the frequency of interaction between breeding male minnows and snakes when compared to the frequency of interaction between either breeding females or nonbreeding fish and snakes. The snakes inhabit the areas around the water's edge, foraging for minnows exclusively in the shallow water rocks (personal observations). Breeding females and nonbreeding fish are primarily pelagic and do not frequent the shallow water rocks. Thus, the males' territoriality likely makes them more vulnerable to predation by the snakes. In addition, males become aggressive towards predators during the breeding season (McMillan, 1972), which is appropriate on the basis of parental care, but dangerous to the individual "care-giver." Breeding males, for example, often attack a person's hand that comes too close to their nest (personal observation).

Garter snakes (*Thamnophis* spp.) eat a variety of prey organisms (Burghardt et al., 1988), although individuals frequently specialize on one prey type (Carpenter, 1952; Drummond, 1983). During several hours of observations at the campus pond we observed western plains garter snakes (*Thamnophis radix*) catch and eat five breeding male fathead minnows, but in no instances did we observe breeding female or nonbreeding fish being caught.

In this study we examine whether breeding activities of male minnows make them more vulnerable to snake predation. We conducted a predator bite survey, testing the hypothesis that if breeding males are at higher risk to snake predation, then they should show significantly more signs of snake attacks than breeding females and both sexes of nonbreeding fish. Predator-induced injuries from which fish have recovered have been documented in fathead minnows (Smith and Lemly, 1986), three-spined stickleback (Reimchen, 1988, 1992), and whitefish (*Coregonus nasus* and *C. clupeaformis*) (Reist et al., 1987). In response to the increased risk of snake predation associated with nest defense, males may have developed a stronger antipredator response to snakes than females since increased predator avoidance ability in risk-prone individuals increases their probability of survival (Holtby and Healey, 1990; Johnsson, 1993). In laboratory experiments, we compared the intensity of antipredator responses of male and female minnows to chemical stimuli from snakes. We predicted that breeding male minnows should exhibit a stronger antipredator response to the chemical stimuli than breeding females.

Magurran and Seghers (1990) demonstrated that populations of Trinidadian guppies skilled in dealing with one type of predator do not necessarily cope well in unfamiliar predation situations. Minnows from Pike Lake (a population originating from the same drainage basin as the campus pond population) are preyed upon heavily by pike (Mathis et al., 1993) but, because of differences in minnow spawning habitat between Pike Lake and the campus pond (personal observations), it is expected that the Pike Lake minnows are exposed to lower snake

predation pressure than the campus pond minnows. In the laboratory, we compared antipredator responses of breeding minnows from both the campus pond and Pike Lake to chemical stimuli from snakes, to test the hypothesis that males from the campus pond will show a more intense response to snake stimulus than males and females from the lake population.

METHODS AND MATERIALS

Predator Bite Survey

We used Gee's Improved Minnow Traps to capture 2609 minnows from the campus pond. They were transported to the laboratory and examined for evidence of predator-induced injuries. The minnows were maintained in three 160-liter tanks, and all uninjured fish were returned to the pond less than 24 hr after capture.

Prior to examination, we anesthetized the minnows with MS 222 (triciane methanesulfonate). Injuries were classified into three categories: (1) snake bites, which were symmetrical, rounded, rasplike lacerations or abrasions on both sides of the fish; (2) crayfish (*Orconectes virilis*) pinches, which were small, deep cuts on either side of the fish; and (3) other injuries, those that could not be classified in either of the aforementioned categories. For the purpose of statistical comparisons, we divided the minnows into three categories: (1) breeding males, (2) breeding females, and (3) other, nonbreeding fish not distinguishable as either sex on the basis of secondary sexual characteristics. We used a chi-square test (Siegel and Castellan, 1988) to compare the frequency of breeding male, breeding female, and nonbreeding fish with snake and crayfish bites.

Responses of Minnows to Chemical Stimuli from Garter Snakes

Collection and Maintenance. We collected fathead minnows from Pike Lake, an oxbow lake of the South Saskatchewan River in south-central Saskatchewan, and from the campus pond. We then transferred the minnows to the laboratory where they were maintained in separate 160-liter tanks at approximately 20°C on a 10:14 light–dark photoperiod and were fed daily with Nutrafin flake food.

Snake Stimulus Preparation. Prior to collecting the stimulus, two garter snakes [one western plains garter snake (*Thamnophis radix*), and one red-sided garter snake (*Thamnophis sirtalis*), both found locally] were each fed, twice a week, approximately equal volumes (range = 4–5 ml, measured by volumetric displacement in water) of previously frozen neonate mice (*Mus domesticus*). We fed the snakes a diet of mice, not fathead minnows, to ensure that alarm substance (AS) would not be ingested by the snakes. This eliminated the pos-

sibility that AS would label the predator as dangerous to naive prey (Mathis and Smith, 1993a,b). One glass bowl containing 900 ml of glass-distilled water was placed into a cage (50 × 30 × 25 cm) that contained both snakes. The snakes bathed and defecated in the water. After 72 hr, the water was removed, filtered through glass wool, pipetted into separate 12-ml polypropylene containers, and frozen at approximately −20°C. As a control stimulus, 12-ml units of glass-distilled water were frozen in separate polypropylene containers.

Testing Protocol. To test for population and sex differences in the responses of minnows to chemical stimuli from the snakes, we divided the minnows (all of which were in breeding condition) into four groups of 10 fish each: (1) campus pond males, (2) campus pond females, (3) Pike Lake males, and (4) Pike Lake females. We arbitrarily placed individual fish into separate Plexiglas acclimation tanks (45 × 45 × 20 cm). Fresh water passed through the acclimation tanks at a constant rate of approximately 250 ml/min, maintaining a constant depth of 4–5 cm. Each tank contained a floating circular shelter measuring 9 cm in diameter. The fish were maintained on a 10:14 light–dark photoperiod and were fed Nutrafin flakes daily. After a two-day acclimation period we transferred the minnows to test tanks. The flow rate in the test tanks was increased to approximately 500 ml/min. Acclimation and testing both occurred at a mean temperature of 19°C.

The test tanks were surrounded by an Opto-Varimex Aqua tracking meter (Columbus Instruments) which laid out a grid of light beams throughout the tanks. A microcomputer scanned the light beam grid for breaks at a frequency of 8 pulses/sec. Lemly and Smith (1986, 1987) described this testing apparatus in detail. Our system differed only in that outflowing water was discarded rather than recirculated. The computer recorded two measures of activity that typically decrease during a fright reaction in fathead minnows: (1) distance travelled (centimeters) and (2) number of stereotypic movements (Lawrence and Smith, 1989; Mathis et al., 1993; Chivers and Smith, 1993). A fright response in fathead minnows includes freezing and increased use of shelter, which the Opto-Varimex apparatus interprets as a decrease in distance traveled and number of stereotypic movements. By decreasing distance traveled and number of stereotypic movements, the minnows likely become less visible to visually oriented predators.

An observer stationed in an adjacent room, to avoid disturbing the test fish, injected chemical stimuli into inflowing water lines that passed through the observation room before entering the test tanks. We conducted experimental observations between 0800 and 1000 hr using control water. Trials lasted for 16 min, with 12 ml of control water stimulus being injected after 8 min. Injection of the test stimulus took approximately 10 sec. A second set of observations, on the same minnows, took place the same day between 1030 and 1230 hr using 12 ml of the snake stimulus. The procedure for injection was the same as for

the water control. The minimum time between tests for an individual fish was at least 150 min.

The minnows' responses were quantified using the two measures recorded by the computer. The significance of the minnows' responses was determined by using a Wilcoxon-Mann-Whitney test (*Wx*; Siegel and Castellan, 1988) for each of the four groups of minnows. The two different stimuli were tested independently.

RESULTS

Predator Bite Survey. Out of 2609 campus pond minnows examined, 30 (1.15%) showed scarring from some type of predation attempt. Fourteen minnows were found with snake bite scarring, 13 minnows were found with crayfish pinches, and three minnows were found with scarring unlike those in the aforementioned categories (Table 1). Male minnows from the campus pond showed significantly more snake- and crayfish-induced injuries than females (χ^2 = 11.565, $df = 1$, $P < 0.01$; $\chi^2 = 7.07$, $df = 1$, $P < 0.01$, for snake and crayfish, respectively) and nonbreeding minnows ($\chi^2 = 47.56$, $df = 1$, $P < 0.001$; $\chi^2 = 22.75$, $df = 1$, $P < 0.001$, for snake and crayfish, respectively). These results suggest that breeding male minnows are more vulnerable to snake and crayfish predation than breeding female and nonbreeding minnows.

Responses of Minnows to Chemical Stimuli from Garter Snakes. In response to snake water, breeding male minnows from the campus pond decreased their distance traveled ($Wx = 65$, $m = 10$, $n = 10$, $P < 0.003$; Figure 1) and numbers of stereotypic movements ($Wx = 63$, $m = 10$, $n = 10$, $P < 0.002$; Figure 2) significantly more compared to exposure to the water control. In contrast, males from the Pike Lake population and females from both popula-

TABLE 1. NUMBER AND PERCENT OF BREEDING MALE, BREEDING FEMALE AND NONBREEDING MINNOWS FROM THE CAMPUS POND POPULATION WITH SNAKE- AND CRAYFISH-INDUCED INJURIES

	Minnows with snake bites		Minnows with Crayfish pinches	
	N	%	N	%
Breeding males	13/417	3.12	9/417	2.15
Breeding females	1/519	0.19	2/519	0.39
Nonbreeding fish	0/1673	0.00	2/1673	0.12
TOTAL	14/2609	0.54	13/2609	0.50%

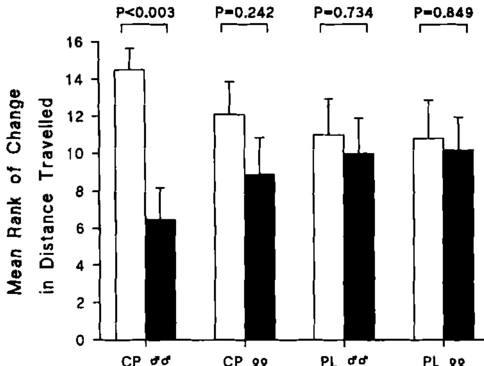

FIG. 1. Mean rank (±SE) of change in total distance traveled (cm) by campus pond (CP) male minnows, CP females, Pike Lake (PL) male minnows, and PL females following exposure to distilled water control (open bars) and chemical stimuli from garter snakes (solid bars).

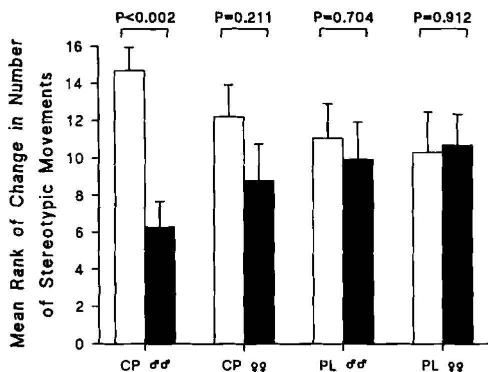

FIG. 2. Mean rank (±SE) of number of stereotypic movements by campus pond (CP) male minnows, CP females, Pike Lake (PL) male minnows, and PL females following exposure to distilled water control (open bars) and chemical stimuli from garter snakes (solid bars).

tions showed no significant decrease in their total distance travelled (cm) ($Wx = 100$, $m = 10$, $n = 10$, $P = 0.734$; $Wx = 102$, $m = 10$, $n = 10$, $P = 0.849$; $Wx = 89$, $m = 10$, $n = 10$, $P = 0.242$, for Pike Lake males, Pike Lake females, and campus pond females, respectively; Figure 1) following exposure to chemical stimuli from snakes compared to exposure to the water control. Likewise, males from the Pike Lake population and females from both

populations showed no significant decrease in number of stereotypic movements ($Wx = 99.5$, $m = 10$, $n = 10$, $P = 0.704$; $Wx = 103$, $m = 10$, $n = 10$, $P = 0.912$; $Wx = 88$, $m = 10$, $n = 10$, $P = 0.211$, for Pike Lake males, Pike Lake females, and campus pond females, respectively; Figure 2) following exposure to chemical stimuli from snakes compared to exposure to the water control.

DISCUSSION

The results of this study suggest that breeding male minnows from the campus pond are more vulnerable to snake predation than breeding females and nonbreeding minnows. This difference likely results from a higher encounter frequency with snakes while defending breeding territories in shallow water. Our results also provide evidence that breeding male minnows are more vulnerable to crayfish predation, probably for the same reason. Such results indicate the cost of reproduction incurred by the individual parent responsible for providing prenatal care, as it may reduce the probability of the parent surviving to future breeding seasons (Gross and Sargent, 1985).

The use of two species of garter snakes in the preparation of the stimulus was not intentional; one snake was initially misidentified. Nonetheless, breeding males from the campus pond did recognize and display appropriate antipredator behavior to the combined chemical stimuli from the snakes, while female campus pond minnows and both male and female Pike Lake minnows did not. From our results we do not know whether the male campus pond minnows' response was a general response to garter snake stimulus or a specific response to western plains garter snake stimulus. Nevertheless, our results demonstrate intra- and interpopulation differences in antipredator behavior.

Our experimental design did not allow us to empirically test whether genetic population differences or learning was responsible for the observed interpopulation differences in ability to recognize snakes as predators. The two minnow populations originated from the same drainage basin and have been separated for only 35 years. This length of time may not be sufficient to develop genetic population differences in ability to recognize snakes. The two populations have not developed genetic population differences in their ability to recognize another predator, the pike (Chivers and Smith, 1994b; unpublished data). It seems likely that learning and not a genetic population difference is responsible for the observed difference in antipredator behavior.

Several mechanisms of learning predator recognition have been demonstrated for fathead minnows. Cyprinid fishes learn to recognize unfamiliar predators when alarm substance, a pheromone released by mechanical damage to epidermal alarm substance cells (ASCs) (reviewed in Smith, 1992), is presented

in conjunction with chemical (Göz, 1941; Magurran, 1989; Chivers and Smith, 1994a) or visual (Chivers and Smith, 1994b) stimuli from an unfamiliar predator. Alarm substance ingested by a predator may chemically label the predator as being dangerous to naive prey (Mathis and Smith, 1993a,b). These mechanisms may not be responsible for the acquisition of predator recognition in the breeding male minnows from the campus pond as ASCs are reduced or eliminated in breeding male fathead minnows (Smith, 1973; Smith and Murphy, 1974). Scarring due to failed predation attempts indicates that a portion of the males attacked by the snakes survive the encounter. These individuals, through the encounter, should learn that snakes are dangerous predators. Since breeding territories are often located in close proximity to one another (McMillan, 1972), other breeding males may witness the snake attack and could learn to recognize garter snakes as dangerous. Pike-naive fathead minnows learn to respond appropriately to pike by observing the reaction of pike-experienced minnows (Chivers and Smith, 1994c). By having a few snake-experienced individuals within visual range, snake-naive males may also learn to regard snakes as potential predators by watching the antipredator reactions of the experienced minnows.

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ISOLATION OF APIGENINIDIN FROM LEAF SHEATHS OF *Sorghum caudatum*

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Abstract—A stable 3-deoxyanthocyanidin, apigeninidin chloride, a potential fungal growth inhibitor and a useful dye, has been isolated with a high yield (10% in dried material) as the major pigment in the sheaths of *Sorghum caudatum*.

Key Words—Dye, 3-deoxanthocyanidin, apigeninidin, *Sorghum caudatum*, fungus inhibitor.

INTRODUCTION

In western and northern Africa, *Sorghum caudatum*, known for its pest resistance quality, is grown mainly for its dyestuff (Sereme et al., 1993a). This paper deals with the isolation of a red dye, apigeninidin, a potential fungal growth inhibitor (Schutt and Netzly, 1991), with a particularly high yield (10% w/w) from the leaf sheaths of *Sorghum caudatum*.

METHODS AND MATERIALS

Plant Material. Sorghum caudatum, variety Monome Kaya, was grown in the 1989 season (August 1–December 10) at the University of Ouagadougou Experimental Station (Burkina Faso). Sheaths of *Sorghum caudatum* were col-

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lected postmaturity (132 days after planting) and air dried before extraction of apigeninidin.

Extraction and Isolation of Apigeninidin. One gram of air-dried and chopped sheaths of *Sorghum caudatum* was extracted with 40 ml of ethanol/HCl (0.1% w/w) at room temperature (35°C) for 3 hr. The extract was concentrated in vacuum to 3 ml. Addition of 0.3 ml of 2 N aq. HCl to the residue gave a brownish precipitate. Apigeninidin was separated by centrifugation and dried.

Characterization of Apigeninidin. MS was performed on a Hewlett-Packard 5985 B(GC-MS) spectrometer. The ^1H NMR spectrum was obtained on a Brucker WM-400 spectrometer with tetramethylsilane as internal standard ($\delta = 0$). UV spectra were recorded on a Perkin-Elmer spectrophometer. TLC was performed on precoated silica gel $60F_{254}$ plates (0.2 mm thickness, 20 × 20 cm) using ethyl acetate–HCO_2H—2N HCl, 85:9:6.

RESULTS AND DISCUSSION

Extraction. The yield of apigeninidin extraction was 10%. This indicates that the main pigment of leaf sheaths of *Sorghum caudatum* is apigeninidin (Sereme et al., 1993a) and is easily extractable.

Characterization. Data of isolated apigeninidin (A): MS *m/e*: 255(M^+). ^1H NMR (0.1% DCl/CD_3OD) showed peaks at δ 6.63 (d, 1H, J = 1.15, C_6—H); 6.91 (d, 1H, J = 1.15, C_8—H); 7.04 (d, 2H, J = 9), $C_{3'}$—H & $C_{5'}$—H); 8.01 (d, 1H, J = 8.6, C_3—H); 8.27 (d, 2H, J = 9, $C_{2'}$—H & $C_{6'}$—H); 9.04 (d, 1H, J = 8.6, C_4—H). UV λ_{max} (0.1% HCl/MeOH)nm(ϵ): 205.2 (30289), 278.5 (15463), 325.3 (4349), 480.8 (27737). TLC (using ethyl acetate–HCO_2H—2N HCl, 85:9:6 as solvent) gave one spot: R_f = 0.5.

A

Data of isolated apigeninidin chloride (A) were confirmed by direct comparison of UV, visible, NMR, and mass spectrometry of an authentic sample (Sweeny and Iacobucci, 1981) from the Coca Cola Company (Atlanta) and with the literature (Harborne, 1966; Nilsson, 1973; Brouillard et al., 1973; Baranac and Amie, 1990). Our findings are well in accordance with the use of *Sorghum*

caudatum as a source of red dye (Sereme et al., 1993a,b) and its pest-resistance quality (Schutt and Netzly, 1991).

Sorghum caudatum is a natural source of apigeninidin and can be a reference for apigeninidin assay in plants.

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Attacus atlas CATERPILLARS (LEP., SATURNIIDAE) SPRAY AN IRRITANT SECRETION FROM DEFENSIVE GLANDS

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Abstract—The ability of *Attacus atlas* caterpillars to spray a defensive secretion seems to be due to the fine structure of the integumental glands that produce it. The giant gland cells are fixed to stable cuticular rings surrounding the gland openings and tightly closed by cuticular lids. Probably by increasing hemolymph pressure, the lids are blasted off and the secretion spouts out. The fluid contains several aromatics, biogenic amines (e.g., acetylcholine, histamine), glycerol, and trehalose and exhibits tyrosinase activity. Deterrent effects of caterpillar secretion and hemolymph on predatory ants could be shown. Presumably the spraying process serves to apply the secretion to sensitive sites of vertebrate target organisms.

Key Words—*Attacus atlas*, biogenic amines, chemical defense, defensive glands of caterpillars, histamine, Lepidoptera, Saturniidae.

INTRODUCTION

Members of the Indo-Australian genus *Attacus* are prominent in the lepidopteran family Saturniidae (= Attacidae) due to their enormous wing size (Peigler, 1989) and their impressive, large caterpillars. These larvae bear series of integumental outgrowths, so-called scoli, on their surface (Figure 1) that occur in two types on last-instar caterpillars. Long, fleshy tubercles in the middle of the body are looked upon as the producers of a white wax layer covering the surface of the larvae (Jones et al., 1982). Flat, hard, and wartlike scoli on thoracic and last abdominal segments excrete a strong-smelling fluid in response to intense irri-

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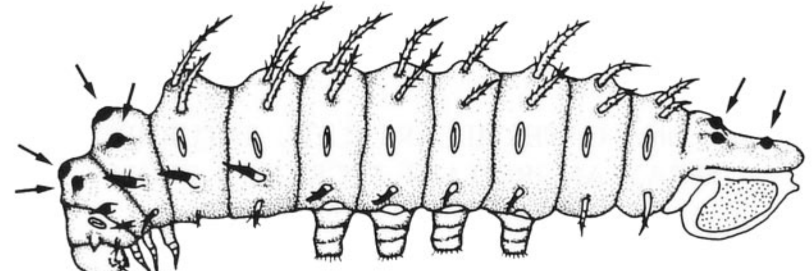

FIG. 1. L6 caterpillar of *Attacus atlas*. The secretion-spraying scoli on thoracic and last abdominal segments are symbolized as black spots and marked by arrows. Between them are elongated nonsecreting tubercles covered with a white wax layer.

tation of the caterpillars, either as a droplet or as a fine stream that can be sprayed up to 50 cm (Paukstadt and Paukstadt, 1991).

It was our aim to understand more of the function and the biological significance of this secretion emission. Therefore, we investigated the functional morphology of the scoli of last-instar Atlas moth caterpillars, *Attacus atlas* (L.). Furthermore, an inventory of the chemical compounds in gland secretion and caterpillar hemolymph was taken and the results compared to the secondary metabolites already found in other saturniid species (Deml and Dettner, 1993). Finally, biological tests with predatory model ant species were performed in the laboratory and in the field.

METHODS AND MATERIALS

Living specimens of *Attacus atlas* from Thailand were obtained commercially and the larvae (ex pupa) were fed with sloe (*Prunus spinosa*) for their whole lives. The animals were reared in plastic boxes at 22°C. Last-instar caterpillars were killed by freezing and stored at −20°C until used. Secretion was obtained by pressing living caterpillars strongly with the fingers or with forceps, or by defrosting the frozen larvae and pressing the scoli and underlying integument gently with forceps. The secretion that flowed out was sucked up with a glass capillary. To sample the hemolymph, a small cut was made with a scalpel in the integument beyond the scoli, and the hemolymph droplets sucked up with a glass capillary.

Scoli were excised from the caterpillar integument, macerated in 5% KOH for 24 hr, dried in acetone, and prepared for scanning electron microscopy (SEM) by critical point drying and sputter coating with gold. Droplets of secretion on glass splinters and also some scoli were not macerated; they were dried

in a vacuum desiccator above P_2O_5 for three days and sputter coated. SEM was carried out with a Stereoscan 90 scanning electron microscope (Cambridge Instruments).

Caterpillar body fluids were chemically analyzed by transferring them from the capillary onto a Solid Injektor SI 1 (SGE) syringe and injecting into a Carlo Erba GC 6000 Vega gas chromatograph containing a 14-m glass capillary column FS-OV-1701 (Chrompack) coupled to a Finnigan-MAT ITD mass spectrometer. Temperature program: 50°C to 260°C (10°C/min), 10 min isotemperature, 260°C to 280°C (5°C/min), 5 min isotemperature; carrier gas: helium. Electron impact ionization (EI) and chemical ionization (CI, reactant gas methanol) mass spectra were obtained in total ion chromatograms and compared to mass spectral data of the NBS library and the mass spectra registries of Stenhagen et al. (1974) and McLafferty and Stauffer (1989). For confirmation of supposed compounds, authentic chemicals were injected and retention times and mass spectra compared. For detecting nonvolatile amines and polyalcohols, the body fluids were acetylated by reaction with a 1:1 mixture (v/v) of acetic anhydride and pyridine for 4 hr under reflux conditions; subsequently the reaction mixture was injected directly into the GC. Authentic substances were treated in the same way.

On account of the sticky characteristics of the secretion, a test for protein was made by dropping secretion on Combur-Test strips (Boehringer, Mannheim). Because the secretion was observed to darken within a few minutes of exposure to air, a tyrosinase assay was performed: the secretion was mixed 1:1 (v/v) with a saturated solution of *p*-cresol in phosphate buffer (pH 7.0) and color development followed (Blaich, 1978).

Effects of hemolymph on ants were demonstrated in a feeding deterrence test: 40 workers of *Lasius niger* (L.) were collected from each of five field nests and placed in Petri dishes (13.7 cm ID), 20 to a dish (total ants, $N = 200$). A glass slide was added in the middle of the dish and a filter paper (12.5 cm diameter) placed on it. Ants were fed only once soon after capture with honey-water; subsequently they were starved and given only water. All ants were tested at the same time in a darkened room. Half an hour before testing, the filter paper and the slide were taken out of the dish. Then a 5-μl drop of a control suspension—triturated entrails of *Tenebrio molitor* larvae (meal worms) diluted 1:3 with water—was placed on one half of the slide. A 5-μl drop of a mixture (1:1, v/v) of this hydrous mealworm suspension and of the *A. atlas* hemolymph was pipetted onto the other half. The trial was started by returning the slide to the middle of the Petri dish. Ants feeding on the drops were counted at 1-min intervals for 10 min. The orientation of the glass slides was changed regularly from dish to dish to avoid side preference of the ants. Statistical evaluation was performed for every minute using the Wilcoxon matched pairs signed rank test for the two-tailed case. Field observations on confrontation of *Formica pratensis*

Retz workers with *A. atlas* secretion and hemolymph were made to supplement the laboratory tests.

RESULTS

The secretion-spraying scoli on meso- and metathorax and abdominal segments 9 and 10 of *A. atlas* caterpillars look like warty, flatly vaulted domes from outside. Nässig (1989) named the gland "Spritzkuppelscolus" ("spraying dome scolus"). The surface of these scoli exhibits several "pores" on low magnification. SEM photographs of these scoli for the first time show that the pores represent openings of the scolus (Figure 2A and B), which are normally closed by round, lidlike structures with a rough surface (Figure 2B above, 2C). These opercula are surrounded by a broad cuticular ring in every case. Some lids were lost or bent up accidentally or intentionally during dissection (Figure 2B below, 2C), revealing inside the opening either a folded membrane (Figure 2C) or a material with a relatively smooth surface. Comparison of the rough lid surface (Figure 2E) with dried, smooth scolus secretion droplets (Figure 2F) definitely established that the opercula do not consist simply of dried secretion but indeed represent specific closures of the gland openings. That the gland secretion is delivered out of these openings is indicated by the clots of smooth material found in some of these pores.

Inside views of macerated scoli showed several cuticular sacs, each of which hangs under one scolus opening and encloses the cuticular ring surrounding the opening (Figure 2D). These sacs seem to represent internal reservoirs of individual gland cells as could be seen in the scoli of other saturniids (Deml and Dettner, 1993). In addition, instead of these sacs giant hyaline structures hanging from the scolus roof can be discerned in nonmacerated preparations; these formations might be the secretion-producing gland cells that apparently reach enormous dimensions of several millimeters. Furthermore, as in *Eudia pavonia* (Deml and Dettner, 1990), the inner surface of *A. atlas* scoli possesses striking ridges between the gland openings, which presumably represent demarcations or attachments of the gland cells. Finally, in nonmacerated preparations, several thin threads or cords were detected that extend from the gland cells to the inner scolus walls and ramify at their ends.

Gland secretion of *A. atlas* caterpillars possesses a typical odor, which is strongly reminiscent of the scolus secretion of the saturniid *Eupackardia calleta*. GC-MS analyses of *A. atlas* L6 caterpillar secretion and hemolymph (Table 1) revealed distinct, quantitative, and qualitative chemical differences between the two fluids. Hemolymph and gland secretion contain many aromatic compounds together with other substances, such as the main compounds glycerol and trehalose. Several substances (especially trace constituents) could be found in only

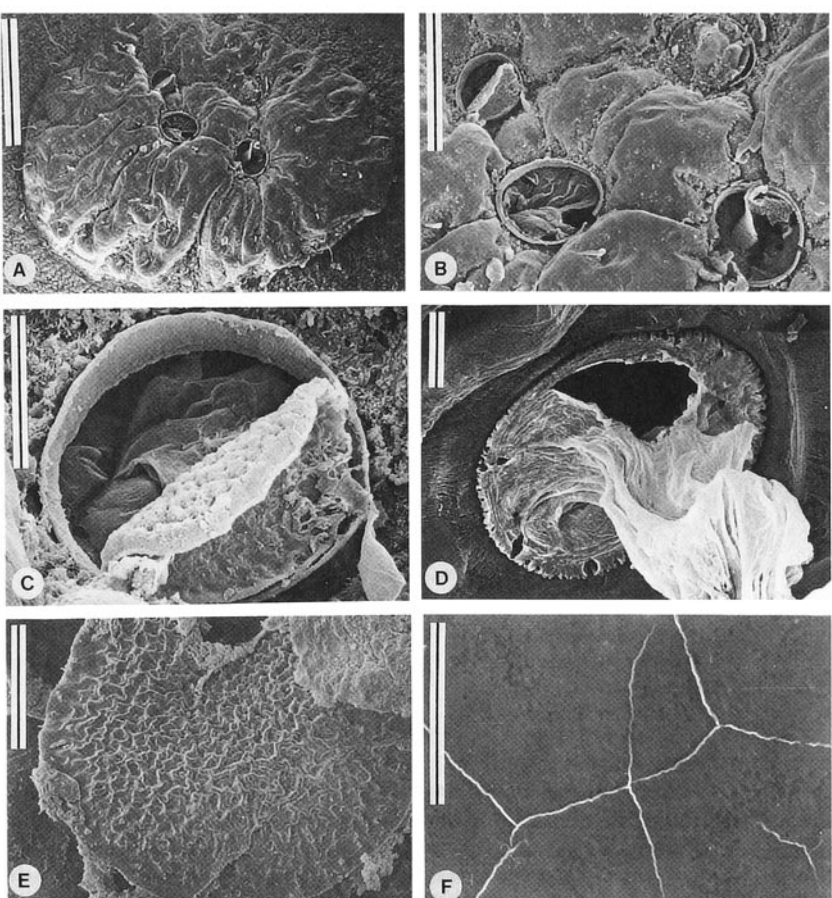

FIG. 2. SEM views of "spraying dome scoli" of *Attacus atlas* L6 caterpillars. (A) Surface view of a whole scolus. (B) Magnified detail of (A) showing four gland openings, two with lids (above). (C) Individual gland opening with surrounding cuticular ring, lid (bent up), and cuticular reservoir (in the interior). (D) Inside view after maceration of a scolus showing the attachment site of an internal gland cell reservoir. The reservoir surrounds the cuticular ring of a gland opening and extends to the lower right corner. (E) Surface of a lid (operculum). (F) Surface of a gland secretion droplet dried on a glass splinter. The droplet is streaked with protein fibrils. Scales: 500 μm (A), 200 μm (B), 50 μm (C–F).

TABLE 1. COMPOUNDS FROM CRUDE AND DERIVATIZED SAMPLES OF HEMOLYMPH (HL) AND GLAND SECRETION (GS) OF *Attacus atlas* L6 CATERPILLARS[a]

Compound [No.]	HL	GS
Phenol [1]	+	+
3,5-Dimethylphenol	?	?
Hydroquinone	?	?
Benzaldehyde	−	?
Phenylacetaldehyde [2]	+	+
Benzonitrile (Cyanobenzene) [3]	−	+
2-Phenetidine [4]	−	+
Dopa	−	−
Dopamine [5]	?	+
Norepinephrine (Noradrenalin) [6]	+	−
Epinephrine (Adrenalin) [7]	−	+
Histamine [8]	−	++
Coumarin [9]	−	+
Pyrazine [10]	−	+
Nicotinamide	?	−
Trehalose	+++	+++
Glycerol [11]	+++	+++
Acetylcholine [12]	−	++
2-(Dimethylamino)ethyl acetate [13]	−	++
2-(Methylamino)ethyl acetate	−	−
2-Aminoethyl acetate	−	?
Choline [14]	+	++
2-(Dimethylamino)ethanol [15]	++	++
2-(Methylamino)ethanol	?	?
2-Aminoethanol	−	?
4-Aminobutyric acid (GABA) [16]	++	++

[a] Semiquantitative data (+/−) are each based on peak areas of 3-5 GC-MS total ion chromatograms (+++ = main compound, ++ = minor compound, + = trace, − = not detectable, ? = trace amounts with corresponding retention times but only incomplete EI mass spectra as compared with authentic chemicals).

some of the individuals examined or were present in all individuals in varying amounts.

For interpretation of mass spectra, mainly the work of Budzikiewicz et al. (1967) was used. Phenylacetaldehyde [2] (M^+ 120) is characterized by a base peak at *m/z* 91 (loss of CHO) (Figure 3). Benzonitrile [3] (M^+ 103) could be identified especially due to its typical fragment at *m/z* 76 (M−HCN). 2-Phenetidine [4] (M^+ 137) revealed main fragments at *m/z* 109, 108 ($M-C_2H_5$), and 80 (further loss of −CO to create the pyridinium cation).

FIG. 3. Compounds detected by GC-MS analyses of the secretion and/or hemolymph of *Attacus atlas* L6 caterpillars. 1 = phenol, 2 = phenylacetaldehyde, 3 = benzonitrile (cyanobenzene), 4 = *o*-ethoxyaniline (2-phenetidine), 5 = 3,4-dihydroxyphenylethylamine (dopamine), 6 = norepinephrine (noradrenalin), 7 = epinephrine (adrenalin), 8 = histamine, 9 = coumarin, 10 = 1,4-diazine (pyrazine), 11 = glycerol, 12 = acetylcholine, 13 = 2-(dimethylamino)ethyl acetate, 14 = choline, 15 = 2-(dimethylamino)ethanol, 16 = 4-aminobutyric acid (GABA).

From the acetylated catecholamines, an M+1 peak (protonated amino group) could be found only in dopamine [5]. Due to a release of acetyl functions from the acetylated amino and hydroxy groups, dopamine further produced several consecutive fragments with intervals of 42 amu, and a corresponding base peak at *m/z* 43; additional typical fragments were at *m/z* 123 and at *m/z* 136. Acetylated norepinephrine [6] and epinephrine [7] were characterized by M−17 fragments (release of H_2O from M+1); consecutive loss of 43 amu started from M−17. Completely acetylated histamine [8] gave an M+1 peak (*m/z* 238) and

fragments at *m/z* 196, 154, and 110 for release of acetyl residues (*m/z* 43; base peak); furthermore, it produces characteristic fragments at *m/z* 136, 94, and 82. The trivalent alcohol glycerol [11] exhibited an M+1 peak (*m/z* 93) and produced typical fragments at *m/z* 75 (loss of H_2O), 61 ($M-CH_2OH$) and 43 (further release of H_2O; base peak). Free acetylcholine [12] and choline [14] produce no mass spectra because both compounds decompose within the ion source chamber of the mass spectrometer or in the GC injection system, to produce tertiary amines by *N*-demethylation (Johnston et al., 1968). Both amines could be determined indirectly by recording mass spectra of their typical decomposition products, which could at the same time represent true caterpillar compounds, however. The simultaneous presence of both quaternary amines and their demethylated counterparts could be verified by the broad peaks of choline and acetylcholine in the total ion current chromatogram on which are superimposed higher, narrow peaks that correspond to (dimethylamino)ethanol and (dimethylamino)ethyl acetate; in addition, decomposition products revealed no M^+ peaks. The tertiary choline precursor 2-(dimethylamino)ethanol [15] gave an M+1 peak, as is typical for many amines in biological materials and produced a distinct M−17 fragment (loss of H_2O). The *m/z* 58 (base peak) is probably due to the formation of $(CH_3)_2N^+{=}CH_2$ and the intensive fragment at *m/z* 44 might correspond to an ethanol residue. 2-(Dimethylamino)ethyl acetate [13] (acetylcholine precursor) gave a peak for M+1 and the following important fragments: *m/z* 87, 72, 58 (base peak) and 43.

Amounts of catecholamines in *A. atlas* caterpillars range as follows: hemolymph: dopamine <5–24 ng/μl, norepinephrine <20–100 ng/μl; and secretion: dopamine 30–60 ng/μl, epinephrine 120–150 ng/μl. Additionally, histamine is present in a concentration of 120–150 ng/μl in the secretion. Other common biogenic amines such as serotonin (5-hydroxytryptamine), octopamine, tyramine, epinine, and ephedrine could not be found in *A. atlas* L6 caterpillars. Acetylcholine could also be detected in the underivatized secretion of an *A. atlas* L3 caterpillar aside from an unidentified, long-chain alcohol.

In *A. atlas* L6 gland secretion (pH range 6–7), protein was detected by a positive reaction of the test strips. In the tyrosinase assay of the secretion, the reaction mixture turned red, which indicates tyrosinase activity and gives evidence of the formation of melanin (darkening of secretion) as well as of the presence of tyrosine in the body fluid.

The results of the ant feeding deterrence test with *Lasius niger* indicate a highly significant ($\alpha \leq 0.01$) deterrent effect of *A. atlas* hemolymph 3 and 4 min after the beginning of the test. The remaining counts showed no significant difference between the number of ants feeding on the sample or on the control but generally less ants were counted at the sample. This weak repellent effect approximately corresponds to the results of a feeding test with *Saturnia pyri* hemolymph (Deml and Dettner, 1993). In the field, defrosted L6 caterpillars of

A. atlas were placed beside an ant trail of the large *Formica pratensis*. If the defensive secretion had previously been removed from the cut caterpillars, the *F. pratensis* workers showed no aversion and licked up the hemolymph that had flowed out, but if squeezed-out secretion was allowed to evaporate on the caterpillars, the larvae were ignored by the ants for up to 3 min. When most secretion droplets had dried, *F. pratensis* workers climbed onto the larvae and anchored themselves by biting into the scoli and integumental wrinkles. Then some ants left the caterpillar again, wiped their mandibles in the sand and showed cleansing behavior, but in comparison to other saturniid caterpillars tested against *F. pratensis* (Deml and Dettner, 1993), *A. atlas* showed a relatively weak repellent effect towards these ants.

DISCUSSION

Many caterpillars of the Saturniidae possess special dermal glands whose phenotypes vary widely within this lepidopteran family (see Nässig, 1989). The type ''Spritzkuppelscolus'' of *Attacus atlas* caterpillars shows several striking morphological similarities to the type ''Sternwarzen'' (''star warts''; Haffer, 1921), i.e., globular scoli with large, hollow bristles as found in other saturniids (Deml and Dettner, 1990, 1993). Several relatively large reservoirs are located inside the scolus, each of which constitutes the interior of one unusually big gland cell. Therefore gland cells of both scolus types belong to the class I gland cells (Noirot and Quennedey, 1974, 1991) that have been found frequently in the pheromone glands of female Lepidoptera. Interiorly arranged cuticular ridges between the gland cells could be found in *A. atlas* scoli just as inside the ''Sternwarzen'' of *Eudia pavonia*. This interior structural identity of both scolus types as well as the ontogenetic emergence of *A. atlas* ''Spritzkuppelscoli'' from ''Sternwarzen'' (Nässig, 1983, 1989; Paukstadt and Paukstadt, 1991) point at a common phylogenetic origin of both types of glands. For this reason, a homology of the bristles (''Sternwarzen'') and the round lids (''Spritzkuppelscoli'') may be postulated.

Frequently *A. atlas* caterpillars discharge their gland secretion as a droplet, but it may also be sprayed up to relatively great distances, about 20–50 cm (Nässig, 1983; Paukstadt and Paukstadt, 1991; our own observations). This property may be attributed to the fine structure of the scoli: the external lids are strongly fixed to the gland openings, which are interiorly surrounded by a thick cuticular ring. The cuticle of the whole scolus is thicker than that of the soft larval integument. In some circumstances a relatively high internal body pressure must be applied via the hemolymph so that the lid can be blasted off; then secretion shoots out of the orifice at a high speed and is sprayed up to 50 cm. The lids may or may not be pulled away, whereas remnants of the secretion

may adhere in the gland opening and harden to clots with a smooth surface; the clots probably mainly consist of protein, but also contain other nonvolatile compounds (e.g., sugars, salts). This clogging of the openings could well explain why individual scoli can deliver secretion only once during each instar after a previous molting (Nässig, 1983; our observations). The tight internal fastening of the reservoirs to the rings in the orifices can be regarded as additional evidence of forceful blast processes. The gland cell retaining cords also observed could prevent the gland cells being torn out of the scoli.

Eisner (1970) assumed that the glands of insects that spray a defensive secretion are sometimes derived from originally stinging structures. As an example, he cites vespid wasps that spray their histamine-containing secretion, which is normally injected. Thus the topically irritant histamine would be applied to the eyes or other sensitive sites of vertebrates. This example seems very appropriate to the *A. atlas* glands, which presumably originate from the stinging "Sternwarzen" and, again, contain histamine. In the Saturniidae, histamine is also a compound in the irritating bristles of *Dirphia* spp. (probably together with irritant proteins; Valle et al., 1954) and in the stinging spines of *Automeris coresus* (together with acetylcholine; Pavan and Dazzini, 1976). In *A. atlas* L6 caterpillar secretion an expanded, synergistic interplay of many compounds seems possible: apart from histamine and acetylcholine, the secretion contains several additional, low-molecular-weight compounds with biological activity, and also proteins having possibly irritant effects.

Jones et al. (1982) found no volatile substances in the gland secretion of *A. atlas* caterpillars by means of gas chromatography. The *A. atlas* compounds detected now (Figure 3) range from active agents like neurotransmitters (e.g., acetylcholine, which is already present in the third-larval instar) to auxiliary substances like glycerol that might serve as solvent (Deml and Dettner, 1993) and assist other compounds to penetrate the skin of target organisms. Considering this diversity of chemicals, aside from the tyrosinase activity, other enzymes necessary for the anabolism of exclusive secretion compounds must be postulated in the glands.

Several *A. atlas* caterpillar compounds have also been detected in other saturniid caterpillars with "Sternwarzen" (Deml and Dettner, 1993) where they produce distinct biological effects. For example, benzonitrile is able to reduce the development of blowflies (*Calliphora vomitoria*) significantly (Deml and Dettner, 1990). Furthermore, this chemical and also phenylacetaldehyde show strong fumigant effects towards adult *Drosophila melanogaster* flies (Dettner et al., 1992). Most saturniid caterpillar secretions and hemolymphs containing these substances act as feeding deterrents against *Lasius niger* and in part against *Formica pratensis* ants (Deml and Dettner, 1993). Now a limited deterrent effect has also been ascertained for *A. atlas* secretion and hemolymph tested against these ants. This fact is of great importance because ants are assumed to be the

main predators of caterpillars, from the Arthropoda. In contrast to our findings, Paukstadt and Paukstadt (1991) observed in the field (Java) that *A. atlas* larvae were not protected from attacking ants, but this need not be true for all ant species cooccurring with *A. atlas* in South-East Asian ecosystems.

However, the main function of *A. atlas* L6 caterpillar secretion seems to be a different one: defense against vertebrates, in particular against birds. Several peculiarities of *A. atlas* caterpillars (e.g., the cryptic coloration of the larvae, the presence of vertebrate neurotransmitters in the secretion, and the delivery of secretion only following strong mechanical irritation of the larvae) suggest this conclusion. The *A. atlas* secretion also causes a burning sensation in man if it is brought into contact with the tongue (Haffer, 1921; Nässig, 1983) or with the eyes (Jones et al., 1982; Paukstadt and Paukstadt, 1991); effects on mucous membranes of birds might be equivalent. It seems plausible that the *A. atlas* caterpillars defend themselves, e.g., against attacking birds from a distance by spraying their irritant secretion into the eyes of the birds. Early larval instars of *A. atlas* possess "Sternwarzen"; maybe in these stages secretion is injected into predators as has been proposed for other Saturniidae (Deml and Dettner, 1993). It seems that the different *A. atlas* caterpillar stages are adapted to different types of predators for which the larvae are suitable as prey at the time, but further biological tests of *A. atlas* body fluids with model birds and (when known) with real tropical target organisms of the larval secretion are imperative. Moreover, investigations of caterpillar body fluids of other *Attacus* species, which in part have inactive scoli, would help to learn more about the chemical defense of this prominent saturniid genus and the phylogenetic development of such an unusual behavior as spraying a defensive fluid.

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WOUND-INDUCED CHANGES IN ROOT AND SHOOT JASMONIC ACID POOLS CORRELATE WITH INDUCED NICOTINE SYNTHESIS IN *Nicotiana sylvestris* SPEGAZZINI AND COMES

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Abstract—Leaf damage by herbivores in *Nicotiana sylvestris* Spegazzini and Comes (Solanaceae) produces a damage signal that dramatically increases *de novo* nicotine synthesis in the roots. The increased synthesis leads to increases in whole-plant nicotine pools, which in turn make plants more resistant to further herbivore attack. Because signal production and the response to the signal occur in widely separated tissues, the speed with which different damage signals exit a damaged leaf can be studied. We propose that electrical damage signals should exit a leaf faster (less than 60 min) than chemical damage signals. Excision of a leaf induces a smaller increase in nicotine production than does puncture damage, so we examined our proposition by excising previously punctured leaves at 1, 60, and 960 min after leaf puncture and quantifying the induced whole-plant nicotine pools six days later when the induced nicotine production had reached a maximum. Significant induced nicotine production occurred only if punctured leaves were excised more than 1 hr after puncture, which is consistent with the characteristics of a slow-moving chemical signal rather than a fast-moving electrical signal. We explore the nature of the chemical signal and demonstrate that additions of 90 μg or more of methyl jasmonate (MJ) in an aqueous solution to the roots of hydroponically grown plants induce *de novo* nicotine synthesis from $^{15}NO_3$ in a manner similar to that induced by leaf damage. We examine the hypothesis that jasmonic acid (JA) functions in the transfer of the damage signal from shoot to root. Using GC-MS techniques to quantify whole-plant JA pools, we demonstrate that leaf damage rapidly (<0.5 hr) increases shoot JA pools and, more slowly (<2 hr), root JA pools. JA levels subsequently decay to levels

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found in undamaged plants within 24 hr and 10 hr for shoots and roots, respectively. The addition of sufficient quantities (186 μg) of MJ in a lanolin paste to leaves from hydroponically grown plants significantly increased endogenous root JA pools and increased *de novo* nicotine synthesis in these plants. However, the addition of 93 μg or less of MJ did not significantly increase endogenous root JA pools and did not significantly affect *de novo* nicotine synthesis. We propose that wounding increases shoot JA pools, which either directly through transport or indirectly through a systemin-like signal increase root JA pools, which, in turn, stimulate root nicotine synthesis and increase whole-plant nicotine pools.

Key Words—Induced defense, *Nicotiana sylvestris*, nicotine, damage signal, jasmonic acid, methyl jasmonate, electric signal.

INTRODUCTION

Leaf damage is known to increase rapidly the concentration of different types of secondary metabolites found in undamaged leaves. Many of these damage-induced increases have proven defense-related roles (Tallamy and Raupp, 1991; Baldwin, 1993). Much of the research on these systemic changes in plant chemistry induced by leaf damage has focused on identifying the chemical signals that activate the chemical changes (Enyedi et al., 1992; Staswick, 1992). For example, jasmonic acid and the polypeptide, systemin (Farmer and Ryan, 1990; Pearce et al., 1991), increase proteinase inhibitor (PI) proteins in tomato. However, chemical damage signals may not be the only type of signal involved. Wounding has long been known to result in electrical signals in plants (Davies, 1987), and a recent study (Wildon et al., 1992) has clearly implicated these electrical signals in the induction of PI proteins in young tomato plants after damage. Hence, in tomato, induced defense responses may be elicited by a combination of both electrical and chemical signals.

Electrical and chemical signals usually differ in the time required for them to exit damaged tissues; this difference may indicate which of the two is the primary damage signal. As reported in Davies (1987) and Wildon et al. (1992), electrical signals in pea and tomato plants travel at 3–5 cm/min and 6–24 cm/min, respectively. In tomato, the electrical signal implicated in the induction of PI proteins exited damaged cotyledons within 5 min (Wildon et al., 1992). Chemical signals, on the other hand, are likely to exit damaged leaves more slowly. Although the phloem transport rates of some chemical signals could reach speeds (Baker and Milburn, 1989) in the same range as the slowest electrical signals, chemical signals require either *de novo* or partial synthesis prior to the formation of the active signal molecule (Raskin, 1992; Farmer and Ryan, 1992; McGurl et al., 1992), which may result in additional delays in signal transduction.

The damage-induced increase in nicotine synthesis in *N. sylvestris* may be

an ideal system in which to use the rate of signal transfer to differentiate between electrical and chemical signals because of the large spatial separation (many centimeters) between the sites of damage (leaves) and sites of nicotine synthesis (roots). We have established that the damage cue is a positive cue that is correlated with the amount of damage a plant receives (Baldwin, 1989; Baldwin and Schmelz, 1994). With ^{15}N pulse-chase experiments, we have established that the synthesis of nicotine with nitrogen derived from $^{15}NO_3$ acquired at the time of damage is significantly higher in damaged plants than in undamaged plants between 10 and 20 hr after leaf damage (Baldwin et al., 1994). Hence, the signal affecting these changes in root nicotine metabolism must travel from leaf to root within this time period. We have established that the damage signal can be blocked by steam girdling the stem (Baldwin, 1989), which suggests that the damage cue is phloem-borne. However, these experiments do not differentiate between a rapidly moving electrical signal and a slower chemical signal.

In order to examine the speed of the shoot-to-root damage signal, we take advantage of the fact that the induced nicotine response of the whole plant to leaf excision is significantly less than it is in response to leaf punctures (Baldwin and Schmelz, 1994). A similar difference in PI protein production in response to leaf excision and punctures has been reported in tomato (Nelson et al., 1983). We propose that if an electrical signal is the primary damage signal, it should exit punctured leaves less than 60 min after leaf puncture. We examine this proposition in small plants by excising previously punctured leaves at 1, 60, and 960 min after leaf puncture and quantifying the whole-plant induced nicotine pools. From the data presented, we conclude that the damage signal is slow moving and hence likely to be a chemical signal. Next we characterize the chemical signal.

Jasmonic acid (JA) has been implicated in the signal-transduction cascade mediating inducible plant defense responses against herbivore attack (Enyedi et al., 1992; Sembdner and Parthier, 1993; Mueller et al., 1993). The best documented example is the transcriptional regulation of proteinase inhibitors (PI) I and II in tomato and alfalfa leaves (Farmer and Ryan, 1990; Farmer et al., 1992). In tomato plants, the exogenous addition of methyl jasmonate (MJ) and JA to leaves or roots results in a dramatic increase in PI I and II transcripts in both treated and untreated leaves in a manner similar to that effected by additions of the polypeptide, systemin (Pearce et al., 1991), or leaf wounding (Farmer and Ryan, 1990). More recently, transitory increases in endogenous JA pools in excised tomato leaves after wounding have been documented (Peña-Cortés et al., 1993). Moreover, JA has been found to increase production of a number of defense-related secondary metabolites in plant cell culture, including benzophenanthridine alkaloids, flavonoids (Gundlach et al., 1992; Mueller et al., 1993), phenolics (Mizukami et al., 1993), and enzymes and transcripts of enzymes thought to be important in regulating secondary metabolic pathways

(Creelman et al., 1992; Mizukami et al., 1993). Here we report that JA is an important component of the damage signal transduction pathway in the wound-induced production of nicotine in *N. sylvestris*. Specifically, we demonstrate that: (1) additions of MJ to the roots of hydroponically grown plants induce *de novo* nicotine synthesis from $^{15}NO_3$ in the same way that leaf damage does; (2) leaf damage rapidly (<0.5 hr) increases endogenous shoot JA pools and, more slowly (<2 hr), endogenous root JA pools; and (3) addition of MJ in a lanolin paste to leaves in quantities that significantly increase endogenous root JA pools also increases *de novo* nicotine synthesis.

METHODS AND MATERIALS

Chemicals. MJ was obtained from Bedoukian Research (Danbury, Connecticut); the manufacturer reports it to be approximately 90.6% 1*R*, 2*R* MJ and 8.1% 1*R*, 2*S* MJ. MJ was deesterified to produce JA by hydrolysis in 0.5 M KOH in 90% aqueous methanol and sonicated for 30 min at room temperature. JA was recovered by extraction into diethyl ether after acidifying the aqueous layer (pH 3) with 1 N HCl. The ether was removed by rotoevaporation and an aliquot was checked for purity by GC-MS after making the trimethylsilyl ester of JA (JA-TMS). Nicotine, ABA, bis(trimethylsilyl)trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS), lanolin, silicic acid (SIL-A-200), and all plant nutrients were purchased from Sigma Chemical Co. (St. Louis, Missouri). ^{15}N-labeled (99.6–99.9 atom %) KNO_3 was purchased from Isotec Inc. (Miamisburg, Ohio).

Plant Growth and Leaf Damage Protocol. Nicotiana sylvestris Spegazzini and Comes seeds were germinated and grown in Cornell mix A (Boodley and Sheldrake, 1977) for 16–20 days, after which they were transferred to 40-liter hydroponic boxes containing a complete nutrient solution [0.292 g/liter Peter's Hydro-sol (W.R. Grace, Inc., Fogelsville, Pennsylvania), 0.193 g/liter $CaNo_3$, and 70 ml/liter of a nitrogen-free nutrient solution (described in Ohnmeiss and Baldwin, 1994)] for five to nine days. To reduce between-plant genetic variance, we used seeds that were full sibs; seeds were from the same collection as those used in Baldwin et al. (1990). The plants were transferred to individual opaque 1-liter containers containing the nitrogen-free nutrient solution supplemented with either KNO_3 or [^{15}N]KNO_3, as described in the individual experiments. Throughout all experiments the original solutions were maintained at 900–1000 ml with distilled water. All plants were grown in a glasshouse and received supplemental lighting from 400-W sodium vapor lamps for 16 hr/day, which provided a minimum PAR of 220 $\mu mol/m^2/sec$. Treatments were randomly assigned to positions on the glasshouse bench within each experiment.

All plants used in each experiment were from the same planting. In order

to estimate accurately the effects of damage on JA and nicotine pools, a high degree of uniformity in plant mass among treatment groups is required. In order to attain this uniformity, plants were sorted within each experiment by initial wet mass and then assigned to treatment groups by consecutive random divisions (as described in Ohnmeiss and Baldwin, 1994).

Reproducible amounts of leaf damage were produced by rolling a fabric pattern tracing-wheel (Dritz, Spartanburg, South Carolina) at least 2 cm from and parallel to both sides of the mid-rib on designated leaves once during each experiment. The damage treatment consisted of four rows of evenly distributed pattern-wheel damage on each side of the mid-rib over the length of the lamina. On average, tracing-wheel damage resulted in 4.5 1-mm^2 punctures/cm of leaf and killed the cells in the puncture zone. Punctures were produced by crushing leaf tissue between the blunt spokes of the tracing-wheel and a plastic card and resulted in minimal vasculature damage with undetectable leaf dry mass loss or reductions in biomass gain (Ohnmeiss and Baldwin, 1994; Baldwin and Schmelz, 1994).

Whole-Plant Nicotine and Biomass Analysis. Plants were separated into roots and shoots (except those of experiment 2 in which leaf cuttings were analyzed separately), frozen in liquid nitrogen, lyophilized, weighed (to 0.1 mg), and ground to fine powder (850-μm mesh) with a Wiley mill (Thomas Scientific). Experiment 4 involved lanolin applications to a small portion of the shoot. The mass of this portion was included in the total biomass and alkaloid determination but was not extracted for alkaloids. Nicotine analysis was modified from Baldwin (1988) to obtain whole-plant nicotine measures. A weighed portion (approximately 50 mg) of lyophilized and homogenized plant material was extracted for 3 days in 10.0 ml of aqueous alkaloid extraction solution (40% methanol–0.1% HCl). Nicotine contents of these extracts were determined by HPLC with external standard curves constructed at least once for each experiment. Whole-plant, leaf cuttings, root, and shoot nicotine pools were calculated from biomass values, and nicotine was expressed as micrograms of nitrogen in nicotine. Since leaf material was removed from some of the plants in experiment 1, the induced nicotine pool was calculated to correct for the nicotine removed from damaged plants in the leaf cuttings. This was calculated as the nicotine pool of a damaged plant minus the mean nicotine pool of undamaged plants less the nicotine pool of the leaf cuttings of the individual damaged plant. Whole-plant quantities of ^{15}N in nicotine were calculated from micrograms of N in nicotine and the measures of ^{15}N incorporation into nicotine as determined by mass spectrometry (Baldwin et al., 1994). Calculations of single and double ^{15}N incorporations into the nicotine molecule were based on the relative abundances of fragment ions 133, 134, and 135 as described in Baldwin et al. (1994).

Jasmonic Acid Extraction and Derivatization. Plants were separated into roots and shoots, immediately frozen in liquid nitrogen, weighed (to 0.1 mg),

and ground separately to a fine powder with a mortar and pestle in liquid N_2. In experiments 2 and 3, a weighed portion, representing 5.0 g and 3.0 g, respectively, of crushed and homogenized material, was placed in a 20-ml extraction vial. The extraction procedure involved the sequential addition of 5.0 ml double-distilled H_2O, 400 μl of 12 N HCl, and 15 ml of dichloromethane (CH_2Cl_2) to each sample. The samples were placed on a shaking table (100 rpm) for 72 hr at room temperature. Seven milliliters of the CH_2Cl_2 layer were removed and loaded onto a silicic acid column (SIL-A-200, for acidic mixtures, 0.775 ± 0.004 g/column). After loading, the columns were air-dried and eluted with 7.0 ml of diethyl ether (containing 0.005% butylated hydroxytoluene as an antioxidant); the eluate was allowed to dry at room temperature and transferred to 2.0-ml vials. The dried residue was derivatized with 300 μl of BSTFA (with 1% TMCS) and analyzed within 48 hr. External standard curves for JA were constructed from air-dried 100-μl aliquots of individual JA–CH_2Cl_2 solutions. These standards consisted of 12 JA concentrations in BSTFA ranging from 0.102 to 4.320 ng/μl; new standards were derivatized and analyzed by GC-MS at least once every 80 plant samples. Methanol was not used in any of the extraction steps to avoid the possibility of forming MJ from JA during the extraction procedure.

In order to avoid the extraction of exogenously applied MJ in experiment 4, leaf portions containing the lanolin applications were weighed separately for biomass measures and were not included in the JA extractions. Moreover, to examine the possibility of deesterifying MJ to form JA during our extraction procedure, a separate experiment was performed. One microgram MJ was extracted from distilled H_2O, loaded onto a column, eluted, dried, and derivatized as outlined above. No JA (detection limit 50 pg) was detected in the three replicates of the MJ-spiked samples, and thus we conclude that our extraction procedure does not convert MJ into JA.

GC-MS Analysis of JA and MJ. JA-TMS and MJ in CH_2Cl_2 samples were separated by GC with reproducible retention times at 13.5 and 11.92 min, respectively, under the following GC conditions: 25-m × 0.20-mm DB-1 column, 0.6 ml/min flow He, injection port 250°C, 0.5 min splitless injection, 100°C for 5.0 min, 100–250°C temperature ramp at 10°C/min. We found no evidence for the separation of 1*R*, 2*R* JA-TMS from 1*R*, 2*S* JA-TMS under these conditions. Ion abundances were determined by a quadrupole MS (70 eV EI with an HP 5971A Mass Selective Detector). The detector was tuned with PFTBA, using the Autotune procedure of the ChemStation software. Compound identification was achieved by scanning all ions from *m/z* 50 to *m/z* 300 with each ion sampled once every 1.3 sec. JA-TMS was positively identified, from a bulk extraction of plant material, with a complete mass spectrum at the correct retention time (Figure 1). To establish the presence of JA in *N. sylvestris*, we extracted 52.3 g of fresh frozen shoot material, using a 10-fold scale-up from

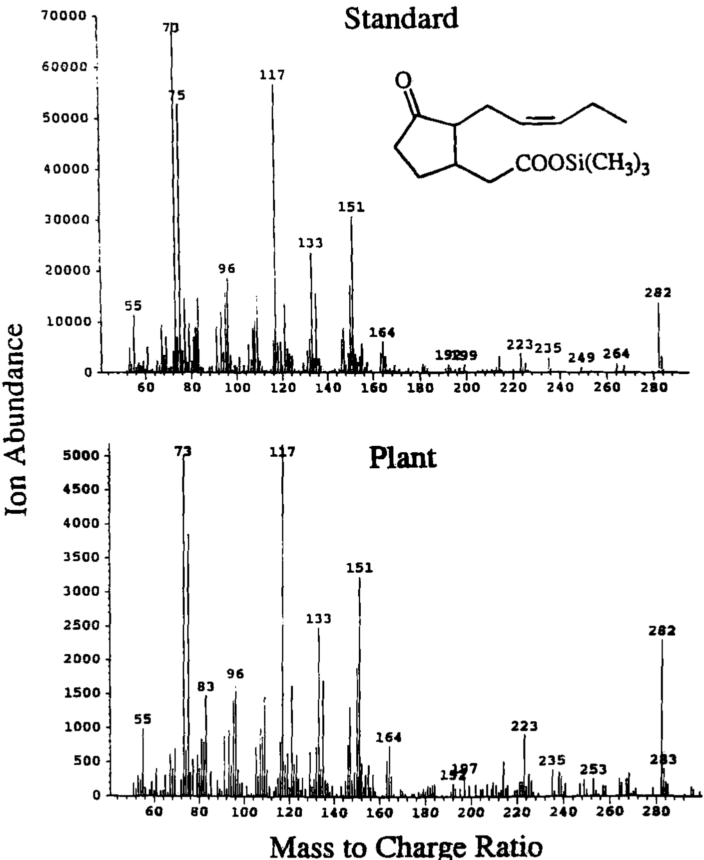

FIG. 1. Complete mass spectrum for JA-TMS in a standard (top) and in a plant sample (bottom) from scans collected at 13.53 min into the GC-MS run.

the previously described JA extraction procedure. The resulting residue was taken up in 5.0 ml CH_2Cl_2, transferred to a 2.0-ml derivatization vial, and allowed to evaporate at room temperature. With 1 ml of solvent remaining, injections were performed with the GC-MS in scan mode for MJ detection. MJ was not detected using the scan mode, and in SIM mode the selected ion ratios at the correct retention time were totally uncharacteristic of MJ. This sample was then dried completely, derivatized in 1 ml BSTFA, and analyzed in scan mode. JA-TMS was found at the correct retention time (13.53 min), and ion ratios for pure JA-TMS and plant-extracted JA-TMS did not differ. Ion abundance ratios for pure JA and plant-derived JA-TMS were *m/z* 282(36, M^+),

151(100), 133(43), 117(54), 96(25) and *m/z* 282(33, M^+), 151(100), 133(42), 117(55), 96(25), respectively (Figure 1). Hence, we confirm the presence of endogenous JA in *N. sylvestris*, but have no evidence for the presence of endogenous MJ. In order to increase our sensitivity for the detection of JA-TMS in individual plant samples, the molecular ion of JA-TMS (*m/z* 282), at the correct retention time was monitored in SIM mode.

Jasmonic Acid Quantification. For experiments 3 and 4, both root and shoot JA pools were quantified with measures of JA recoveries calculated from individual standard addition experiments using plant material from the same planting as the experiment and extracted under the same conditions as the individual plant samples. Because an external standard technique was used for the calculation of JA pools, it is important to rigorously quantify the extraction efficiency of JA. Moreover, because plant ontogeny may influence the extraction efficiency, we deemed it important to determine the efficiency of our extraction protocol for each experiment. The standard addition experiment used to calculate the JA recoveries for experiment 3 used 5-g samples of homogenized root (N = 7) and shoot (N = 20) material to which a range of weighed JA (0–398.7 and 0–463.5 ng for root and shoot, respectively) quantities were added. These samples were extracted and processed as described above. The slope of the regression between *m/z* 282 ion abundance in 1 μl of sample and the JA ng/μl added to the sample was calculated (Figure 2A). The percent recovery was obtained by dividing the standard addition slope by the slope of an external JA-TMS standard curve and multiplying it by 100. The slope of the external JA standard was 305.83 (r^2 = 0.999), and the results of the standard additions for experiments 3 and 4 are reported in the caption of Figure 2. The percentage recoveries calculated for the root and shoot tissues were 76.3% and 63.9%, respectively.

A similar experiment was performed with experiment 4. A range of JA quantities (0–463.5 and 14.4–463.5 ng JA, for root and shoot samples, respectively) were added to individual 3.0-g samples of homogenized root (N = 16) and shoot (N = 14) tissues, and these samples were prepared for JA quantitation with the individual samples of experiment 4. Again the slope of the regression between *m/z* 282 ion abundance in 1 μl of sample and the nanograms of JA per microliter added to the sample was calculated (Figure 2B). Individual root and shoot JA external standards had slopes of 227.41 (r^2 = 0.998) and 158.11 (r^2 = 0.999), respectively. The calculated root and shoot percent recoveries were 72.9% and 86.7%, respectively. The range of JA amounts added in these standard addition experiments was greater than that found in the plant material, and hence we were confident that we did not exceed the capacity of the silicic acid columns.

Statistical Analysis. One-way analysis of variance (ANOVA) was performed to analyze treatment effects on nicotine, JA pools, and biomass. Con-

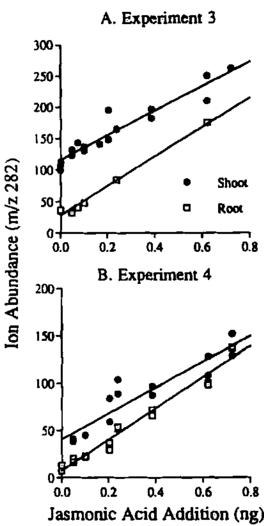

FIG. 2. (A) A standard addition experiment for experiment 3 resulted in regressions of $y = 233.3 \pm 12.5x + 28.4 \pm 3.2$ ($r^2 = 0.986$) and $y = 195.5 \pm 14.3x + 116.1 \pm 4.3$ ($r^2 = 0.912$) for root and shoot tissues, respectively. (B) In experiment 4, root and shoot standard additions of JA yielded regressions of $y = 165.7 \pm 7.7x + 7.2 \pm 2.9$ ($r^2 = 0.969$) and $y = 137.1 \pm 16.1x + 40.7 \pm 6.5$ ($r^2 = 0.847$), respectively. Ion abundance and JA additions are expressed as amount per 1 μl of a 300-μl sample. Slopes were used to calculate experiment and tissue-specific extraction efficiencies.

trasts were performed if the main effects were significant. One-tailed t tests were performed on JA pools of damaged plants and undamaged plants harvested at the same time. All data analysis was performed with the MGLH module of the SYSTAT statistical package (Evanston, Illinois).

Experiment 1. Here we examined our proposition that electrical damage signals should exit a leaf faster (<60 min) than chemical damage signals. Because leaf excision induces a smaller increase in nicotine production than does puncture damage of the same leaf (Baldwin and Schmelz, 1994), we examined our proposition by excising previously punctured leaves at 1, 60, and 960 min after leaf puncture and quantifying the induced whole-plant nicotine pools six days later when the induced nicotine production is known to have reached a maximum (Baldwin et al., 1994). The experiment consisted of the following five treatment groups: three damage treatments in which plants had the same leaf positions (phylotactic positions 6–9 with the apical leaf designated as position 1) punctured and excised at either 1, 60, or 960 min after the puncture treatment, one damage treatment in which plants had the same leaves excised, and one control group in which the plants were not damaged. Leaf position 6

on all plants was always a fully expanded and photosynthetically active leaf. Leaves were excised with a clean razor incision at the junction of the petiole to the stem. The four damage treatment groups were designated by the time delay (in minutes) between the puncture treatment and leaf excision (e.g., 0, 1, 60, 960). The plants in damage treatment 0 did not have the puncture damage treatment; leaves were only excised.

The plants used in this experiment were grown in individual 1-liter containers for 10 days, during which time they each received a 4 mM KNO_3 addition to the nitrogen-free hydroponic solution. One day before the experiment, the nitrogen-free solution was replaced, and 40 plants of uniform size and shape were weighed and assigned to five equal treatment groups by wet mass (grand mean = 13.43 ± 0.43 g; one-way ANOVA, $F_{4,35} = 0.009$, $P = 1.000$).

Experiment 2. This experiment examines the effect of exogenous additions of MJ to the roots on changes in whole-plant nicotine production and pools. Nicotine production, from the time of MJ addition and damage, was determined by adding [^{15}N]KNO_3 as the nitrogen source and quantifying the whole-plant pools of ^{15}N in nicotine five days later, the time when maximum damage-induced nicotine production has occurred (Baldwin et al., 1994). In similar experiments, we (Baldwin et al., 1994) determined that nicotine turnover was so low as to be undetectable during this time period, and hence nicotine production is accurately estimated by the size of the ^{15}N-labeled nicotine pool. The experiment consisted of nine treatment groups: six MJ treatments, one atmospherically isolated control, one nonisolated control, and a damage treatment. All plants were grown in 1-liter hydroponic chambers and MJ was added to the nutrient solutions of these chambers as an aqueous solution. Three MJ-treatment groups received a total of 0.5, 90, and 450 μg of MJ to the plants' individual growth chambers, in two additions of 1.0-ml aqueous MJ solutions of 1.1, 200.0, and 1003 μM MJ, respectively, on days 1 and 3. Likewise, three additional MJ-treatment groups received a total of 1, 180, and 900 μg of MJ over the course of the experiment by the daily additions of 1.0-ml aqueous MJ solutions of 1.1, 200, and 1003 μM MJ, respectively, on days 1–4. Two groups of untreated plants were randomly assigned to positions on the greenhouse bench among the six groups of MJ treated plants: an undamaged control group and a damaged group. An additional undamaged control group was created to test for potential contamination of untreated plants by nearby MJ-treated plants. This control group—the atmospherically isolated control group—was placed in a sealed Plexiglas (UF-3; Almac Plastics, Rochester, New York) box (measuring 61 cm × 25 cm × 41 cm) located on the glasshouse bench. It was fitted with a fan and ducting that replaced the volume of air in the box every minute with air from outside of the glasshouse, which was presumably free of MJ contamination.

The plants used in this experiment were grown in individual 1-liter containers for 12 days, during which time they each received a 2 mM KNO_3 addition

to the nitrogen-free hydroponic solution. One day before the experiment, the nitrogen-free solution was replaced, and 72 plants of uniform size and shape were weighed and assigned to nine equal treatment groups by wet mass (grand mean = 5.38 ± 0.08 g; one-way ANOVA, $F_{8,63}$ = 0.0007, P = 1.00). The start of the experiment was designated as time zero, when all plants received 7.5 mg of $[^{15}N]KNO_3$ (0.5 mM in the 1-liter containers) and the MJ-treated plants received their first MJ additions, and damage protocol was applied to the first four fully expanded leaves of the plants in the damage treatment.

Experiment 3. This experiment was designed to quantify changes in shoot and root JA pools of plants that had received damage only to their shoot. The design included damaged and undamaged treatments, destructively harvested at five sequential times (0.5, 2, 10, 24 hr, and five days) after damage. The plants of these 10 treatment groups were harvested for the analysis of individual root and shoot JA pools through the 24-hr harvest and whole-plant nicotine total pools and production on the five-day harvest. Plants used in this experiment were grown individually in 1-liter containers for 23 days, and each received additions of KNO_3 bringing their hydroponic solutions to 2.0, 0.5, 1.0, and 2.0 mM on days 1, 11, 14, and 16, respectively. On day 23, the nitrogen-free solutions were replaced and 50 plants of uniform size and shape were weighed and assigned to 10 equal treatment groups by wet mass (grand mean = 25.57 ± 0.34 g; $F_{9,40}$ = 0.06, P = 1.00). The experiment started the next day (time zero), when all plants received 7.5 mg of ^{15}N as $[^{15}N]KNO_3$ (0.5 mM in the 1-liter containers), and plants in the damage treatments were damaged on the first six fully expanded leaves. A standard addition experiment (see Jasmonic Acid Quantification section) to measure the recovery of JA from root and shoot tissues was performed in tandem using plants from the same planting.

Experiment 4. This experiment examines whether the addition of MJ to the shoots of plants results in changes in the root and shoot JA pools and nicotine production of treated plants. MJ in a lanolin paste was applied to the adaxial surface of leaves occupying positions 6 and 7 from the apical leaf. The lanolin (0.186 ± 0.001 g per plant) was spread with a plastic applicator in a thin layer spanning the proximal 8 cm of the lamina and was divided equally between the two leaf positions. The five lanolin treatments consisted of a pure lanolin treatment, three different levels of MJ in lanolin (19, 93, and 186 μg MJ), and a pattern-wheel damage coupled with the 186-μg MJ treatment (186 μg MJ-D). With one exception, each of the five lanolin treatments was destructively sampled at four different harvest times: 0.5, 2, and 10 hr for JA analysis and five days later for nicotine analysis. The 186-μg MJ-D group did not contain a 10-hr JA harvest.

Plants used in this experiment were grown in individual 1.0-liter containers, and each received additions of KNO_3 bringing their hydroponic solutions to 2.0 mM on each of days 1, 4, and 9. On day 16, 95 plants of uniform size and

shape were weighed and assigned to 19 groups of five by wet mass (grand mean = 15.94 ± 0.40 g; $F_{18,76} = 0.005$, $P = 1.00$). As in experiment 3, a standard addition experiment (see Jasmonic Acid Quantification section) to measure the recovery of JA from root and shoot tissues was performed in tandem using plants from the same planting. When the experiment started (time zero), all plants received 7.5 mg of ^{15}N as $[^{15}N]KNO_3$ (0.5 mM in the 1-liter containers), and the appropriate treatment groups received lanolin applications and damage to leaves occupying positions 4–7.

RESULTS

Experiment 1. Whole-plant nicotine pools differed significantly among the five treatments ($F_{4,35} = 5.491$; $P = 0.002$) as determined by the ANOVA. Contrasts constructed from the ANOVA among the treatments demonstrated that plants with punctured leaves excised at 0, 1, and 60 min after damage had only marginally higher nicotine pools than undamaged controls ($F_{1,35} = 3.599$; $P > 0.065$) and that plants with punctured leaves excised 960 min after damage had significantly greater nicotine pools than all other damaged treatments ($F_{1,35} = 12.841$; $P < 0.001$) and the undamaged treatment ($F_{1,35} = 20.024$; $P < 0.0001$; Figure 3B). The estimates of wound-induced nicotine pools (Figure 3C) of plants in treatment groups 0, 1, and 60 were significantly higher ($F_{1,35} = 5.902$; $P = 0.020$) than those of undamaged plants, and again the induced pools of plants in treatment 960 were significantly larger than the other damaged treatment groups ($F_{1,35} = 14.236$; $P = 0.001$). The amount of leaf mass (grams) removed in the leaf cuttings (±SEM) and the amount of nicotine (milligrams) in the cuttings were 0.30 ± 0.02 and 0.53 ± 0.04, respectively. Leaf cuttings comprised only 10% of the plants' final dry mass and 5% of the nicotine pool present in the undamaged plants (Figure 3A). As expected, total plant biomass of the damaged groups was significantly lower than the undamaged control group ($F_{4,35} = 4.495$; $P = 0.005$), but among the damaged plant treatments final biomass did not differ ($F_{3,28} = 1.374$; $P = 0.271$). These results demonstrate that significant wound-induced nicotine production occurred only if punctured leaves were excised more than 1 hr after puncture, which is consistent with the characteristics of a slow-moving chemical signal activating root nicotine production rather than a fast-moving electrical signal.

Experiment 2. The results of this experiment clearly demonstrate that MJ is a potent inducer of *de novo* nicotine synthesis and dramatically increases whole-plant nicotine accumulations when introduced to the roots of hydroponically grown plants. Four levels of MJ addition (90, 180, 450, and 900 μg/plant), as well as leaf puncture damage, significantly increased both nicotine production and accumulation above levels found in undamaged control plants

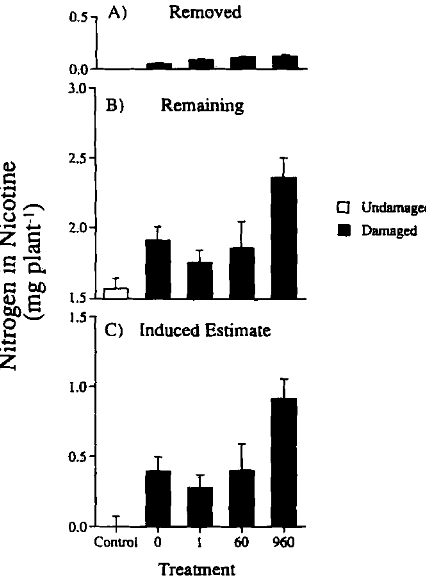

FIG. 3. Mean (±SEM) nicotine (expressed as milligrams N in nicotine) removed in leaf cuttings (A) and remaining in shoots and roots (B) and the whole-plant wound-induced amount of nicotine (C) calculated by correcting for the nicotine removed in the leaf cuttings. Undamaged controls and damaged plants with particular leaves punctured at time 0 and excised at 1, 60, or 960 min after the damage treatment are represented by open and solid bars, respectively.

(all contrast $F_{1,63}$s > 11.3, Ps < 0.002), whereas the two lower levels of MJ addition (0.5 and 1.0 μg/plant) did not affect nicotine production or accumulation (all contrast $F_{1,63}$s < 0.65, Ps > 0.422; Figure 4A and B). Damaged plants had whole-plant N in nicotine pools 1.4 times as large as the pools in undamaged controls (a damage-induced difference of 260 μg N in nicotine per plant) and over the five-day experiment incorporated 3.6% of the ^{15}N pool given each plant at the beginning of each experiment into nicotine; undamaged control plants, on the other hand, incorporated only 1.3%. MJ-induced plants had whole-plant N in nicotine pools 1.9–2.7 times larger than the pools of undamaged controls (induced differences between 586 and 1059 μg N in nicotine per plant), and incorporated between 6.1 and 9.6% of the ^{15}N pool given at the time of treatment into nicotine. These results suggest that a lower threshold exists for this induced defense response—between 1.0 and 90 μg of exogenously added MJ—and that an upper threshold exists above 450 μg as the saturation of the nicotine response (Figure 4A and B) suggests. How these exogenously applied amounts compare to the changes in endogenous JA pool size is unclear, but

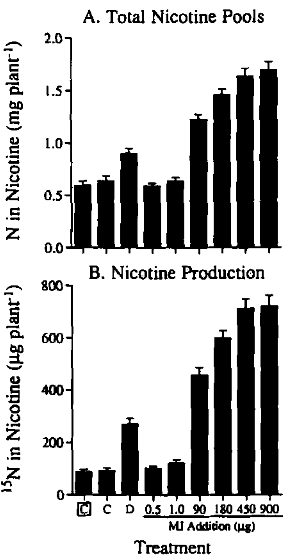

FIG. 4. Mean (±SEM) whole-plant (A) milligrams of N in nicotine and (B) micrograms of ^{15}N in nicotine for atmospherically-isolated control ([C]), undamaged control (C), and damaged (D) plant groups, and for groups denoted by the total μg of MJ added to the hydroponic solution of each plant during the course of the five-day experiment (0.5, 1, 90, 180, 450, and 900 μg/plant).

they are likely substantially larger given that only a portion of the added MJ is likely to be the most active isomer for the nicotine response (e.g., Koda et al., 1992) and that only a portion of the amount added to the hydroponic solutions is likely to be taken up by the plant.

MJ-induced plants did not differ significantly in plant dry mass from damaged plants at the end of the experiment (contrast $F_{1,63} = 0.05$, $P > 0.832$), but both damaged and MJ-induced plants were significantly smaller (by 11%) than undamaged control plants ($F_{8,63} = 14.6$, $P < 0.001$). This decrease in growth caused by MJ and damage may, in part, reflect a growth-related cost of nicotine production and other defense responses, such as PIs that are likely to be induced by these quantities of MJ. The two control treatments did not differ in any measure of nicotine production (all contrast $F_{1,63}$s < 0.32, Ps > 0.571), and thus the amount of MJ present in the glasshouse atmosphere was not sufficient to induce untreated plants.

Experiment 3. The results of this experiment demonstrate that leaf damage results in a transient and coordinated increase in shoot and root endogenous JA pools. Within 0.5–2 hr after damage, the shoot and root JA pools of damaged

plants were significantly greater ($t_8 = -2.841$, $P < 0.025$ and $t_8 = -2.519$, $P < 0.050$ for shoots at 0.5 and 2 hr, respectively; $t_7 = -1.898$, $P < 0.050$ for roots at 2 hr) than the corresponding JA pools of undamaged plants. The observed maximum in damage-induced shoot JA pools (a 1.7-fold increase equaling 550 ng JA) occurred 0.5 hr after damage (Figure 5A), while the damaged-induced root JA maximum (a 17-fold increase equaling 1390 ng JA) was observed 2 hr after damage (Figure 5B); the large standard error associated with the root JA pool of damaged plants at the 2-hr harvest may reflect the transitory nature of this portion of the signal cascade. JA pools of damaged plants had decreased to levels found in undamaged plants by the 10-hr harvest (t_8s = -1.348 and 0.437, Ps > 0.250 for shoot and root tissues, respectively). The rapid increases in shoot and root endogenous JA pools so soon after damage correlate with the induced response five days later, as measured by a statistically significant ($F_{1,8} > 10.09$, $P < 0.014$) increase (286 μg/plant) in the incorporation of ^{15}N into nicotine in damaged plants (Figure 5C).

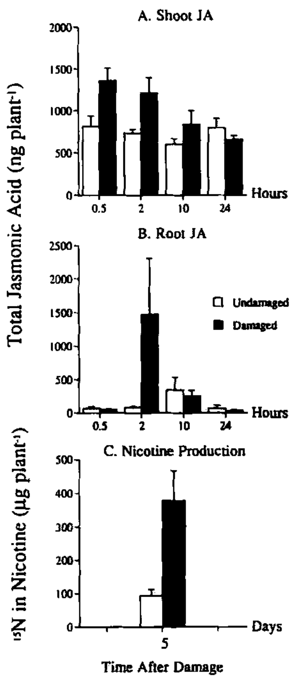

FIG. 5. Mean (±SEM) total JA at 0.5, 2, and 10 hr after damage for (A) shoot and (B) root tissues, and (C) whole-plant ^{15}N in nicotine pools (micrograms) at day 5 for the undamaged (open) and damaged (solid) plants of experiment 3.

Experiment 4. Since leaf damage caused a coordinated increase in both root and shoot JA pools, this experiment was designed to introduce exogenous MJ into a plant in a manner that mimicked the spatial pattern of damage-induced changes in JA pools. The addition of MJ to leaves increased both shoot and root JA pools and increased *de novo* nicotine production. Additions of three levels of MJ (19, 93, and 186 μg/plant) in lanolin to the shoot did not lead to statistically different JA pools in the shoot at any harvest time (all contrast $F_{1,19}$s < 2.18, Ps > 0.1559; Figure 6A); however, an addition of 186 μg of MJ in lanolin to a shoot with leaf puncture damage resulted in significant increases in the shoot's JA pool at 0.5 and 2 hr (both contrast $F_{1,19}$s > 4.44, Ps < 0.049; increases of 1160 ng JA over lanolin controls), which is consistent with the damage-induced increase in endogenous shoot JA in experiment 3. Only plants treated with 186 μg MJ had significant increases in their root JA pools (0.5, 2, and 10 hr; all contrast $F_{1,19}$s > 6.82, $P < 0.019$; Figure 6B); plants treated with a combination of the 186 μg MJ treatment and leaf puncture damage had a significantly (contrast $F_{1,19} = 17.7$, $P < 0.001$) larger root JA pool (109 ng JA) than did lanolin controls at the 2-hr harvest. Moreover, they were the only plants to produce a statistically significant increase in de novo nicotine production (contrast $F_{1,20} > 14.55$, $P < 0.002$; Figure 6C). The MJ treatments of 93 and 186 μg in lanolin resulted in marginally significant increases in *de novo* nicotine production (contrasts $F_{1,20} = 4.19$, $P = 0.054$ and $F_{1,20} = 3.89$, $P = 0.063$, respectively).

DISCUSSION

In summary, the results from these experiments demonstrate that: (1) the primary damage signal requires more than 1 hr to exit punctured leaves: (2) additions of MJ to the roots of hydroponically grown plants induces *de novo* nicotine synthesis from $^{15}NO_3$ in the same way that leaf damage does; (3) leaf damage rapidly (<0.5 hr) increases endogenous shoot JA pools and, more slowly (<2 hr), endogenous root JA pools; (4) addition of MJ in a lanolin paste to leaves in quantities sufficient to significantly increase root JA pools also increases the rate of *de novo* nicotine synthesis. The timing of the increases in endogenous root JA pools induced by leaf damage (namely, within 2 hr) is consistent with the time required for the damage signal to exit damaged leaves (namely, greater than 1 hr).

The nicotine-based induced defense described here appears to have many components in common with the more thoroughly described PI-based defense system of tomatoes (Farmer and Ryan, 1990; Farmer et al., 1992). We propose that damage-induced increases in shoot JA pools either directly through transport or indirectly through a systemin-like signal increase root JA pools, which affect

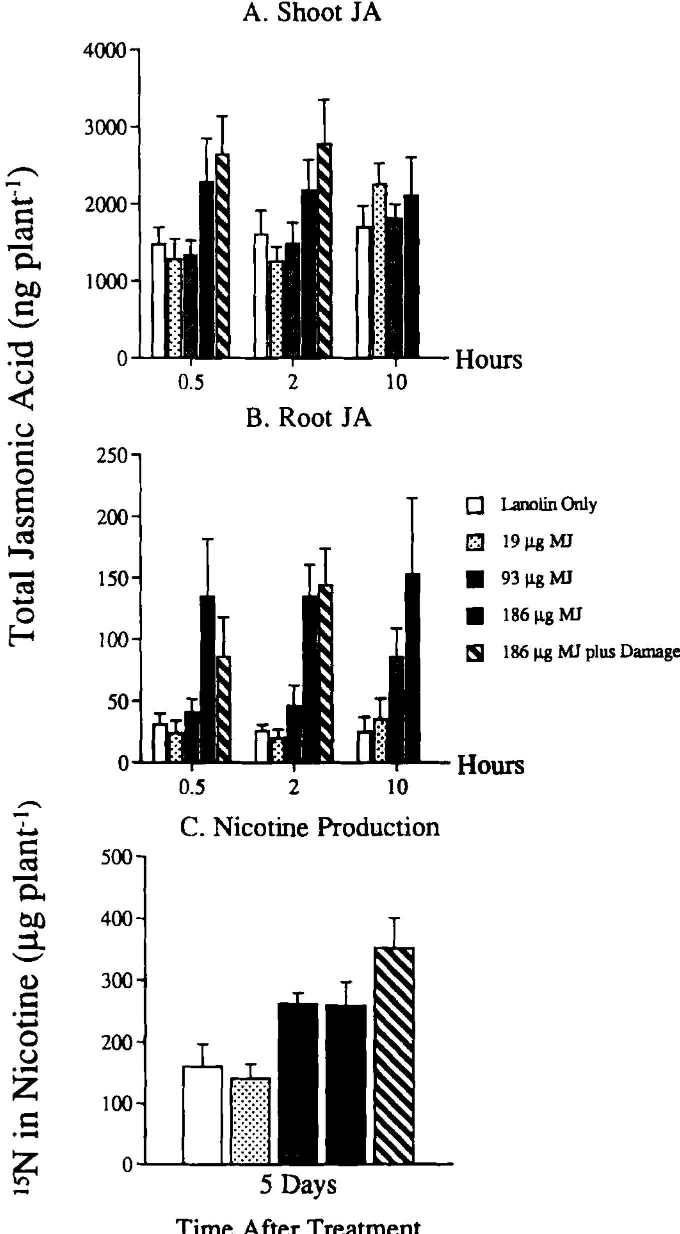

FIG. 6. Mean (±SEM) total JA at 0.5, 2, and 10 hr after damage for (A) shoot and (B) root tissues, and (C) whole-plant ^{15}N in nicotine pools (micrograms) at day 5 for plants of experiment 4. Refer to legend for quantities of MJ applied to shoots in lanolin pastes.

the increase in root nicotine synthesis and whole-plant pools. These experiments do not rule out the possibility that an electrical signal plays a role in inducing the smaller alkaloidal response observed in plants with leaves excised ≤1 hr after damage.

The results of experiment 2 clearly demonstrate that these plants are physiologically capable of producing two to three times more nicotine in response to an MJ treatment than they produce in response to our leaf-damage protocol. This difference may reflect a failure of our damage protocol to produce a full-blown response. For example, given that nicotine is produced in the roots, the introduction of MJ directly to the roots may heighten the nicotine response simply by delivering the damage signal to the site of nicotine synthesis more efficiently than is possible by the normal intraplant route. The discovery that JA plays a critical role in this induced plant defense will greatly facilitate the exploration of the ecological constraints on this induced defense and allow its activation without wounding the plant. Being able to uncouple the response to damage from damage itself represents a major experimental achievement in the examination of the costs and benefits of these defense responses.

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FIELD RESPONSE OF MARITIME PINE SCALE, *Matsucoccus feytaudi* DUC. (HOMOPTERA: MARGARODIDAE), TO SYNTHETIC SEX PHEROMONE STEREOISOMERS

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Abstract—The absolute configuration of the primary component of the maritime pine scale (*Matsucoccus feytaudi*) pheromone (i.e., (8*E*,10*E*)-3,7,9-trimethyl-8,10-dodecadien-6-one) was determined as 3*S*,7*R* by field-trapping experiments using synthetic stereoisomers and according to previous NMR considerations. The 3*R*,7*R* isomer showed similar activity to 3*S*,7*R*, whereas *M. feytaudi* males responded very weakly to the two other candidates (3*R*,7*S* and 3*S*,7*S*). Further studies were conducted to optimize scale trapping for monitoring scale populations. Results of these studies showed that the trapping efficiency was related to pheromone dose, trap area, and wind speed but not to trap height.

Key Words—Maritime pine scale, *Matsucoccus feytaudi*, Homoptera, Margarodidae, sex pheromone, field trapping, (8*E*,10*E*)-3,7,9-trimethyl-8,10-dodecadien-6-one, absolute configuration.

INTRODUCTION

Matsucoccus feytaudi Duc. (*Homoptera: Margarodidae)* is a specific pest of the maritime pine tree (*Pinus pinaster* Ait) in Europe and Africa (Riom and Ger-

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binot, 1977). This scale caused considerable damage in southeast France in the late 1950s (Schvester, 1971), which resulted in an increasing interest in the biology of this species in southwest France, where the scale is endemic (Riom, 1979). Recent studies on the sex pheromone emitted by *M. feytaudi* females have led to the identification of two compounds with the following isomeric structures: (8*E*,10*E*)-3,7,9-trimethyl-8,10-dodecadien-6-one (major compound) and (8*Z*,10*E*)-3,7,9-trimethyl-8,10-dodecadien-6-one (minor compound) Einhorn et al., 1990).[4] The major compound (ca. 97%) might be considered in a first approach as the primary component of the pheromone according to its strong attractiveness in laboratory bioassays.

At that stage, no information was available regarding the stereochemistry of the chiral carbons (i.e., C_3 and C_7) in any of these compounds. However, the synthetic analogs of the major compound were subsequently obtained by enantioselective routes, and the comparison of their ^{1}H NMR spectra with those of the natural compound led Cywin and Kallmerten (1992) to conclude that a priority reflective stereochemistry existed (e.g., either 3*S*,7*R* or 3*R*,7*S*) (Figure 1). The same conclusion was previously reported (Cywin et al., 1991) for "matsuone," the sex pheromone of *Matsucoccus resinosae*, *Matsucoccus matsumurae*, and *Matsucoccus thunbergianae* (Lanier et al., 1989).

In order to unambiguously establish the absolute configuration of the natural pheromone of *M. feytaudi*, the attractiveness of the four possible stereoisomers (3*S*,7*R*; 3*R*,7*S*; 3*R*,7*R*; and 3*S*,7*S*) of the major component was investigated in the field. These trapping experiments were conducted using compounds prepared independently by Mori and Harashima (1993).

Specific experiments were also carried out in order to determine the importance of some experimental factors in field trapping of *M. feytaudi*, such as weather effects. Concentration of the compounds, duration of their attractiveness, trap design, and trap height were also studied in order to establish the optimal methods for monitoring this scale species.

[4] Initially, these compounds were incorrectly named as (2*E*,4*E*)- and (2*E*,4*Z*)-4,6,10-trimethyl-2,4-dodecadien-7-one.

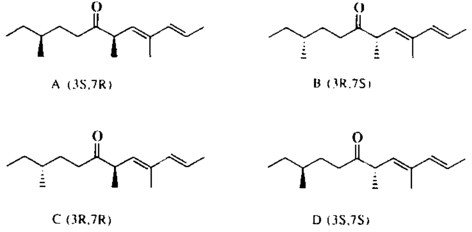

FIG. 1. Structure of the four possible stereoisomers of the major component of the *Matsucoccus feytaudi* sex pheromone.

METHODS AND MATERIALS

Chemicals

Synthetic stereoisomers of the major components of maritime pine scale pheromone were prepared and applied as hexane solutions to standard red rubber septa (SNL, France) at load rates of 1–200 μg/septum. Throughout the text, these isomers are referred to as 3*S*,7*R* (A), 3*R*,7*S* (B), 3*R*,7*R* (C), and 3*S*,7*S* (D).

Trapping Design

The sticky traps consisted of a piece of plasticized cardboard, coated on one side. The first trap model had a square shape (21.5 × 21.5 cm) and was subdivided into 16 squares (5 × 5 cm) outlined in its central part. It was tied to the trunk of a tree. The second model, of the same area, had a circular shape (12 cm radius) and was subdivided into four concentric circles (ring width of 3 cm) outlined in its central part. It was fastened vertically to a 1.30-m wooden stake. For both models, the rubber septum was pinned to the center of the trap.

According to the subdivisions outlined on the trap surface, concentric zones were defined. For the square traps, the central zone corresponded to the four inner 5-cm × 5-cm squares, the medium zone to the 12 other squares, and the external zone to the edges. For the circular traps, the central zone corresponded to the inner disk, the internal, medium, and external zones corresponded to the first, second, and third rings, respectively. Catch density in each zone could thus be obtained by dividing the total catches of a zone by its proper area. In all plots, the traps were oriented east or south in order to protect them from dominant winds and rain.

Field Bioassays

The field experiments were conducted in stands of maritime pine at the INRA Forest Research Centre, Bordeaux, France, from March 3 to 24, 1992.

Experiment 1: Relative Attractiveness of Four Isomers. Five treatments were compared: A, B, C, D (200 μg each) and a solvent blank. Twenty-five baited traps were placed in the same stand in a Latin square design (i.e., five replicates of the five treatments, 20 m minimum distance between traps). Traps were tied 1.3 m above ground level. All traps were assessed in the same afternoon, nine times between March 5 and 23. Pheromone caps were renewed on March 17. The nine counts were summed for each trap. Experiment 1 was thus analyzed with five replicates for each treatment.

Experiment 2: Relative Attractiveness of Isomers A and B at Lower Doses. Based on structure, determined previously by NMR, isomers A and B were considered to be the best candidates for the major component of the natural

pheromone. Three treatments were compared: 100 μg A, 100 μg B, and a solvent blank. Eighteen baited traps were placed in the same stand in a randomized block design (six replicates of the three treatments, 20 m minimum distance between traps). Traps were tied to the trunks 1.3 m above ground level. All traps were assessed in the same afternoon, 10 times between March 4 and 24. Pheromone caps were renewed on March 17. The 10 counts were summed for each trap. Experiment 2 was thus analyzed with six replicates for each treatment.

Experiment 3: Dose Effect of Isomer A on Its Own Attractiveness. Circular sticky traps were used. Four treatments were compared: 50, 10, and 1 μg of A and a solvent blank. Twenty-four traps were placed in a randomized block design (six replicates of the four treatments, 20 m minimum distance between traps). Traps were placed 1.30 m above ground level, between two pine trees. Capture counts were taken in the same afternoon, nine times between March 19 and April 22. The nine counts were summed for each trap. Experiment 3 was thus analyzed with six replicates for each treatment.

Experiment 4: Duration of Attractiveness of Isomer A. Three treatments were compared: rubber septa impregnated with 50 μg of A and previously aged in the field for two weeks, caps freshly impregnated with 50 μg of A, and a control (solvent blank). The treatments were tested in six experimental units of three adjoining trees. Each tree was associated with only one treatment, and all the sticky traps were tied at 1.30 m above ground level. Four units were placed in the 15-year-old stand and two units in the 16-year-old stand with a minimum distance between units of 50 m. All the traps were assessed in the same afternoon, four times between March 18 and 25. The four counts were summed for each trap and data from the two plantings pooled. Experiment 4 was thus analyzed with six replicates for each treatment.

Experiment 5: Pheromone Efficiency and Trap Height. An experimental unit of three adjoining trees, 4 m apart from each other, was designed. On the first tree, one sticky trap, baited with two distinct rubber septa impregnated with A (50 μg) and B (50 μg) respectively, was placed on the trunk at 1.3 m above ground level. On the second tree, two sticky traps, baited with one septum impregnated with the solvent only (test trap), were placed at 1.3 and 3 m, respectively, the latter corresponding to the height of the first living branches. On the third tree, a pheromone-baited sticky trap (50 μg A + 50 μg B) was placed at 3 m above ground level. Four replicates of this experimental unit, at least 50 m apart from each other, were set in three different stands (15, 16, and 19 years old) at least 500 m apart from each other. Male captures were counted in the afternoon, seven times between March 4 and 13. The seven counts were summed for each trap and data from the three stands pooled. Experiment 5 was thus analyzed with 12 replicates of each type of trap × height combination.

Statistical Analyses

All statistical analyses were carried out using SAS Software (SAS Institute, 1987). All confidence intervals of the mean were given at the 5% level and calculated as $CL = t_{\alpha/2} \times \frac{s}{\sqrt{n}}$ (Scherrer, 1984).

For variables expressed in percent, analyses of variance (ANOVA) were computed following arcsin $(\sqrt{x})$ transformation.

Data for male catch per trap were tested for homoscedasticity with Bartlett's test. They were found to be heteroscedastic with chi-square as follows: 23.7 ($df = 4$), 40.6 ($df = 2$), 29.0 ($df = 4$), 29.9 ($df = 2$), 20.4 ($df = 2$) in experiments 1, 2, 3, 4, and 5, respectively. Since the standard errors were consistently correlated with the means, raw data were log-transformed (Dagnelie, 1973).

RESULTS AND DISCUSSION

Relative Attractiveness of Isomers A–D. In field tests (Table 1), at a dose of 200 μg, traps baited with A or C isomers exhibit high attractiveness towards

TABLE 1. MEAN (± CONFIDENCE INTERVAL) NUMBER OF *Matsucoccus feytaudi* MALES ATTRACTED TO SYNTHETIC ISOMERS OF PHEROMONE IN DIFFERENT TREATMENTS (e.g., ISOMER ARRANGEMENTS × RATES × AGES)

	Treatment				
Experiment	Attractant	Rate (μg)	Age (week)	Mean male catch/trap[a]	ANOVA[b]
1	A	200	0	307.8 ± 124.2[a]	
	C	200	0	261.4 ± 176.7[a]	
	D	200	0	40.8 ± 40.9[b]	
	B	200	0	20.0 ± 17.3[b]	
	solvent blank	0	0	2.02 ± 1.2[c]	$P = 0.0001$
2	A	100	0	406.7 ± 168.1[a]	
	B	100	0	9.5 ± 13.0[b]	
	solvent blank	0	0	1.8 ± 2.5[b]	$P = 0.0001$
3	A	50	0	129.2 ± 47.3[a]	
	A	10	0	38.7 ± 7.2[b]	
	A	1	0	15.8 ± 3.7[c]	
	solvent blank	0	0	1.2 ± 0.8[d]	$P = 0.0001$
4	A	50	0	380.0 ± 221.7[a]	
	A	50	2	161.2 ± 107.6[a]	
	solvent blank	0	0	6.5 ± 1.3[b]	$P = 0.02$

[a] Means followed by the same letter are not significantly different at $P = 0.05$ (Scheffe's multiple range test)

[b] Variance analyses were made after log transformation.

M. feytaudi males, whereas these insects respond weakly to B and D (experiment 1). At a lower dose (100 μg), A shows the same high activity, whereas B shows no activity (experiment 2).

Since A and C do not differ significantly in their activity (experiment 1) and B and D are both weakly attractive, it may be concluded that *R* stereochemistry at C_7 is necessary for attractiveness. Furthermore, this activity does not depend on the absolute configuration of the second asymmetrical carbon (C_3). The conclusions are in complete agreement with the very high stereochemical purities of the synthesized samples, which can be deduced (Mori and Harashima, 1993) as follows:

A:3*S*,7*R* (99.3%) and 3*S*,7*S* (0.7%); B:3*R*,7*S* (98.3%), 3*S*,7*S* (1.3%), and 3*R*,7*R* (0.4%); C:3*R*,7*R* (98.4%), 3*S*,7*R* (1.3%), and 3*R*,7*S* (0.3%); and D:3*S*,7*S* (98.2%) and 3*S*,7*R* (1.8%).

In particular, it must be noted that the activity of C is not due to the contaminating 3*S*,7*R*-isomer since an even higher content appears in D, which has almost no activity. On the other hand, the weak activity observed for samples B and D at high doses (Table 1, experiment 1) could result from the presence of the 3*R*,7*R* or 3*S*,7*R* isomer, respectively.

Enantiomeric specificity has also been demonstrated previously in the case of diaspids such as California red scale, *A. aurantii* (Gieselmann et al., 1980), yellow scale, *Aonidiella citrina*, (Roelofs et al., 1982) and white peach scale, *Pseudaulacaspis pentagona* (Heath et al., 1980). However, the present study provides the first evidence of such an effect in the *Margarodidae* family.

This high activity shown by A in the presence of B (Table 2, experiment 5), if confirmed by further experiments would indicate the absence of any inhibitory effect (at least at the doses used) by the latter compound. According to

TABLE 2. MEAN (± CONFIDENCE INTERVAL) NUMBER OF *Matsucoccus feytaudi* MALES CAUGHT AT TRUNK (1.3 m HEIGHT) AND BRANCH (3 m HEIGHT) LEVELS IN PHEROMONE (A 50 μg + B 50 μg) AND CONTROL (SOLVENT BLANK) TRAPS

Trap	Trap height	*N*	Mean catch/trap[a]	ANOVA[b]
Pheromone	trunk	12	141.1 ± 51.8[a]	
Pheromone	branch	12	119.3 ± 42.0[a]	
Control	trunk	12	3.9 ± 2.8[b]	
Control	branch	12	6.8 ± 5.3[b]	
				$P = 0.0001$

[a] Means followed by the same letter are not significantly different at $P = 0.05$ (Scheffe's multiple range test).

[b] Variance analyses were made after log transformation.

experiments 1 and 2, A and C exhibit similar high activities and B and D similar very low activities. These observations suggest the future use of a racemic material, probably accessible by a more economic synthetic route for practical applications.

Response of Males to Dilutions of Isomer A. In field tests (experiment 3), the concentration of formulation A showed a significant positive effect on trap catch efficiency when studied in the 1- to 50-μg range (Table 1). Even when baited with 1 μg, pheromone traps exhibited a higher trap efficiency than controls. These observations suggest the use of low pheromone doses in estimating population levels, since they usually provide the best correlation between catch and population densities (Audemard and Sauphanor, 1980).

On the other hand, the range of doses tested in experiment 3 is too small to allow the determination of the optimal dose of the attractive compound. However, experiments 1 and 2, which took place within the same period, in nearby stands of the same age, show similar attractiveness of the traps baited with A at 100 μg and 200 μg. The effect of concentration may therefore reach a maximum at about 100 μg. *Matsucoccus resinosae* (Bean and Godwin) males exhibit a similar rapid initial increase of response to female crude extracts and a similar decline thereafter (Park et al., 1986). Ortu and Delrio (1982) note that the use of a very high pheromone dose does not significantly increase the catches of *Planococcus citri* (Risso) males. Likewise, using live females, catches of California red scale males were shown to reach a maximum (Rice and Moreno, 1970). Thus, above a certain dose (or female equivalents), excessive release of pheromone molecules could significantly lessen the attraction of the emitting caps or females.

Spatial Distribution of Captures on Trap Surface. In experiment 3, pheromone-baited circular traps did catch numerous males although these traps had not been tied to the trees. Thus, the main contribution to the captures probably involves flying insects instead of males walking on the trunks.

The relative catch density in each zone of these traps is calculated as the quotient of male catch in a trap zone by the total catch in the trap and the trap zone area (Figure 2). Then the variance of the relative catch density is analyzed (ANOVA) according to pheromone dose, trap zone, and the interaction of these two factors (six replicates). The relative catch density varies significantly with the trap zone ($P = 0.001$). It consistently increases to the external zone (Figure 2), except for control traps. This characteristic distribution of males in the trap zones would then be related to the presence of pheromone. The relative catch density also varies significantly according to the interaction between zone and pheromone dose ($P = 0.01$). The relative proportion of capture in the external zone of the trap differs more from the others with higher doses (Figure 2).

Thus, a repulsive effect of the septa may exist, occurring in the first few centimeters around them. The flying males, being attracted by the pheromone

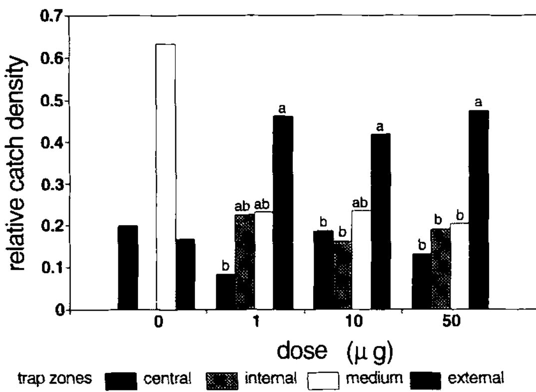

FIG. 2. Relative densities of *M. feytaudi* males caught in the different concentric zones of the trap (calculated as number of males caught in a zone/total number of males caught in the trap per trap zone area), according to the dose of isomer A (experiment 3). Bars (mean relative catch densities) with the same letter are not significantly different at $P = 0.05$ (Scheffe's multiple range test, after log transformation).

odor at a distance, could thus remain on the periphery of the trap surface by too high a concentration of pheromone. A similar effect has been observed with San Jose scale at the beginning of the trapping period with high rates of pheromone (Rice and Hoyt, 1980). According to these authors, a large amount of released pheromone may confuse responding males before they reach the traps. In the same manner, in laboratory bioassays, Lanier et al. (1989) have reported a decrease in the attractiveness of *M. resinosae* pheromone at >0.01-μg rates. At these values, walking males move sideways from the odorous source. One could also hypothesize that a certain concentration threshold of pheromone may have induced landing behavior in the flying insect. As the pheromone dose increases, flying scales would reach this concentration threshold progressively further from the releasing septa, thus inducing a higher catch in the more external zones of the traps.

Whatever the reason, pheromone septa would therefore prevent males from being caught in their vicinity. This indicates that the trapping efficiency can be related to the trap area as well as to the pheromone dose.

Duration of Synthetic Pheromone Attractiveness. When recorded weekly (experiment 4), the attractiveness of 2-week-old septa baited with 50 μg A is not significantly lower than that of fresh septa (Table 1). The male catch related to the 2-week-old septa is two fold less than the catch obtained with new septa, but 25-fold more than in the control traps. Moreover, in experiments 1 and 2, the male catches in the A pheromone traps are always significantly higher than in the test traps when analyzed at 12- to 13-day periods. It may be concluded

therefore that the attractiveness of the lures baited with synthetic *M. feytaudi* pheromone lasts at least two weeks.

As a comparison, rubber septa kept in the field show no loss of attractiveness before five weeks with *P. citri* pheromone (250 μg) (Ortu and Delrio, 1982), before six weeks with the California red scale pheromone (10–100 μg) (Tashiro et al., 1979), and before 10 weeks in the case of the San Jose scale (33–900 μg) (Rice and Hoyt, 1980; Anderson et al., 1981). As a rule, the pheromone attractiveness of scale appears to be very persistent.

In experiments 1, 2, and 5, we computed the yield of capture for each counting date as follows:

$$\text{yield of capture} = \frac{\text{mean catch in A traps} - \text{mean catch in test traps}}{\text{mean catch in A traps}}$$

In traps baited with 50 μg and 100 μg, the yield of capture (y) significantly decreases with the days of weathering (x) according to the curve: $y = 1 - e^{ax+b}$ (Figure 3.). In traps baited with 200 μg, the yield of capture does not significantly differ from 1, during the same trapping period. Extrapolating from these models, the duration of weathering necessary to bring the yield of capture to 50% would be 13 days for 50 μg of A, which is consistent with the results of experiment 4. Thus, the decrease in the attractiveness of the pheromone traps

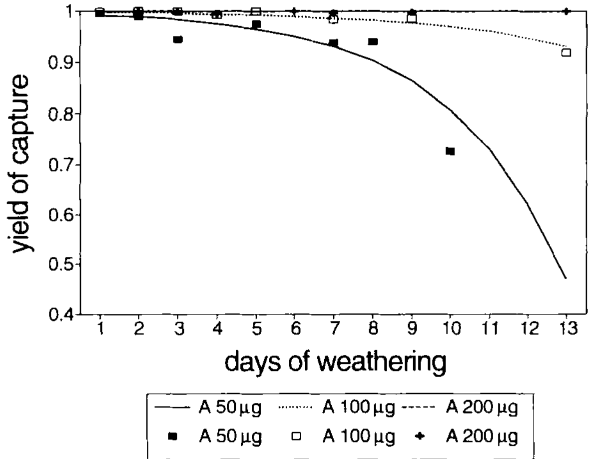

FIG. 3. Regression of the yield of capture against the days of weathering, according to the dose of isomer A (experiments 1, 2, and 5).
$y = 1 - e^{0.339x - 5.038}$ ($r^2 = 0.79$, $P = 0.01$) for dose of 50 μg; $y = 1 - e^{0.264x - 6.106}$ ($r^2 = 0.94$, $P = 0.01$) for dose of 100 μg; and $y \approx 1$($r^2 = 0.29$, $P = 0.64$) for dose of 200 μg.

appears to be slow and continuous. It may also be dependent on the load rates as the duration of efficiency is shorter with the 50 μg formulation than with the 100 μg one, and even shorter when compared to the 200 μg one.

Determination of Optimal Trap Height and Support. Statistical analysis shows no significant difference between the number of males caught at the trunk (1.3 m) or branch level (3 m) for both pheromone-baited traps and solvent blank traps (experiment 5, Table 2). Since the test traps catch passively dispersing males, it means that these insects are to be found equally at the two levels. In contrast, *Aonidiella aurantii* (Coquillett) (Rice and Moreno, 1970) and *P. citri* (Moreno et al., 1984) are mainly caught on the middle third of their host trees, at the crown level. Scale males may prefer to fly at the level where the probability of meeting the females is higher, such as the trunk and the main branches in the case of *M. feytaudi* (Riom, 1980).

Effects of Weather on Trapping Efficiency. Scale capture and weather con-

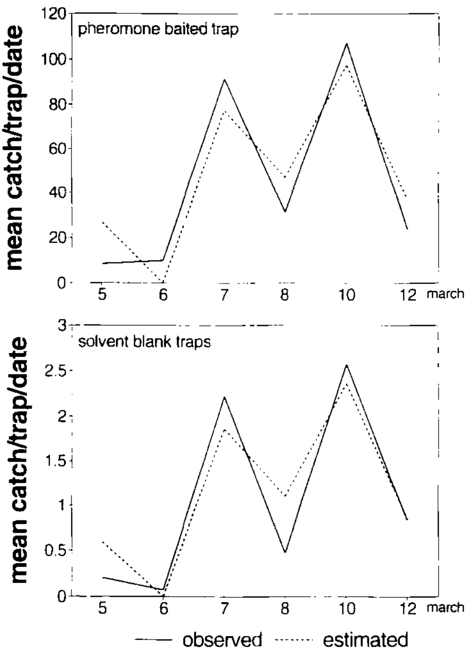

FIG. 4. Average number of male *M. feytaudi* caught per trap per date in the counting period (solid line) in experiment 5. Estimated numbers of male *M. feytaudi* caught per trap per date according to the wind speed of the preceding day (dashed line): $y = 137.57 - 100.78x$ ($r^2 = 0.83$, $P = 0.05$) for the pheromone baited traps, and $y = 3.37 - 2.53x$ ($r^2 = 0.83$, $P = 0.05$) for the solvent blank traps.

ditions were recorded almost daily in experiment 5. The number of daily test trap captures did not increase or decrease unvaryingly (Figure 4); we could thus assume that their fluctuation did not represent variation in population level. Since the pattern of daily captures is similar for the pheromone baited traps and the control test traps, the fluctuations in trapping efficiency could not be related to the development of cap attractiveness. Accordingly, the daily patterns of trap efficiency have been studied in relation to the weather conditions. Six parameters have been computed: average temperature, relative humidity, and wind speed of the real day and the day before the date of counting. Stepwise regression procedures only entered the wind speed of the preceding day, and this variable had a negative influence on the number of male catches.

Wind speeds greater than 1 m/sec would prevent males from flying up to the pheromone source and drastically reduce trapping efficiency. Doane (1966) reported that *M. resinosae* males are forced to fly downward when airspeed exceeds 0.5 m/sec, the same value that prevents upwind flight of *A. aurantii* (Rice and Moreno, 1970). Scale, as poor flyers, would wait for calmed winds before taking off. The weather conditions that correlate best with the trap catches were recorded the day preceding counting, which began at midday. Thus, the greater part of the captures would have been made on the afternoon preceding counting; high trap catches could depend on a combination of low wind speeds and temperatures higher than the flight threshold (Rice and Hoyt, 1980; Ortu and Delrio, 1982).

CONCLUSION

Field experiments have shown the importance of the stereochemistry of the main component of the sex pheromone of *M. feytaudi*, particularly regarding the absolute configuration of C_7.

The efficiency of sticky traps baited with the synthetic pheromone, as demonstrated with endemic populations, proved to be sensitive enough to allow further developments of this trapping method in the field of pest monitoring.

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ERGOVALINE AND PERAMINE PRODUCTION IN ENDOPHYTE-INFECTED TALL FESCUE: INDEPENDENT REGULATION AND EFFECTS OF PLANT AND ENDOPHYTE GENOTYPE

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Abstract—Peramine and ergovaline have ecological and economical significance because they possess insect and/or mammalian toxicity properties. The relationship between these endophytically derived alkaloids in tall fescue (*Festuca arundinacea* Schreb.) is unknown. Seasonal and plant tissue effects on the concentration of peramine and ergovaline was investigated in field and greenhouse experiments. The relationship between the alkaloids and the regulatory effects of endophyte and plant on their content were investigated among progeny of reciprocal crosses between high- and low-ergovaline and peramine plant–endophyte combinations. Variation in peramine concentration ranged from 750 to 1742 μg/kg in greenhouse-grown plants, and there was no seasonal trend in peramine content. There was a correlation (r = 0.69) between peramine and ergovaline content among leaf tissues of field-grown plants, but there was no correlation between the alkaloids in the culm (r = 0.20) or panicle (r = 0.17) tissues. Mean leaf ergovaline concentration of progeny derived from the low-ergovaline parent (163 μg/kg) was less than the mid-parent value (228 μg/kg), but mean of progeny from the high-ergovaline parent was not different from the mid-parent value. Ranges within each progeny set were approximately double their mean. Mean leaf peramine concentrations of the progeny sets were similar to the mid-parent value (3354 μg/kg) but ranges were from 1716 to 8763 μg/kg. There was no correlation between leaf peramine and ergovaline (r = 0.01). These data suggest that endophytically produced alkaloids are independently regulated and are controlled by both plant and endophyte genotype.

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Key Words—Tall fescue, fescue toxicosis, endophyte, alkaloids, peramine, ergovaline, *Acremonium coenophialum*.

INTRODUCTION

Three classes of alkaloids are responsible for resistance to grazing animals in endophyte-infected (*Acremonium coenophialum* Morgan-Jones and Gams) tall fescue. Of the three alkaloids, ergot and peramine are produced by the endophyte (Rowan and Gaynor, 1986; Siegel et al., 1990) but loline alkaloids are produced by the plant in response to the endophyte (Bush et al., 1982; Belesky et al., 1989). Each alkaloid affects specific animal species that feed on the host plant. Loline alkaloids confer resistance to *Schizaphis granum* (Rondani) and *Rhopalosphum padi* (L.) aphids. Peramine confers resistance to *R. padi* in tall fescue (Siegel et al., 1990) and the Argentine stem weevil [*Listronotus bonariensis* (Kuschel)] in perennial ryegrass (*Lolium perenne* L.) (Rowan et al., 1986). Ergot alkaloids confer resistance to ungulate grazers (Hill et al., 1994). Drought (Elmi et al., 1989) and disease (Clay, 1988) resistances are also associated with the endophyte in tall fescue.

The positive effects of the endophyte on the tall fescue host, combined with its deleterious effects on grazing animals, present livestock producers with a biological dilemma. If they utilize endophyte-infected tall fescue for pasture, livestock suffer toxicosis conditions (Hoveland et al., 1983; Read and Camp, 1986), but when they utilize endophyte-free tall fescue, they risk loss of the pasture due to environmental and/or insect stresses (Siegel et al., 1990; Rowan et al., 1986; Read and Camp, 1986; Elmi et al., 1989).

An alternate approach would be to select and breed endophyte-infected cultivars that are not toxic to grazing livestock (Hill, 1993). Ergovaline, the predominant ergot alkaloid in endophyte-infected tall fescue (Lyons et al., 1986), varies within individual plants depending upon the genotype of the host plant (Agee and Hill, 1994). A breeding strategy designed to reduce toxicity of endophyte-infected tall fescue seems plausible if other beneficial endophytic products, including peramine, are not concomitantly reduced. Therefore, an understanding of the relationship between ergovaline and peramine contents of endophyte-infected tall fescue is needed before manipulation of the plant–endophyte association for reduced livestock toxicity can be pursued.

Seasonal, plant genotypic, and plant morphological effects on peramine content in endophyte-infected tall fescue are unknown, complicating the design of meaningful experiments for determining the relationship between peramine and ergovaline. The objectives of this study were to determine: (1) seasonal variation in peramine concentration in endophyte-infected tall fescue; (2) peramine and ergovaline content in endophyte-infected tall fescue tissues; and

(3) peramine and ergovaline content within progeny sets from a reciprocal cross between high- and low-ergovaline-content tall fescue genotypes.

METHODS AND MATERIALS

Experiment 1. Seasonal Variation in Peramine Concentration in Tall Fescue. Five endophyte-infected tall fescue genotypes, known to vary in ergovaline content, were selected from a Kentucky-31 population (Agee and Hill, 1994). Five plants from each genotype were vegetatively propagated, under greenhouse growing conditions, by placing individual tillers in 4-liter pots containing a 1 : 1 : 1 soil mix of Cecil sandy clay loam (Typic Hapludult, mixed, mesic, thermic)–peat–perlite. On November 1, 1991, three plants of uniform size from each genotype were selected and arranged on the greenhouse bench in a randomized complete block design using three replications. Greenhouse conditions consisted of 25/20°C day/night temperature in the fall, winter, and spring, and a 32/24°C day/night temperature from June through mid-September. Plants were fertilized weekly with 0.3, 0.13, and 0.25 g/pot of N, P, and K, respectively, using a commercial greenhouse liquid fertilizer.

All plants were harvested beginning January 1991 and every 28 days thereafter for 14 months, whenever sufficient plant tissue was present for alkaloid analysis. Herbage was hand-clipped to a height of 2 cm above the soil surface, packed in ice, transported to the laboratory, and frozen at −70°C. Samples were freeze-dried, ground in a Wiley mill to pass a 1-mm screen, and stored at −70°C until analyzed for peramine.

Plant genotype and harvest date effects were determined by analysis of variance using PCSAS (SAS Institute, Cary, North Carolina). Genotypes were treated as whole plots and harvest dates as splits of the whole plot. The mean square for genotypes within replications was used as the error term to test for significance of plant genotypes. The residual error term was used to determine the significance of harvest dates and the genotype × harvest date interaction. Treatments were separated with a Fisher's protected LSD.

Experiment 2. Peramine and Ergovaline Content in Tissues of Endophyte-Infected Tall Fescue. Five endophyte-infected tall fescue genotypes were selected based on diverse morphology from a pasture of Kentucky-31 tall fescue growing at the USDA-ARS Southern Piedmont Soil and Water Conservation Lab in Watkinsville, Georgia. Each of the five genotypes was vegetatively propagated by planting five individual tillers in 4-liter pots containing a 1 : 1 : 1 soil mix of Cecil sandy clay loam soil (Typic Hapludult, mixed, mesic, thermic)–peat–perlite. On January 14, 1991, three uniform plants from each genotype were selected and planted in the field. The experimental site was located on a Pacolet soil (clayey, kaolinitic, thermic, typic Kanhapludult) at the UGA Plant Sciences

Farm near Watkinsville, Georgia. The soil had been plowed, disked, and fumigated with methyl bromide on January 3, 1991. Plant genotypes were randomly assigned and planted on 0.67-m centers in three complete blocks, with each block serving as a replication. At planting, the plot was fertilized with N, P, and K at a rate of 80, 35, and 66 kg/ha, respectively. Plots were fertilized again on March 5, 1991, with N, P, and, K at a rate of 150, 66, and 125 kg/ha, respectively, and again in March 1992 and 1993. Plants were permitted to establish in 1991.

In 1992, significant quantities of ergot [*Claviceps purpurea* (Fr.) Tul.] and leaf rust (*Puccinia coronata* Cda.) were present on some plant genotypes, which confounded plant health with susceptible or resistant genotypes to each of these diseases. It was felt that the presence of ergot in the panicles of specific genotypes might artificially inflate ergovaline content of those plants. It also was felt that plants susceptible to leaf rust may have artificially reduced alkaloid concentration. Therefore, tissues were not analyzed for alkaloids during 1992.

Plants were harvested when seed of all plant genotypes were at the hard dough stage on June 6, 1993. Herbage was hand-clipped to a height of 10 cm above the soil line. Leaf blades were removed from the sheaths at the ligule, and the panicles were removed from the culms by cutting the peduncle 2 cm below the bottom pedicel. Leaf blades, panicles, and culms with leaf sheaths were frozen at −70°C and freeze-dried. The separate plant parts were ground in a Wiley mill to pass a 1-mm screen and stored at −70°C until analyzed for ergovaline and peramine. Dry weights for each tissue type were not recorded. Genotypic values for alkaloid concentration were, therefore, based upon arithmetic means of the tissue types. As a consequence, genotypic means of alkaloid concentration will not be reported for this experiment. However, genotypic alkaloid concentration was included into the mathematical model for appropriate statistical analysis for the effects of the various plant tissue types and their interactions with plant genotypes.

Effects of genotype and tissue types on ergovaline and peramine content were determined by the PROC ANOVA subroutine of PCSAS. The model was a factorial arrangement between plant genotypes and tissue types within the randomized complete block design. Means were separated using a Fisher's protected LSD. Correlations between ergovaline and peramine were determined within each tissue type using the PROC REG subroutine of PCSAS.

Experiment 3. Peramine and Ergovaline Content in Progeny from Reciprocal Cross between High- and Low-Ergovaline-Containing Tall Fescue Genotype. Two genotypes of tall fescue, PDN15 and PDN12, were previously identified as high- and low-ergovaline-producing plants, respectively (Agee and Hill, 1994). The plants were reciprocally pollinated to provide one set of progeny from each maternal parent, each being of homologous genetic content. Because endophytes are passed maternally through the seed (Bacon et al., 1977), the

endophyte in plants within a progeny set was identical. However, the two endophytes were genetically unique for the two progeny sets.

A subset of 40 endophyte-infected plants from each progeny set was vegetatively propagated on August 20, 1992. Ramets of three to five tillers from each of the 80 genotypes were planted into 4-liter pots. The parent plants, PDN12 and PDN15, were also vegetatively propagated. The progeny and parent plants were randomly assigned to each of four greenhouse benches, with each bench serving as a replication. Plants were fertilized weekly with 0.3, 0.13, and 0.25 g/pot of N, P, and K, respectively, with a commercial greenhouse fertilizer and maintained in the greenhouse with a 25/20°C day/night temperature regime.

Plants were harvested November 13, 1992, by hand clipping to a height of approximately 10 cm above the soil line. Care was taken to harvest leaf tissue only. Harvested tissue was frozen at −70°C, freeze-dried, and ground in a Wiley mill to pass through a 1-mm mesh. Samples were stored at −70°C until analyzed for alkaloids.

Treatment effects on ergovaline and peramine concentration were determined using the PROC ANOVA subroutine of PCSAS. Significances of parental means were determined by assigning the parents to a randomized complete block model and means were separated using a Fisher's protected LSD. The maternal parent effect, which was assumed to be that of the endophyte, was tested by assigning each of the 80 progeny genotypes to their maternal parent in a randomized complete block design, and differences between means of the two populations were determined with a Fisher's protected LSD. Genotypes within each progeny set were assigned to a randomized complete block design. Analyses of variance were conducted on the progeny sets independently, and means for plant genotypes within each progeny set were tested for differences in alkaloid concentration, using a Fisher's protected LSD. To test for associations between ergovaline and peramine, all progeny were included into one statistical model. Ergovaline was assigned to the dependent variable and peramine to the independent variable in a regression analysis using the PROC REG subroutine of PCSAS.

Laboratory Analysis. Extraction and analysis of plant tissue for ergovaline were conducted using the procedures of Hill et al., (1993). One-gram subsamples of tissue were extracted with 40 ml chloroform–0.01 M sodium hydroxide (9 : 1 v/v). The mixture was passed through a generic coffee filter and 20 ml of eluate were then passed through a HL silica gel solid-phase chromatography column. Plant pigments were eluted with 1 ml chloroform–acetone (75 : 25). The column was eluted with 2 ml methanol and the eluate injected directly into the HPLC for ergovaline detection. Extraction efficiency averaged 91% as determined by HPLC of recovered ergotamine tartrate-spiked internal standards.

The HPLC configuration was a Shimadzu (Columbia, Maryland) model

LC-600 liquid chromatograph equipped with a SIL-9A auto injector and a RF-525 fluorescence spectrophotometer (excitation wavelength: 250 nm; emission wavelength: 420 nm) with a 100 × 4.6-mm Alltech (Deerfield, Illinois) column packed with 3-μm Adsorbosphere HS C_{18}. An isocratic mobile phase of 38% acetonitrile in a 200 mg/liter aqueous solution of ammonium carbonate.

Peramine was extracted using a method modified from Tapper et al., (1989). One-half gram subsamples of tissue were extracted in a two-phase solvent system, first in 6 ml of methanol–chloroform (1 : 1) for 30 min, followed by 6 ml each of hexane and distilled water. Following centrifugation, a 1-ml portion of the aqueous phase was passed through a 100-mg Analytichem Bond Elut CBA column (Varian, Harbor City, California). The column was washed with 1 ml of water, after which the bound peramine was eluted with 0.5 ml of 5% aqueous formic acid. Extraction efficiency averaged 81% as determined by HPLC from five peramine sulfate-spiked endophyte-free tall fescue samples.

Peramine detection was conducted using a Shimadzu model LC-600 liquid chromatograph equipped with a SIL-9A auto-injector and a SPD-6AV multi-wavelength UV detector at 280 nm with a 100 × 4.6-mm alltech Adsorbosphere HS C_{18}, 3-μm column. Standard solutions were made from peramine sulfate (donated by G.C.M. Latch, Palmerston North, New Zealand) in 20% (v/v) aqueous propan-2-ol. An isocratic mobile phase of 18% (v/v) acetonitrile in a guanidine carbonate–formic acid buffer was used (Tapper et al., 1989).

RESULTS AND DISCUSSION

Experiment 1. Seasonal Variation in Peramine Concentration of Tall Fescue. No harvests were made between November 1991 and February 1992, because plants became pot-bound and did not grow vigorously. During the remainder of the experimental period, differences in peramine concentration were found among genotypes and harvest dates in the greenhouse-grown tall fescue (Table 1, Figure 1). Plant genotypes PDN14, PDN15, and PDN16 had the highest concentrations of peramine and were not different from one another (Table 2). Plant genotypes PDN12 and PDN13 were lower than the other plant genotypes, but PDN13 was lower in peramine concentration than PDN12.

Peramine concentrations differed among dates, but there were no seasonal trends as to when peramine would be at a maximum or minimum concentration within the plant tissue (Figure 1). Mean maximum and minimum peramine values varied by as much as 132% depending upon the date of harvest. The relative concentrations of peramine among plant genotypes within harvest dates was the same because there was no interaction between plant genotype and harvest date. This differed from what others have found for ergovaline. Using the same genotypes, Agee and Hill (1994) found that ergovaline in greenhouse-

TABLE 1. MEAN SQUARES (MS) FOR PERAMINE CONCENTRATION IN 5 GREENHOUSE-GROWN GENOTYPES OF ENDOPHYTE-INFECTED TALL FESCUE (EXPERIMENT 1)

Source	*df*	MS (μg peramine/kg tissue)
Genotype	4	7782967.0[a]
Rep	2	233541.0
Error A	8	726181.1
Date	9	1978068.0[a]
Genotype × date	36	562871.0
Error B	83	419930.3

[a] Significant at the 0.05 level of probability.

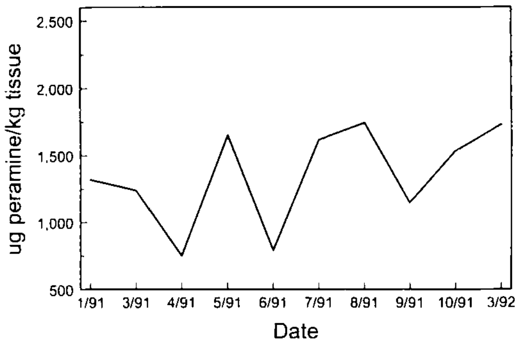

FIG. 1. Mean seasonal distribution of peramine concentration in five, greenhouse-grown, tall fescue genotypes (experiment 1).

TABLE 2. MONTHLY AND GENOTYPIC MEANS FOR PERAMINE CONCENTRATION OF GREENHOUSE GROWN ENDOPHYTE-INFECTED TALL FESCUE (EXPERIMENT 1)

Genotype	Peramine (μg/kg tissue)
PDN12	989
PDN13	513
PDN14	1776
PDN15	1674
PDN16	1729
LSD (0.05)[a]	453

[a] Least significant difference at the 0.05 level of probability.

grown tall fescue peaked in spring and fall. In addition, they determined that high-ergovaline-producing plants do not necessarily express ergovaline production and were not different from low-ergovaline-producing genotypes during the summer months. Therefore, if comparisons are to be made between peramine and ergovaline, harvest scheduling should be based upon the likelihood that ergovaline will be at a maximum, assuming that harvest date will have no effect on the relative concentrations of peramine.

Experiment 2. Peramine and Ergovaline Content in Tissues of Endophyte-Infected Tall Fescue. Peramine content differed among tall fescue genotypes and tissue types, but there was no genotype × tissue type interaction (Table 3). Therefore, peramine content of any one tissue type is indicative of the relative concentration of peramine in the plant. Stem tissue had a greater concentration of peramine than did leaf blades or panicles (Table 4).

There was a significant interaction among plant parts and genotypes in ergovaline concentration (Table 3). For example, genotype PDN2 had the low-

TABLE 3. MEAN SQUARES FOR ERGOVALINE AND PERAMINE CONCENTRATION IN PLANT TISSUES OF 5 GENOTYPES OF ENDOPHYTE-INFECTED TALL FESCUE (EXPERIMENT 2)

Source	*df*	Mean square (μg alkaloid/kg tissue)	
		Peramine	Ergovaline
Genotype	4	2603139[a]	1137287[a]
Rep	2	732768	241830
Tissue type	2	8828838[a]	3493908[a]
Genotype × tissue type	8	1333877	252534[a]
Error	28	695971	102186

[a] Significant at the 0.05 level of probability.

TABLE 4. MEAN PERAMINE CONCENTRATION IN PLANT TISSUE OF 5 ENDOPHYTE-INFECTED TALL FESCUE GENOTYPES

Plant part	Peramine (μg/kg tissue)
Leaf blade	2729
Culm	4210
Panicle	3118
LSD (0.05)[a]	624

[a] Least significant difference at the 0.05 level of probability.

est, and PDN11 the highest, concentrations of ergovaline in the leaf blade (Figure 2). Ergovaline concentrations among these two genotypes were not different from one another in the stem tissue but differed in the panicle. Conversely, ergovaline concentrations in leaf blades of PDN14 and PDN15 were not different, but PDN14 had more than twice the concentration of ergovaline in the culms compared to PDN15. Similar comparisons of ergovaline concentrations between other plant tissues could be made among genotypes (e.g., PDN12 vs. other genotypes).

Inasmuch as there was no plant genotype × tissue type interaction for peramine and there was a genotype × tissue type interaction for ergovaline, correlations between the two alkaloids were conducted within each tissue type across all genotypes. No correlations were found between ergovaline and peramine concentrations within culm (r = 0.20) or panicle (r = 0.17) tissue. However, within leaf tissue, the alkaloids were positively correlated (r = 0.69). While the number of plant genotypes in this study was limited, the data suggest that leaf tissue should be used to determine associations between the two alkaloids in a larger population.

Experiment 3. Peramine and Ergovaline Content in Progeny from Reciprocal Cross between High- and Low-Ergovaline-Containing Tall Fescue Genotype. Mean ergovaline concentration of PDN12 was lower than that of PDN15, verifying that the parents differed in this trait (Table 5). The progeny mean of genotype PDN15 was not different from the mid-parent, but that of PDN12 was lower than the mid-parent. This suggests that a maternal trait of PDN12 limited ergovaline production in those progeny. It is possible that the endophyte is serving as that maternal characteristic because: (1) it is passed through the seed and is present in all progeny (Bacon et al., 1977); and (2) endophytes vary in their ability to produce ergovaline (Bacon, 1988). Although the endophyte is

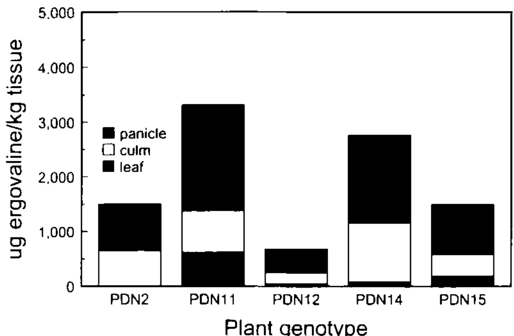

FIG. 2. Ergovaline concentration in leaf, culm, and panicle tissues of field-grown tall fescue (experiment 2).

likely to affect ergovaline concentration within the PDN12 progeny, differences between the extremes in the range of the progeny from that parent suggest that the plant also affects the endophyte's ability to produce ergovaline. The progeny mean from the PDN15 parent was not different from the mid-parent, but differences between the extremes of the progeny also suggest that the genotype of the host plant affected the endophyte's capability of producing ergovaline. These results are similar to those of Agee and Hill (1994).

As with ergovaline, parent PDN15 was higher in peramine concentration than was parent PDN12 (Table 6). The progeny means from the two parents

TABLE 5. ERGOVALINE CONCENTRATIONS OF PARENTS AND PROGENY FROM RECIPROCAL CROSS BETWEEN LOW- AND HIGH-ERGOVALINE-CONTAINING TALL FESCUE GENOTYPE (EXPERIMENT 3)

	Ergovaline (μg/kg tissue)		
	Maternal genotype		
	PDN12	PDN15	LSD (0.05)[a]
Parental mean	50	405	155
Midparent value	228		
F_1 progeny means	163	218	17
F_1 range	36-348 (94)[b]	71-435 (118)[b]	

[a] Least significant difference at the 0.05 level of probability.
[b] LSD for comparisons of individuals within each progeny set.

TABLE 6. PERAMINE CONCENTRATION OF PARENTS AND PROGENY FROM RECIPROCAL CROSS BETWEEN LOW- AND HIGH-PERAMINE-CONTAINING TALL FESCUE GENOTYPE (EXPERIMENT 3)

	Peramine (μg/kg tissue)		
	Maternal genotype		
	PDN12	PDN15	LSD (0.05)[a]
Parental mean	2382	4326	1566
Midparent	3354		
F_1 progeny means	3319	3594	413
F_1 range (LSD)	1716-5433 (2474)[b]	1767-8763 (2893)[b]	

[a] Least significant difference at the 0.05 level of probability.
[b] LSD for comparisons of individuals within each progeny set.

were not different, nor were they different from the mid-parent. However, genotypes within each progeny set differed from one another. Because the endophytes within progeny sets were identical, progeny from each parent should have had identical alkaloid contents as their parents if endophyte alone were responsible for peramine production. Inasmuch as progeny differed from the parent, the logical conclusion is that the plant genotype affected the degree to which peramine is produced by the endophyte.

There was no correlation between peramine and ergovaline contents in the progeny from the reciprocal cross ($r^2 = 0.01$). This suggests that the plant independently modified endophytic production of the two alkaloids. One mechanism for independent regulation could be specific nutrient requirements of the endophyte for production of each alkaloid. L-Tryptophan is essential for ergovaline production by endophytes, and the fungal enzyme dimethylallyl tryptophan synthase (DMATase) is the first pathway-specific enzyme for ergot alkaloid biosynthesis (Lee et al., 1976; Cress et al., 1981). If tryptophan is of limited supply to the endophyte, ergovaline biosynthesis would be inhibited. An alternate mechanism for ergovaline regulation is the availability of calcium to the endophyte. Calcium serves as a coenzyme to DMATase and would limit ergovaline synthesis if unavailable to the endophyte (Lee et al., 1976; Cress et al., 1981).

Peramine biosynthesis by the endophyte is not well understood, but peramine has been synthesized under laboratory conditions (Dumas, 1988; Brimble and Rowan, 1990). It is speculated that peramine biosynthesis involves acquisition of a cyclic dipeptide of proline and arginine (Tapper, 1993). If either amino acid were limiting to the endophyte, peramine synthesis may be inhibited.

This study indicates that peramine and ergovaline are produced by the endophyte independent of one another. Therefore, by selecting and breeding for a low ergot alkaloid concentration in endophyte-infected tall fescue, it is unlikely that a concomitant reduction in peramine will occur. If the mechanisms for alkaloid regulation are as postulated, specific nutrient effects on alkaloid biosynthesis are not likely to have an effect on the general nutrition of the endophyte. If either regulatory mechanism were affecting the general nutrition of the endophyte, both alkaloids would be lower in concentration as a consequence of a less vigorous endophyte. Our data suggest that this is not the case and, therefore, reduction of either may not affect the fitness of the endophyte. Hence, breeding endophyte-infected tall fescue with reduced toxicity to livestock is not likely to affect peramine-mediated insect resistance.

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RESPONSE OF *Matsucoccus thunbergianae* MALES TO SYNTHETIC SEX PHEROMONE AND ITS UTILIZATION FOR MONITORING THE SPREAD OF INFESTATION

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Abstract—Attraction of (2*E*,4*E*,6*R*,10*R*)-4,6,10,12-tetramethyl-2,4-tridecadien-7-one [**1**; (6*R*,10*R*)-matsuone] and its antipode [**2**; (6*S*,10*S*)-matsuone] to *Matsucoccus thunbergianae* males was studied in the laboratory and in the field. They showed stronger response to **1**. In laboratory bioassays, the threshold concentrations for male attraction with **1** and **2** were 16 fg and 150 pg, respectively. In the field, during the first two days after traps were set, traps baited with 50 μg of **1** on rubber septa or filter paper rolls caught more males than control traps. Between the sixth day and the tenth day after traps were set, in a daily trap catch experiment, the traps with 50 μg of **1** on filter paper rolls caught more males than control traps on one day only, whereas those on rubber septa were always effective. The shape of the traps did not affect male catches. Males were caught on pheromone traps in locations where no scales were found by the customary detecting procedures.

Key Words—*Matsucoccus thunbergianae*, Homoptera, Coccoidea, Margarodidae, black pine bast scale, sex pheromone, attractant, (2*E*,4*E*,6*R*,10*R*)-4,6,10,12-tetramethyl-2,4-tridecadien-7-one, matsuone, monitoring, detection.

INTRODUCTION

The black pine bast scale, *Matsucoccus thunbergianae* Miller and Park, seriously damages natural forests of Japanese black pine, *Pinus thunbergiana*, the

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most dominant tree species in southern coastal areas of the Korean peninsula. The scale may kill all sizes of *P. thunbergiana*; in heavy infestations a whole forest is virtually destroyed. It also attacks Japanese red pine, *P. densiflora*, but mortality of this species is negligible. Since its discovery in 1983, survey results have shown that the zone of infestation has been expanding at a rate of about 5 km each year (Park, 1991).

Among 26 species of *Matsucoccus* that presently infest pine trees worldwide (Park, 1991), *M. thunbergianae* in Korea, *M. resinosae* on *P. resinosa* in the United States (Anderson et al., 1976), *M. matsumurae* on *P. tabulaeformis* in China (Cheng and Ming, 1979), *M. feutaudi* on *P. pinaster* in France (Riom and Gerbinot, 1977), and *M. josephi* on *P. halepensis* in Israel (Mendel and Rosenberg, 1988) cause significant economic damage. *M. thunbergianae* is morphologically most similar to *M. matsumurae* and *M. resinosae* (Miller and Park, 1987). McClure (1983) suggested that *M. resinosae* was a junior synonym of *M. matsumurae*. This opinion was supported by Young et al. (1984) based on their morphological similarity and the cross-attractiveness between the species, but synonymy has not been formally proposed.

Park et al. (1986) isolated the sex pheromone of *M. resinosae* in the United States and demonstrated that the species has cross-attractiveness with *M. thunbergianae*. Pheromones of coccoids prior to *Matsucoccus* had proven to be species specific, as with pairs of closely related species within the genera *Aonidiella* (Moreno et al., 1972) and *Planococcus* (Rotundo and Tremblay, 1975). There was no cross-attraction between *M. josephi* and *M. resinosae* or *M. thunbergianae* (Mendel, personal communication to G.N. Lanier, 1987). Lanier et al. (1989) identified the sex pheromone of *M. matsumurae* in China, *M. resinosae*, and *M. thunbergianae* as (2*E*,4*E*)-4,6,10,12-tetramethyl-2,4-tridecadien-7-one and gave it the trivial name matsuone. Matsuone was not available for testing then, and its isomers were bioassayed. Hibbard et al. (1991) tested matsuone with unknown stereochemistry to males of the above three species and demonstrated its attractiveness in both laboratory and field experiments. Cywin et al. (1991) synthesized (6*R*,10*R*)-matsuone and its 6*R*,10*S* diastereomer and confirmed that the natural pheromone was (6*R**,10*R**)-matsuone. Accordingly, Mori and Harashima (1993b) synthesized (6*R*,10*R*)- and (6*S*,10*S*)-matsuone, pheromones **1** and **2**, respectively. We report comparative laboratory bioassay results of these two compounds with *M. thunbergianae* males in Korea. Field bioassays with (6*R*,10*R*)-matsuone and trials for utilizing this synthetic pheromone as a means of monitoring the spread of the scale infestation are also reported.

METHODS AND MATERIALS

Pheromone, Insect Sources, and Field Sites. Synthetic pheromones (Figure 1) were provided by K. Mori, University of Tokyo, Japan. The synthetic chemistry procedures were reported by Mori and Harashima (1993b). *M. thunber-*

1: (6*R*,10*R*)-matsuone 2: (6*S*,10*S*)-matsuone

FIG. 1. Structures of the synthetic pheromones tested.

gianae is univoltine and mostly emerges in the morning hours in early April (Park, 1988). Virgin females possess and release the maximum amount of sex pheromone between 8 AM and 2 PM during the first three days after emergence (Park and Abrahamson, 1991). Thus, for laboratory bioassays, second-instar nymphs on pine branches, collected from a Japanese black pine forest in the middle of January, were reared at room temperatures. Cocoons from this laboratory colony were placed in emergence cages similar to those described by Lanier et al. (1989). A crude female extract was prepared from the same colony by placing 20 newly emerged virgin females into a screw-cap glass vial containing 1 ml of hexane at 10 AM on February 6, 1993. After three days at 25°C, the extract was diluted with hexane to 1% of the original concentration.

Male trapping field experiments with various treatments were conducted in *P. thunbergiana* forests in Naju County, Chonnam Province, Korea. The forests, invaded by the scale in 1989, have several open areas with diameters of more than 30 m. Average height and crown diameter of overstory trees were 8 and 4 m, respectively. Tree density was sparse: crown closure was approximately 40%. Field sites for the trials utilizing the synthetic pheromone as a means of monitoring the spread of the scale infestation were located south, north, and east of the infestation loci. To each direction, sample plots were located at 2, 4, and 6 km from the boundary of known scale distribution.

Laboratory Bioassays. The bioassay procedures for testing the attraction of pheromone samples generally corresponded to those of Part et al. (1986), Park (1988), Lanier et al. (1989), and Hibbard et al. (1991). Each of approximately 10 test males was kept under a separate, numbered, and inverted glass cup (ca. 5 ml) until tested. The diluted crude female extract or a serially diluted synthetic pheromone sample in 1 μl hexane was deposited by a micropipet (Microcaps: Drummond Scientific Co.), just inside the tip of a medicine dropper. The amount of female pheromone in 1 μl of the diluted crude female extract was 2×10^{-4} female equivalents (FE). Excess hexane was expelled by depressing the bulb 20 times, and the dropper tip was positioned about 8 mm to one side of a male walking on a sheet of white paper. Puffs of air were delivered to the antennae of the walking male by gently pressing the bulb at 1.5-sec intervals. Relative attraction was measured as degree of following towards the

retreating dropper tip, which maintained approximately the same distance from the male. Males that followed one or more sides of a 4-cm-equilateral triangle were given scores of 1–3, according to the number of triangle sides completed; males responding to the sample but following less than one full side were given a score of 0.5, and those who did not follow were given a score of 0. Pheromone samples were bioassayed in a random order, using each test male repeatedly at more than 5-min intervals in successive tests. Constantly walking males respond to pheromone more positively and consistently than those walking with frequent launching attempts, walking with limping motion, or that appear to have become sluggish (Park, 1988). Therefore, those that did not walk constantly on the paper sheet were replaced. Bioassays were conducted between 9:30 AM and 1:00 PM on February 10 and 11, 1993. Temperature was 17 ± 1°C, and air humidity was 55 ± 10% relative humidity.

Field Experiments. Following the laboratory bioassay results, only pheromone **1** was tested in the field. Four kinds of baits were used: 1, 10, and 50 μg on rubber GC septa (Supelco Inc., Bellefonte, Pennsylvania) and 50μg on a filter paper roll (10 × 1 cm filter paper rolled: 4 mm diameter, 1 cm long). For protection from solar radiation, baits were placed within a paper cap (1.5 × 1.5 × 1.5 cm) with white outside and black inside. Two types of white plastic traps were used: a card trap (12 × 12 cm) and a delta-shaped trap (15 × 20 cm, folded at the centerline). The traps were coated with Tanglefoot (The Tanglefoot Company, Grand Rapids, Michigan) on both sides. Each of the four kinds of baits was placed in a hole (2 × 2 cm) at the center of the card trap, and a rubber septum with 50 μg was also suspended at the center of the delta-shaped trap. Therefore, including the control (card traps without baits), there were six different treatments. Three traps with the same treatment were affixed vertically to a bamboo pole at 0.5, 1.5, and 2.5 m above the ground. These six bamboo poles, each with a different treatment, were posted in an open area adjacent to pine groves infested by the scale, approximately 10 m from one another and at least 5 m from the trees. Within the pine forest, two bamboo poles were posted: one with card traps baited with 50μg on a rubber septum, and the other with control traps. The experiment was laid out in a split plot design with treatments as main plots and heights as subplots, and four replications were conducted. Traps were set between 7 and 10 AM on April 3, 1993. The peak of daily male flight is between 11 AM and 1 PM (Park, 1988), and the sticky traps were collected for male counts between 4 and 7 PM on April 4.

Another field bioassay, the daily trap catch experiment, with 1 and 50 μg on rubber septa and 50 μg on a filter paper roll was conducted. Card traps (12 × 12 cm) were affixed to bamboo poles at 0.5 m above the ground in open areas. The distance of bamboo poles from the nearest pine grove was approximately 10 m. Treatments were 5 m apart, and there were at least 10 m between replications. Seven replications were conducted in a randomized block design.

Traps were set between 5 and 7 PM on April 4. Trapped males were counted daily, from April 5 to 14, between 4 and 6 PM by removing males from the traps.

The locations of male landings within a trap in response to the pheromone dispenser was studied at the same experiment sites. A rubber septum with 50 μg was placed at the center of a round sticky panel 40 cm in diameter. In open areas, eight traps were placed at 1–2 m above the ground. Trapped males were counted by the distance from the dispensers: 0–5 cm, 5–10 cm, and 10–20 cm. Traps were set between 7 and 10 AM on April 3 and collected between 4 and 7 PM on April 4.

Trials for Utilizing Pheromone for Detecting New Infestations. The scale is most readily observed during the egg stage, with white cottony egg sacs in bark crevices and on nodal area of trunks (Miller and Park, 1987). Therefore, the current procedure for detecting new infestations involves examining more than 30 tree samples from each survey plot in late April, when most eggs are laid but before they hatch. At each sample plot (see Table 2), 10 card traps (12 × 12 cm) baited with 50 μg on a rubber septum or on a filter paper roll were affixed to Japanese black pine saplings 1 m above the ground, approximately 10 m apart. Traps were set on April 6 and 7, and trapped males were counted between 6 and 14 days after setting the traps. The customary scale-detecting procedures were conducted at the same locations in late April, and the efficiency of finding new infestations by ovisac counts was compared with the pheromone traps.

Statistical Analysis. In laboratory bioassays, the data were rank transformed and analyzed with one-way analysis of variance (ANOVA). Field trapping data were log $(x + 1)$ transformed to meet the assumption of equal variance, and the transformed data were analyzed with ANOVA of a split plot design and two-way ANOVA. Each data set was followed by Duncan's multiple range test when significant F values were found in the ANOVA.

RESULTS AND DISCUSSION

Laboratory Bioassays. Attraction of pheromone **1** was approximately 10^4 times stronger than pheromone **2.** The threshold concentrations for male attraction with pheromones **1** and **2** were 16 fg and 150 pg, respectively. Maximum responses were elicited by 2×10^{-4} FE of virgin female crude extract, between 1.6 and 160 pg of **1,** and at 15 ng of **2** (Table 1). The attraction of **1** appeared stronger than matsuone with unknown stereochemistry. In the latter, the threshold concentration and the lowest dosage required for maximum responses, tested with the same insect species, were 400 fg and 4.0 pg, respectively (Hibbard et al., 1991). The attraction of **1** decreased at higher concentrations; in supplementary bioassays, at 16 ng, all test males became paralyzed for a few seconds.

TABLE 1. RESPONSE OF MALES TO DILUTIONS OF SYNTHETIC PHEROMONES AND CRUDE EXTRACT OF VIRGIN FEMALES[a]

Material	Quantity[b]	Males tested (N)	Response score ($X \pm$ SE)[c]
Control		25	0 D
Crude extract	2×10^{-4} FE[d]	26	2.88 ± 0.08 A
1: (6*R*, 10*R*)-matsuone	1.6 ng	5	2.20 ± 0.33 B
	160 pg	5	2.80 ± 0.18 A
	16 pg	5	3.00 A
	1.6 pg	29	2.93 ± 0.05 A
	160 fg	35	2.09 ± 0.15 B
	16 fg	35	0.54 ± 0.13 C
	1.6 fg	30	0 D
2: (6*S*, 10*S*)-matsuone	15 ng	25	2.96 ± 0.04 A
	1.5 ng	27	2.13 ± 0.19 B
	150 pg	26	0.52 ± 0.11 C
	15 pg	21	0.02 ± 0.02 D
	1.5 pg	16	0.03 ± 0.03 D

[a] Response scores: 0 = no following; 0.5 = followed for less than one full triangle side; 1, 2, or 3 = number of triangle sides completed.
[b] Each in 1 μl hexane.
[c] Statistical analysis was performed on rank transformed data ($F = 116.8$; $df = 13{,}296$; $P < 0.01$). Means followed by the same letter are not significantly different ($P < 0.05$, Duncan's multiple range test).
[d] Female equivalents.

Field Experiments with Various Treatments and Heights. In trap catch data, interaction between treatments and heights was found by ANOVA ($F = 5.7$; $df = 14{,}48$; $P < 0.01$). Thus, significant tests were performed between treatments within each height and between heights within each treatment.

The largest number of males were caught on traps with 50 μg of pheromone **1** on a rubber septum placed at 0.5 m above the ground within forests; an average of 9114.8 males were caught on a trap (Figure 2). Males appeared to respond better to pheromone traps near the ground. In control traps, both in open areas and within forests, male catches were not different between heights. However, in most baited traps, traps at 0.5 m above the ground caught more males than those at 2.5 m high. Tree density was rather sparse in this field experiment. In forests with higher crown closure, baited traps near the ground might not catch more males than at higher ones.

Females on trees did not appear to significantly hamper the efficiency of synthetic pheromone traps placed within forests. At all heights, baited traps within forests caught more males than those in open areas, whereas male catches

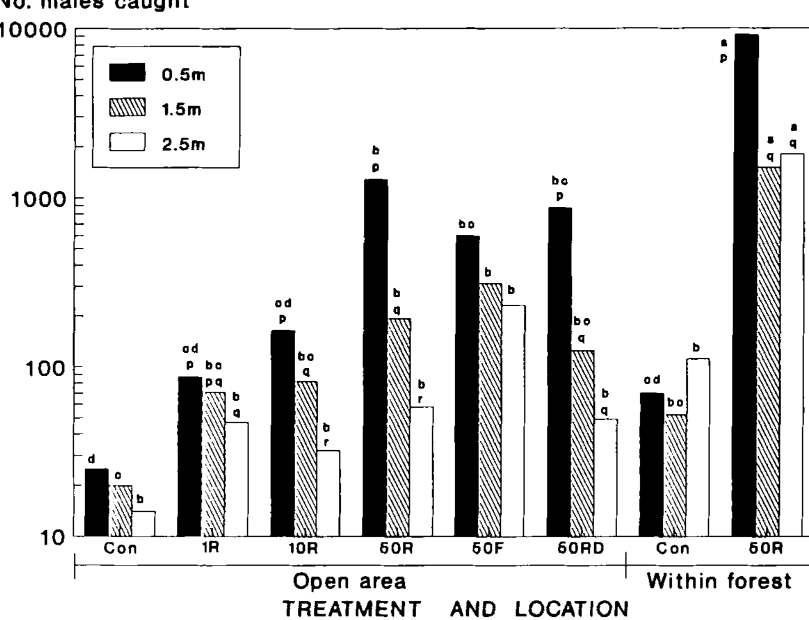

FIG. 2. Number of males caught on traps with various treatments and heights during the first two days after traps were set (four replications). Although raw data are shown, significance tests were performed on log (x + 1)-transformed data. Bars topped by the same letter are not significantly different ($P < 0.05$, Duncan's multiple-range tests). Letters a–d are comparisons between treatments within each height, and p–r are between heights within each treatment. Treatments without letters p–r are those where significant F values were not found in the ANOVA. Con: card trap without bait. 1R: card trap with 1 μg on rubber septum. 10R: card trap with 10 μg on rubber septum. 50R: card trap with 50 μg on rubber septum. 50F: card trap with 50 μg on filter paper roll. 50RD: delta-shaped trap with 50 μg on rubber septum.

on control traps placed within forests and in open areas were not different from each other. Furthermore, baited traps within forests were effective at all heights, whereas the same traps in open areas did not catch more males than control traps at 2.5 m above the ground. Males flying in open areas may be less responsive to pheromones than those flying within forests. As suggested by Doane (1966) and Park (1988), males may have a preliminary flight period before they actively respond to pheromone. Many males flying in open areas may be doing their preliminary flights. Dispensers and the shape of the traps did not affect trap catches: at all heights, the number of males caught by card traps with

50 μg on rubber septa, those on filter paper rolls, and delta-shaped traps with 50 μg on rubber septa were not different from one another.

Daily Trap Catches. During the first day of trapping, traps with 50 μg on rubber septa and on filter paper rolls caught more males than control traps and those with 1 μg on rubber septa. On the second day, traps with 50 μg on rubber septa were the most effective, and those with 50 μg on filter paper rolls and 1 μg on rubber septa caught more males than control traps. Between April 7 and 9, there were no differences between treatments due to low temperatures and few insect flights. After that period, traps with 50 μg on rubber septa were always effective, and those on filter paper rolls were effective on April 13 only. The peak of adult activity in *M. thunbergianae* is early April, and it may be responsible for the smaller number of males on traps after April 10 (Figure 3).

Male Landings in Response to Pheromone. Numbers of males landing on section 1–5 cm, 5–10 cm, and 10–20 cm away from the dispensers were 56.6, 238.3, and 1246.8, respectively (Figure 4). Male landings per unit area were not significantly different from one another ($F = 0.42$; $df = 2,14$; $P > 0.05$). Males do not appear to land on the exact spot of the pheromone source, but

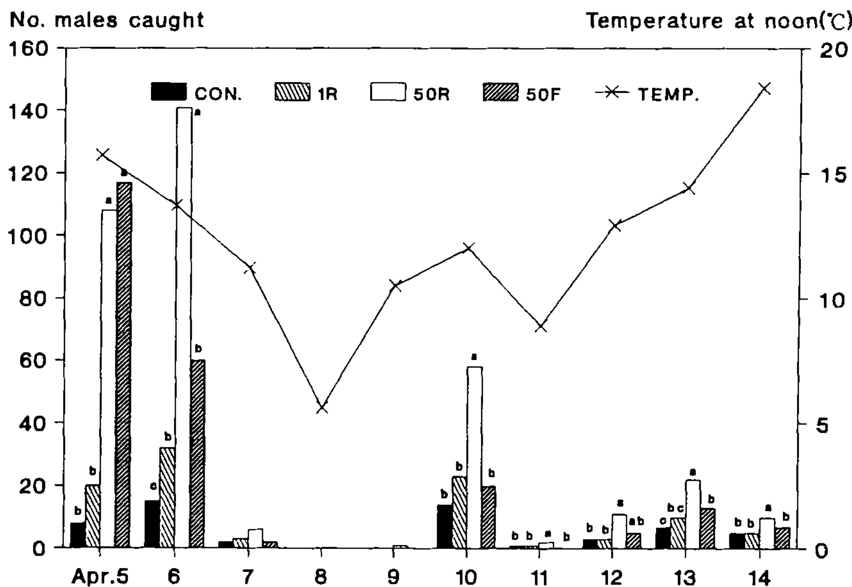

FIG. 3. Daily trap catches during 10 days after traps were set (seven replications). In each date, bars topped by the same letter are not significantly different. Dates without letters are those where signficant F values were not found in the ANOVA. See Figure 2.

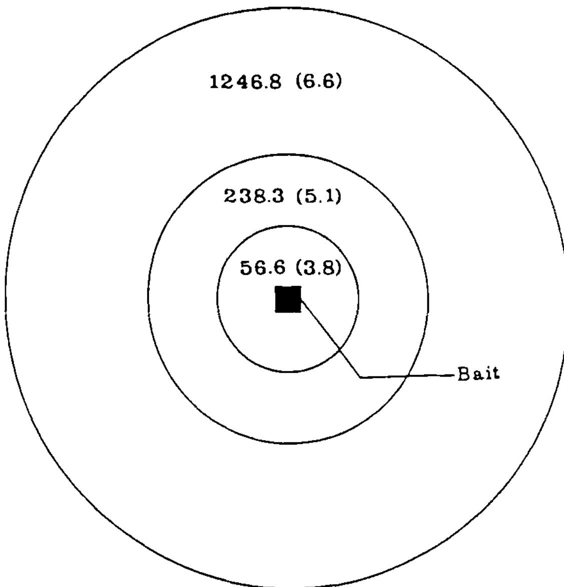

FIG. 4. Number of males trapped on both sides of each section by the distance from a bait (eight replications). Diameters of the circles are 10, 20, and 40 cm. In parentheses are males per 10 cm^2.

rather fly around the pheromone source and land on the substrate encountered. It corresponds to the hypothetical illustration of male locomotion, from adult emergence to mate-finding, by Park (1988). He hypothesized that male alighting on pine branches is followed by mate searching on foot and demonstrated that males followed a virgin female scent trail on a filter paper sheet. After alighting, males appear to locate females by simply searching for higher pheromone gradients and/or following female scent trails.

In field experiments, traps with 1 μg or 10 μg, except on April 6 and 13 of the daily trap catch data, did not catch more males than control traps. These low-dose treatments could have been more effective with larger trap spacing, thus reducing effects of traps with higher pheromone evaporation rates and/or haphazard landing on control traps by males flying within the active space. However, long distances between treatments or replications can confound experiments when there are great differences in natural male flight density. In order to minimize the interaction between treatments, as well as the variation in trap catches caused by differences in flight density, preliminary data will be needed on the distribution of natural male flights in potential experiment sites and on

the size of the active space of a pheromone trap with the highest dose to be tested.

Trials for Utilizing Pheromone in Detecting New Infestations. In the three locations outside the known scale distribution, several males were caught on traps where no egg sacs were found (Table 2). In Chonnam Province, where the scale's expanding distribution is towards the south, egg sacs were found by the customary scale surveys at 2 km, and males were caught on traps at 2 and 4 km outside the known distribution. In Chonbuk Province, where the spread is towards the north, no egg sacs were found, whereas males were trapped at 6 km outside. In Kyungnam Province, egg sacs were found at 2 km out to the east; males were caught at 2 and 4 km outside. As in Chonbuk Province, the first spread of infestation can be sporadic rather than continuous. Apparently, the scale increases its territory by dispersing from each of the new infestation loci, and thus appears in continuous populations over a wider area.

Early detection of *M. thunbergianae* is important because the scale kills trees within a few years of infestation. Severe tree mortality could be avoided by applying timely control measures such as the trunk implantation of systemic insecticides (Lee et al., 1991). With several examples of utilizing synthetic sex pheromones for detection and monitoring the populations of some scale insect species, as referenced by Hibbard et al., (1991), it appears that male trapping by pheromone **1** can be used as a component of the scale control program. It

TABLE 2. TRAP CATCHES AND EGG SAC SURVEY RESULTS AT LOCATIONS OUTSIDE OF KNOWN DISTRIBUTION

		Trap[a]				
		Rubber septum		Filter paper roll		
Location (direction of expansion)	Distance from known distribution (km)	Traps (*N*)	Males caught (*N*)	Traps (*N*)	Males caught (*N*)	Results of egg sac survey
Jindo County,	2	6	481	4	49	found
Chonnam Prov.	4	6	50	4	11	not found
(south)	6	6	0	4	0	not found
Gochang County,	2	6	0	4	0	not found
Chonbuk Province	4	5	0	5	0	not found
(north)	6	6	3	4	4	not found
Gosung County,	2	5	22	5	12	found
Kyungsang Province	4	5	292	5	224	not found
(east)	6	5	0	5	4	found

[a] In each trap, the dispenser was treated with 50 μg of **1**.

may also be used for managing the populations of *M. resinosae* and *M. matsumurae*, which share the same pheromone with *M. thunbergianae*. Sex pheromones of *M. feytaudi* (Cywin and Kallmerten, 1991; Mori and Harashima, 1993a) and *M. josephi* (Mori, personal communication, 1994) have also been synthesized. It is possible that these species share similar aspects of reproductive behavior with *M. thunbergianae*, *M. resinosae*, and *M. matsumurae*, and pheromone research on the *Matsucoccus* species mentioned in this paper could be mutually helpful in managing this economically important group of insect pests.

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IDENTIFICATION OF BRANCHED ALKANES IN LIPIDS OF *Leptinotarsa decemlineata* SAY AND *Tribolium destructor* BY GC-MS: A COMPARISON OF MAIN-BEAM AND LINK-SCANNED SPECTRA

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Abstract—Branched hydrocarbons were identified in the lipids of *Leptinotarsa decemlineata* Say and *Tribolium destructor* by gas chromatography, ordinary electron impact mass spectrometry, and linked, scanned, daughter-ion monitoring. This methodology allowed us to revise our earlier results based only on GC-MS data confirming the existence of only monomethyl-, dimethyl-, and trimethylalkanes in the hydrocarbons of *L. decemlineata* Say. The hydrocarbons from *Tribolium destructor* consist of *n*-alkanes, 3-methylalkanes, internally branched monomethylalkanes and dimethylalkanes. Daughter-ion monitoring can be particularly important for distinguishing between incidentally overlapped GC peaks of hydrocarbons from different series. A trace, for example, of dimethylalkane coeluating with *n*-alkane was easily identified in GC peak of hydrocarbon mixture of *T. destructor*. Link scans confirmed also molecular weights for the compounds without molecular ions in the mass spectra. Structural assignment of the compounds were verified by comparison of the experimental and calculated values of the GC retention Kovats indexes (KI).

Key Words—Insects hydrocarbons, alkanes, Colorado beetle, *Leptinotarsa*

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decemlineata Say, *Tribolium destructor*, Coleoptera, Teneboionidae, choysomelidae, mass spectrometry, linked scanning.

INTRODUCTION

There are two kinds of lipids in insect cuticles: structural and free lipids. The main function of free cuticular lipids is to minimize transpiration and to protect terrestrial insects from desiccation (Lockey, 1988). The lipid layer also protects the insects from being infected by microorganisms (Kervin, 1984). Some of the lipid components of cuticle are also involved in chemical communication within and between species (Lockey, 1988; Blomquist et al., 1987).

The composition of free cuticular lipids of insects varies from species to species, being a chemical tool for their taxonomy (Lockey, 1988; Blomquist et al., 1987). Nevertheless, the following chemical classes could be specified: hydrocarbons, fatty acids, long-chain alcohols, esters, glycerides, sterols, aldehydes and ketones (Lockey, 1988; Blomquist et al., 1987).

Cuticular lipids of all insects investigated so far contain significant amounts of hydrocarbons. The identification of the components requires their separation and recording their electron-impact mass spectra. Additionally, the branched hydrocarbon structures must be confirmed by the proper values of Kovats retention indices (KI) and must be supported by rational biosynthetic pathways.

Thus, knowledge of the molecular weight (MW) of a compound is crucial for application of KI values to structure elucidation because ΔKI is calculated as the difference of KI of a straight chain isomer and branched one, both with the same total carbon numbers. Direct proof of MW could be obtained from chemical ionization spectra generated with methane where quasimolecular ion $(M-H)^+$ is observed or from linked scanned spectra.

This paper presents the identification of branched alkanes and the comparison of main-beam mass spectra with linked scans of individual hydrocarbons of two Coleoptera: *Leptinotarsa decemlineata* Say and *Tribolium destructor*.

Summons (1987) first reported an application of GC-MS with link scans for multiple reaction monitoring of branched and isoprenoid hydrocarbons for the study of ancient and recent sediment compositions. He found that the intense metastable peaks with even masses are diagnostic for specific branched hydrocarbons.

METHODS AND MATERIALS

Female *Leptinotarsa decemlineata* Say beetles (150 beetles) chosen from wild population were extracted with chloroform (150 ml) in a Soxhlet apparatus for 1.5 hr. The extract was filtered and the solvent evaporated under vacuum at

a temperature not exceeding 50°C, providing 1.1538 g of crude lipids. Hydrocarbons were separated from the polar lipids by preparative column chromatography on a neutral aluminum oxide eluted with cyclohexane.

Beetles of mixed sexes of *Tribolium destructor* were collected from laboratory breeding carried out at 20°C. A mixture of cuticular lipids was obtained by immersing 450 beetles in 200 ml of CH_2Cl_2 for 5 min. The solvent was removed under reduced pressure. *n*-Alkanes and branched hydrocarbons were separated from the other lipids by preparative TLC using standard plates covered with silica gel (Kieselgel 60, Merck). *n*-Hexane was used as development solvent. A fraction with $R_f = 0.8$ was collected which contained normal and branched hydrocarbons.

Hydrocarbon fractions were analyzed by gas chromatography on a 40-m glass capillary column coated with Dexsil 300 liquid phase (0.25 mm ID, film 0.1 μm) and on a 40-m OV-1 column. The separation was carried out with a Varian Aerograph model 1400 gas chromatograph, which had been modified to accept a glass capillary column with a splitter (1:30) and makeup system. The GC oven was programmed from 200 to 310°C at 2°/min. Injector temperature was kept 10°C above the upper GC oven temperature. Argon was used as a carrier gas with a flow rate that provided 1 min solvent retention time per each 10 m of the column. KIs were determined at 250 and 260°C. The error was ± 1.0 unit for KI greater than 5.

Mass spectra (70 eV) were measured using a VG 7070E mass spectrometer controlled by a data system. The samples were introduced via gas chromatography. The Dani 3800 GC was equipped with 25-m × 0.32-mm-ID fused silica column coated with OV-1 liquid phase. The flow rate of helium was 1 ml/min. The column was programmed from 100°C to 280°C at 2°/min. (*L. decemlineata* Say) or from 180°C to 280°C at 2°/min. (*T. destructor*). It was directly coupled with the ion source of the instrument. The ion source was operated at 250°C with an ionization energy of 70 eV.

For daughter-ion recording, a metastable ion scan, with B/E constant, was applied. The mass spectrometer was normally scanned downfield over its calibrated range with a magnet scan. At a chosen mass, the electrostatic analyzer voltage was unlinked from the constant accelerating voltage and scanned simultaneously together with the magnetic field such that the ratio B/E was constant. Metastable scan was controlled by the PDP 11/24 computer with a software version System VG II-250.

RESULTS AND DISCUSSION

Hydrocarbons of Leptinotarsa decemlineata *Say.* Hydrocarbons of *Leptinotarsa decemlineata* Say are equal to 1 mg/insect, which makes 13% of the total lipid extract. Hydrocarbons constitute a complex mixture with more than

30 components (Figure 1) in the range 2800–3600 units of KI. Identified chemical compounds are included in Table 1 together with their retention indices and characteristic ions in the mass spectra. The major components consist of 29, 30, 31, 32, and 37 carbon atoms. As no *n*-alkanes were found, the mixture seems to be composed solely of branched hydrocarbons. Thus, the hydrocarbons of *L. decemlineata* Say differ considerably from most of the other insects so far investigated.

The most informative in the structural assignment of branched hydrocarbons were the electron-impact mass spectra. For methylalkanes, each α-cleavage at the branch point provided two prominent ions at C_nH_{2n+1} and C_nH_{2n} (Table 1). The mass spectrum of a hydrocarbon, whether straight-chain or branched, shows the fragment ions separated by 14 amu, which corresponds to carbon–carbon bond cleavages.

Mono-, di-, and trimethylalkanes were found in lipids of *Leptinotarsa decemlineata* Say (Table 1). More critical discussion of the spectra together with comparison of experimental and calculated KI values and with the assistance of link-scanned metastable ions spectra helped us to correct our previous structures of hydrocarbons (Maliński et al., 1986).

It is well known that the mass spectra of 2,*X*-dimethylalkanes do not obey all empirical rules of fragmentation for dimethylalkanes under electron impact ionization. The first branch point at C-2 is not revealed in the spectra (Pomonis et al., 1989).

In the EI spectrum of GC peak 5 (Figure 1) the ions *m/z* 407 $(M-15)^+$, *m/z* 337 $(M-85)^+$ and *m/z* 113/112 are observed (Figure 2). Thus, the compound could be identified as 7-methylnonacosane, but ΔKI, measured as a difference of KI of *n*-alkane and isoalkane, both with the same total number of carbons, is 95 (Table 1), which significantly exceeds that of mono-branched alkanes, suggesting two branching points. The only structures of the hydrocarbon are then 2,6- or 7,22-dimethyloctacosanes. The other proof of the structure was supplied by the ΔKI calculation (Kissin et al., 1986). Two structures, viz., 2,6- and 7,22-dimethyl-octacosane were taken into account. Calculated ΔKIs were 89 and 114, respectively, which selects the 2,6-dimethyl structure. Retention indexes needed for calculation were taken from the literature (Hebanowska et al., 1989). The metastable ion spectrum shows mainly the transition of $M^{+\cdot} \rightarrow$ 337/336 (M−85) accompanied by the weaker $M^{+\cdot} \rightarrow$ 309/308 (M−113) (Figure 3).

The EI spectrum of the peak 6 (Figure 4) reveals only the ions *m/z* 295/294 (M−141) and *m/z* 169/168 (M−267). Again, the spectrum could be assigned to 11-methyltriacontane or 11,19-dimethylnonacosane. The retention index of the compound was found to be 2918 (Table 1). Link-scanned spectrum (Figure 5) proved the molecular weight of 436 amu, which corresponds to a hydrocarbon with 31 carbon atoms. It shows the most abundant ions corresponding to the

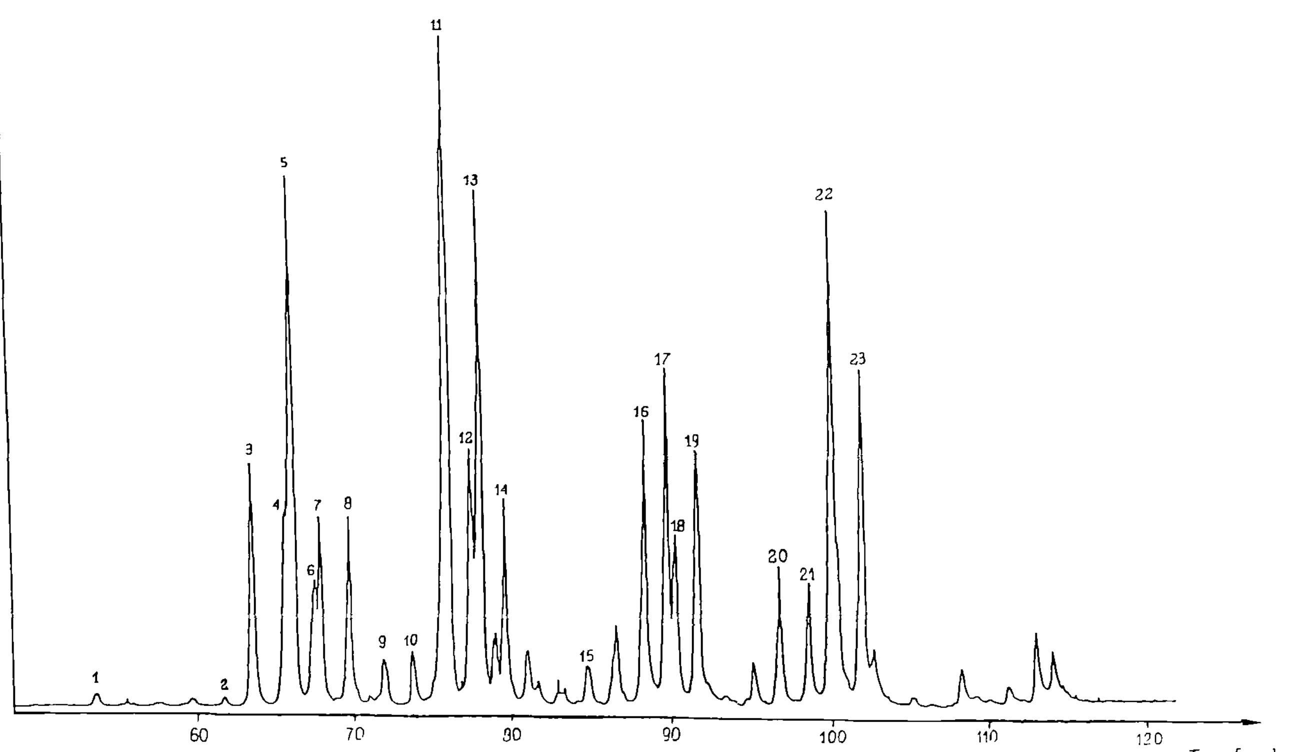

FIG. 1. TIG chromatogram of cuticular hydrocarbons of *Leptinotarsa decemlineata* Say, 25-m fused silica capillary column coated with OV-1 liquid phase, column temp. 100–280°C programmed at 2°C/min, then held isothermally for 30 min.

TABLE 1. RETENTION INDEXES OF HYDROCARBONS OF *L. decemlineata* SAY

TIC peak	Identification	Kovats retention indexes on Dexsil 300	Kovats retention indexes on OV-1	ΔKI[a]	Main beam mass spectral data (*m/z*)
1	2,6-dimethylhexacosane	2700.0	2704.2	96	112/113, 308/309
2	10-methyloctacosane	2832.1	2836.1	64	154/155, 280/281
3	2-methyloctacosane	2860.2	2864.2	36	365, 393
4	2,10- or 2,18-dimethyl-octacosane	2892.2	2899.2	101	168/169, 280/281
5	2,6-dimethyloctacosane	2900.0	2905.3	95	112/113, 336/337
6	2,10,18-trimethyloctacosane	2918.0			168/169, 294/295
7	11-methyloctacosane	2931.3	2934.3	66	168/169, 280/281
8	11,19-dimethylnonacosane	2959.5	2966.9	133	168/169, 294/295
9	2,6-dimethylnonacosane				112/113, 350/351
10	12-methyltriacontane	3031.1	3033.9	66	182/183, 280/281
11	2-methyltriacontane	3062.2	3065.1	35	393, 421
12	2,10- and 2,12-dimethyl-triacontane	3091.1	3096.5	103	168/169, 308/309; 196/197, 280/281
13	2,6-dimethyltriacontane	3100.0	3105.2	95	112/113, 364/365
14	11- and 13-methyl-hentriacontane	3133.0	3137.2	63	168/169, 308/309; 196/197, 280/281
15	12-methyldotriacontane	3227.4	3233.7	66	182/183, 308/309
16	2,10-dimethyldotriacontane	3290.6	3297.2	103	168/169, 336/337
17	2,10,16- and 22-trimethyl-dotriacontane	3310.6	3324.0	176	168/169, 252/253, 266/267, 350/351; 168/169, 350/351
18	11-, 13- and 17-methyl-tritriacontane	3326.9	3331.7	68	168/169, 336/337; 196/197, 308/309; 252/253
19	11,17-dimethyltritriacontane	3350.1	3361.1	139	168/169, 252/253, 266/267, 350/351
20	10,18- and 12,18-dimethyl-tetratriacontane	3449.3			154/155, 252/253, 280/281, 378/379; 182/183, 252/253, 280/281, 350/351
21	2,10-dimethyltetratriacontane				168/169, 364/365
22	2,10,16- and 2,16,24-tri-methyltetratriacontane	3510.3	3524.2	176	168/169, 266/267, 280/281, 378/379; 168/169, 252/253, 294/295, 378/379
23	11,19- and 11,23-dimethyl-pentatriacontane	3550.3	3560.2	140	168/169, 252/253, 294/295, 378/379; 168/169, 196/197, 350/351, 378/379

[a] $\Delta KI = KI_{n\text{-alkane}\, C_n} - KI_{\text{isoalkane}\, C_n}$ on OV-1.

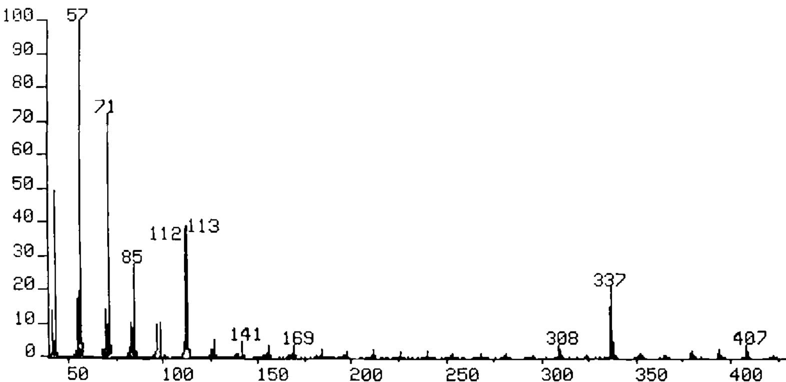

FIG. 2. EI mass spectrum of 2,6-dimethyloctacosane.

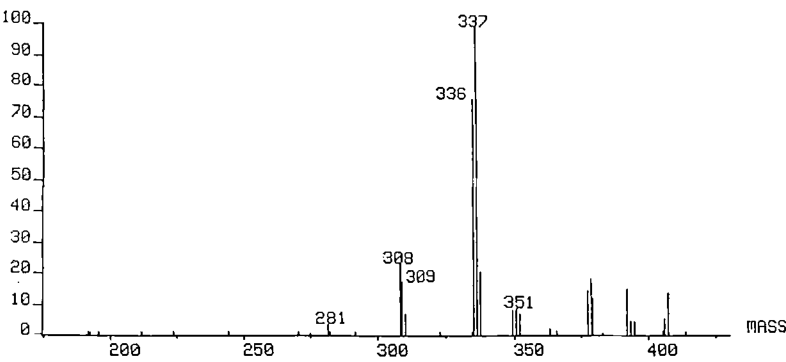

FIG. 3. Link scan daughter ions of *m/z* 422.

transitions $M^{+\cdot} \rightarrow 421$ (M−15); $M^{+\cdot} \rightarrow 407$ (M−29), and $M^{+\cdot} \rightarrow 295$ (M−141). The value of experimental ΔKI (182; Dexsil 300) suggests a triple-branched hydrocarbon. 2,10,18-Trimethyloctacosane with the sites of branching on even carbon numbers but with odd spacings between them explains the spectrum. The calculated ΔKI value for this compound is 176 (Kissin et al., 1986).

169/168 295

$C-C(C)-C_7-C(C)-C_7-C(C)-C_{10}$ 168 > 169

169/168

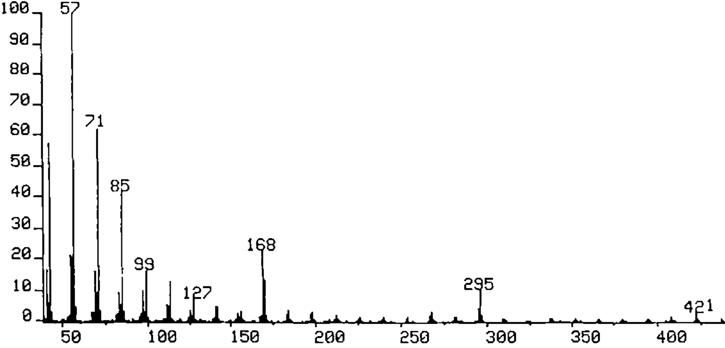

FIG. 4. EI mass spectrum of 2,10,18-trimethyloctacosane.

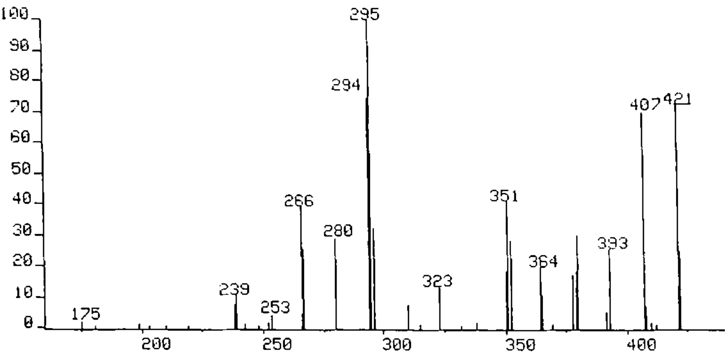

FIG. 5. Link scan daughter ions of *m/z* 436.

GC peak 13 shows the ions *m/z* 365 (M−85) and 113/112 (M−337) in the EI spectrum (Figure 6), generated by the cleavages at both sides of the second branching point, but again without a direct proof for the first branch site. Similar to 2,6-dimethylhexacosane and -octacosane, ΔKI was found to be 95 for OV-1 liquid phase. The link scanned metastable ion spectrum (Figure 7) points out the molecular weight (450 amu) and the transition $M^{+\cdot} \rightarrow 365/364$ (M−85). Keeping in mind the results and retention index value KI = 3105 with ΔKI = 95, one can assign the compound as 2,6-dimethyltriacontane.

Hydrocarbons of Tribolium destructor. Hydrocarbons of *T. destructor* (Hebanowska et al., 1989) were separated by TLC from the total cuticular lipid fraction. Gas chromatography analysis of high boiling hydrocarbons is presented in Figure 8. Retention indices and partial mass spectra of the components are listed in Table 2.

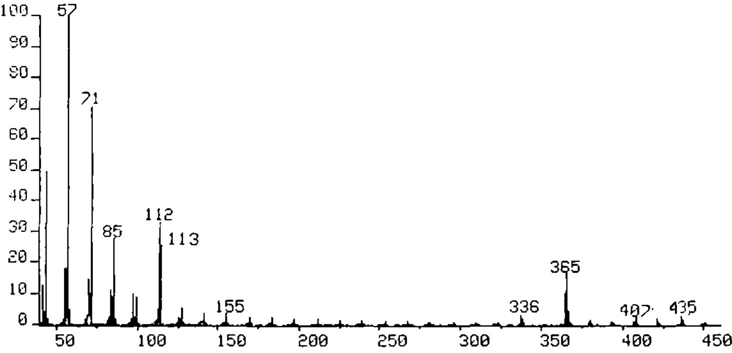

FIG. 6. EI mass spectrum of 2,6-dimethyltriacontane.

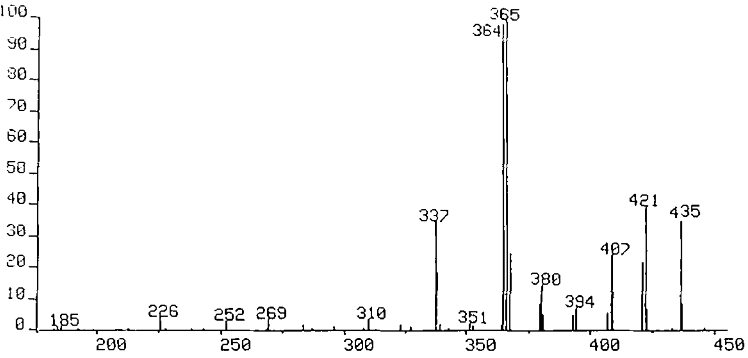

FIG. 7. Link scan daughter ions of *m/z* 450.

The hydrocarbon mixture of *T. destructor* contains *n*-alkanes, branched monomethylalkanes, and dimethylalkanes. *n*-Alkanes form a homologous series ranging from C_{26} to C_{29}. They were identified by coinjection with the standards.

The electron-impact mass spectrum of *n*-heptacosane (Figure 9) represents a typical pattern of the ions for *n*-alkane with intensive ones at lower masses. Figure 10 shows a B/E constant spectrum of the molecular ion of *n*-heptacosane (*m/z* 380). The pairs of the peaks in each cluster represent the formation of alkyl $(C_nH_{2n+1})^+$ and alkene $(C_nH_{2n})^{+\cdot}$ fragment ions with typical for *n*-alkanes distribution of the intensities.

Internally branched 13-methylheptacosane (Figure 11) has two pairs of medium intensity ions at *m/z* 197/196 and 225/224 in the EI spectrum without proof of a molecular ion. In turn, the daughter ion spectrum (Figure 12) of molecular ion *m/z* 394 is dominated by two transitions: $M^{+\cdot} \rightarrow 225/224$

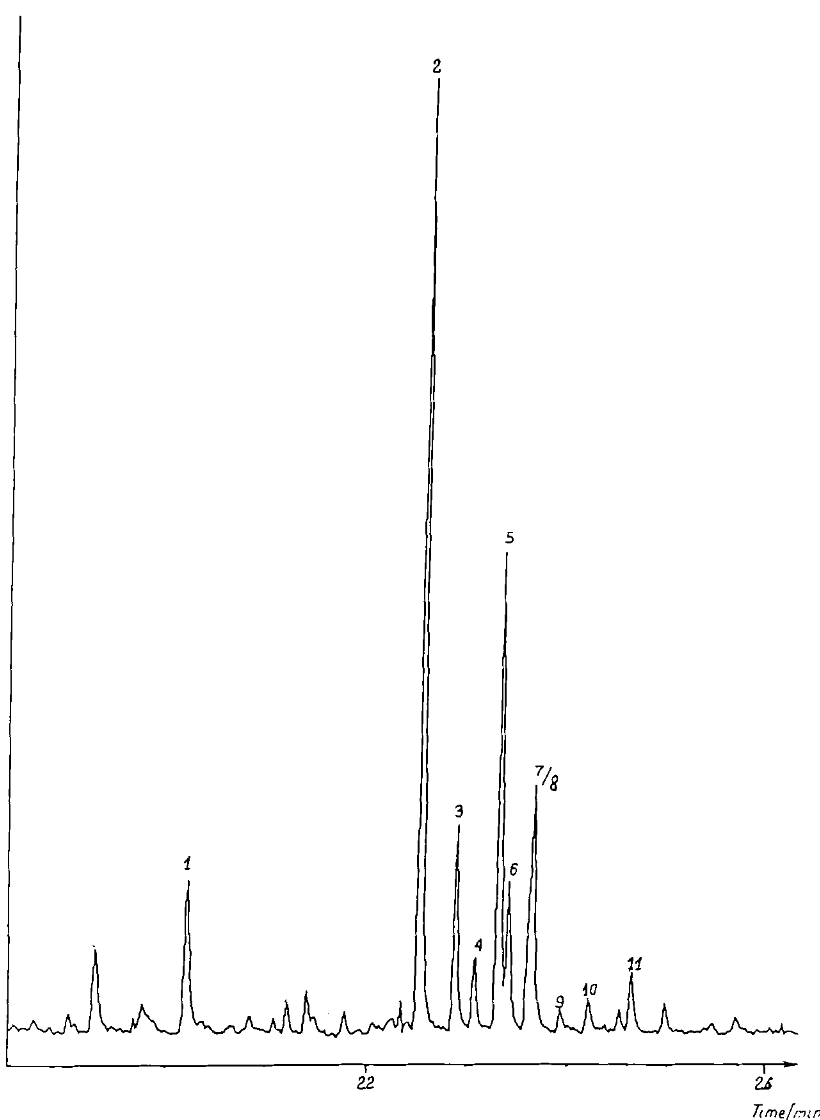

FIG. 8. TIC chromatogram of cuticular hydrocarbons of *Tribolium destructor*, 25-m fused silica capillary column coated with OV-1 liquid phase, column temp. 180°C programmed at 2°C/min.

TABLE 2. RETENTION INDEXES OF HYDROCARBONS OF *Tribolium destructor*

TIC peak	Identification	Kovats retention indexes on Dexsil 300	Kovats retention indexes on OV-1	ΔKI[a]	Main beam mass spectral data (m/z)
1	*n*-hexacosane	2600	2600		366 ($M^{+\cdot}$)
2	*n*-heptacosane	2700	2700		380 ($M^{+\cdot}$)
3	13-methylheptacosane	2728	2734	66	196/197, 224/225
4	5-methylheptacosane	2747	2753	47	84/85, 337
5	3-methylheptacosane	2772	2775	25	364/365
6	5,15-dimethylheptacosane	2772	2785	115	196/197, 238/239, 351
7	*n*-octacosane	2800	2800		394 ($M^{+\cdot}$)
8	3,13-dimethylheptacosane	2800	2800	100	210/211, 224/225, 379
9	14-methyloctacosane	2828	2834	66	210/211, 224/225
10	4-methyloctacosane	2856	2860	40	70/71, 364/365
11	*n*-nonacosane	2900	2900		408 ($M^{+\cdot}$)

[a] $\Delta KI = KI_{n\text{-alkane } C_n} - KI_{\text{isoalkane } C_n}$ on OV-1.

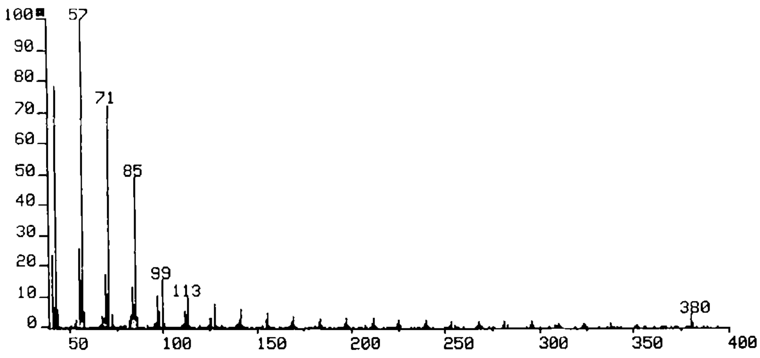

FIG. 9. EI mass spectrum of *n*-heptacosane.

(M−169) and $M^{+\cdot}$ → 197/196 (M−197). The retention index (KI = 2728) and its difference from the straight chain isomer index (ΔKI = 72) supports the internally mono-branched structure.

5-Methylheptacosane has complementary ions (those generated by cleavage at both sides of branching points) at *m/z* 337 (M−57) and *m/z* 85 (M−309) accompanied with a significant ion at *m/z* 84 (Figure 13). The link-scanned metastable-ion spectrum (Figure 14) shows two abundant ions of the transitions $M^{+\cdot}$ → 337/336 and $M^{+\cdot}$ → 309/308, corresponding to α-cleavages at the branching point accompanied with hydrogen elimination.

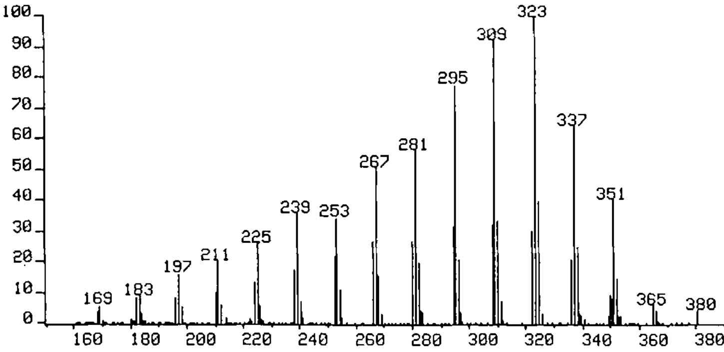

FIG. 10. Link scan daughter ions of m/z 380.

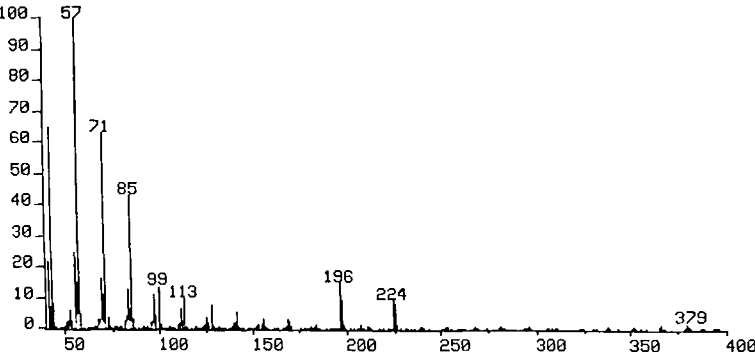

FIG. 11. EI mass spectrum of 13-methylheptacosane.

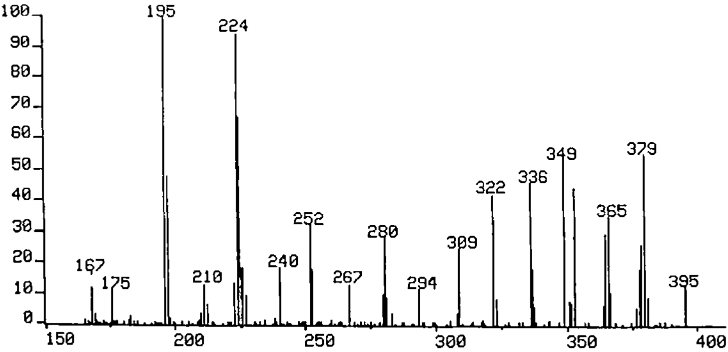

FIG. 12. Link scan daughter ions of m/z 394.

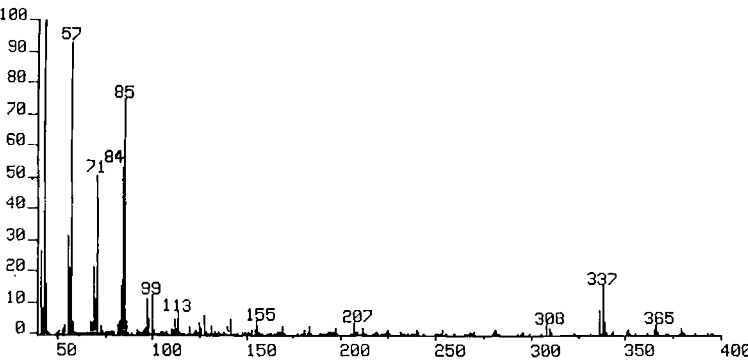

FIG. 13. EI mass spectrum of 5-methylheptacosane.

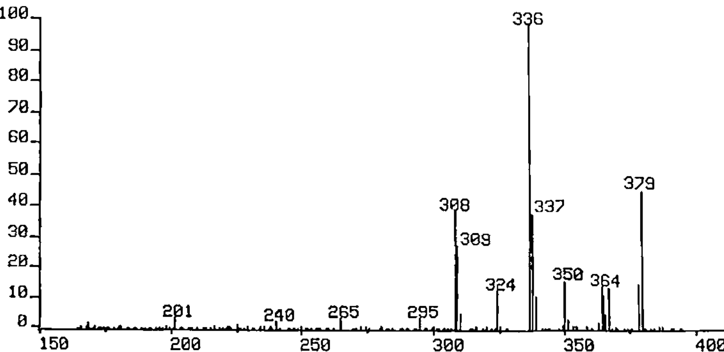

FIG. 14. Link scan daughter ions of *m/z* 394.

According to the literature data (Summons, 1987) for unresolved gas chromatography peaks, application of link-scanned spectra should provide unambiguous direct proof for the presence of the components in the mixture. The electron-impact mass spectrum on one side of GC peaks 7/8 suggests an *n*-alkane structure (Figure 15). Link scan (Figure 16) performed with tuning to *m/z* 394 as a parent ion of *n*-octacosane (C_{28}) provides a daughter spectrum entirely characteristic for *n*-alkane with slightly disturbed intensities of *m/z* 295 and *m/z* 337. The ordinary mass spectrum recorded on the other slope of the GC peak suggests the 3,13-dimethylalkane structure (Figure 17) (Hebanowska et al., 1989). In fact, tuning the instrument for the parent ion *m/z* 408 (C_{29}) and scanning its daughter ions (Figure 18) indicates a double branched structure. Fortunately, both slopes of peak 7/8 provided the main beam spectra characteristic for the

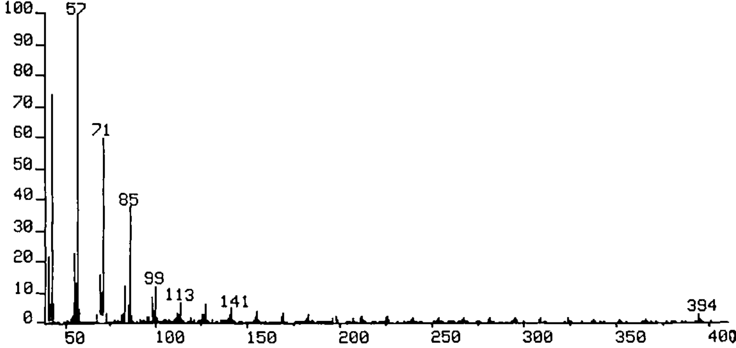

FIG. 15. EI mass spectrum of *n*-octacosane.

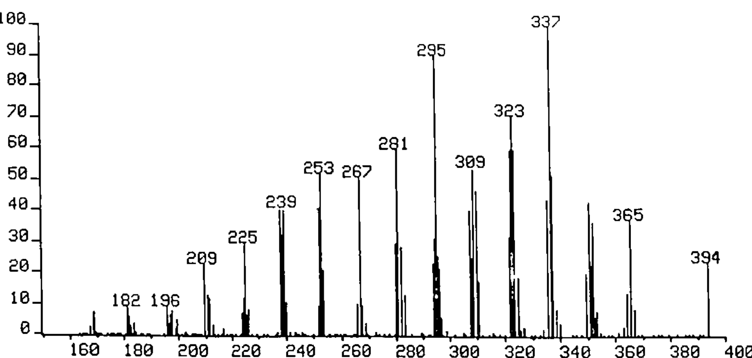

FIG. 16. Link scan daughter ions of *m*/*z* 394.

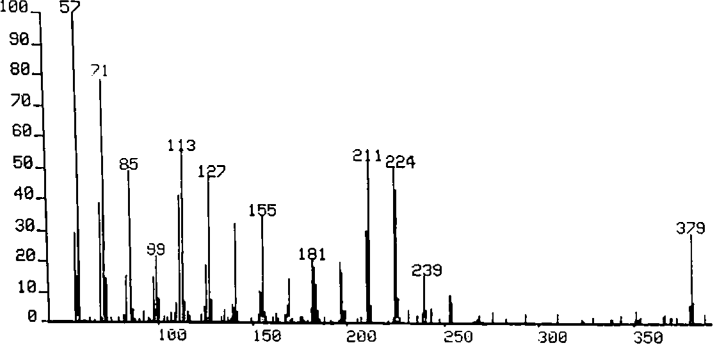

FIG. 17. EI mass spectrum of 3,13-dimethylheptacosane.

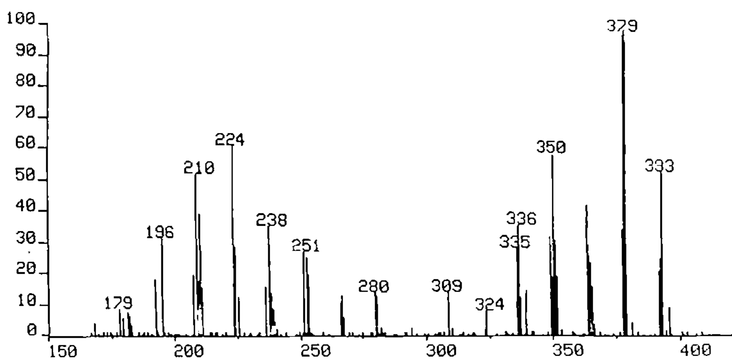

FIG. 18. Link scan daughter ions of m/z 408.

assigned structures, but link scans would reveal the components even in the case of total overlapping of the peaks.

The results presented above demonstrated that recording of metastable ions with B/E constant is a practical and useful technique for identifying branched hydrocarbons admitted to the instrument via GC, particularly where many isomers are present in the peaks.

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DORSAL ABDOMINAL GLANDS IN NYMPHS OF SOUTHERN GREEN STINK BUG, *Nezara viridula* (L.) (HETEROPTERA: PENTATOMIDAE): CHEMISTRY OF SECRETIONS OF FIVE INSTARS AND ROLE OF (*E*)-4-OXO-2-DECENAL, COMPOUND SPECIFIC TO FIRST INSTARS

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Abstract—We investigated the exocrine secretions from the five nymphal instars in the southern green stink bug, *Nezara viridula*, by analyzing separately the contents of the three dorsal abdominal reservoirs. All DAGs 1 produced a mixture of five alkanes with 12, 13, 14, 15, and 16 carbons. No differences were found between DAGs 2 and DAGs 3, for the five instars: the glands of first instars produce the same alkanes as DAGs 1, *n*-tridecane, traces of (*E*)-2-decenal, and a specific compound: (*E*)-4-oxo-2-decenal. In the other instars (second to fifth), (*E*)-4-oxo-2-decenal is absent from the secretion but another compound is present: (*E*)-4-oxo-2-hexenal. The kinetics of production of the different compounds were studied, the maximum amounts produced occurring 36 hr after hatching. The biological function of (*E*)-4-oxo-2-decenal was investigated. Using olfactometry, we showed that this compound acts as an attractant and an arrestant on second instars, at physiological doses. Moreover, this semiochemical was shown to be repellent to the fire-ant *Solenopsis geminata*, a potential predator of *N. viridula* and we established the dose-response curve for the repellent activity.

Key Words—*Nezara viridula*, Heteroptera, Pentatomidae, dorsal abdominal

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glands, nymphs, (*E*)-4-oxo-2-decenal, aggregation pheromone, repellent, predation.

INTRODUCTION

The role and structure of the dorsal abdominal glands (DAGs) in nymphs of Heteroptera have been reviewed by Staddon (1979), Pavis (1987), and Aldrich (1988). In most of the Pentatomidae, three glands formed by an invagination of the intersegmental membrane open medially between the abdominal tergites III–IV (DAG 1), IV–V (DAG 2), and V–VI (DAG 3) (Aldrich et al., 1978). The DAGs 1 are minute and often remain active in adults (Aldrich, 1988). In response to a disturbance or an aggression, a part of the content of the glands is discharged by the insect, in different ways depending on the species.

The main function of these nymph secretions is to defend against predators, but other functions, such as aggregation or alarm pheromones are proposed by some authors (Calam and Youdeowi, 1968; Levinson et al., 1974; Schmuck, 1987; Gunawardena and Herath, 1991; Farine, 1989; Farine et al., 1992a).

In the southern green stink bug, *Nezara viridula* (L.), the secretions reported in DAGs of nymphs were identified from whole-body extracts of first (Lockwood and Story, 1985), second, and third instars (Ishiwatari, 1974). Recently, Borges and Aldrich (1992) studied the composition of whole-body extracts in first and second instars of different species of Pentatominae, including *Nezara viridula*. Except in one species, first instars produce a specific compound identified as (*E*)-4-oxo-2-decenal, probably biosynthesized via oxidation of (*E*)-2-decenal, but its biological activity was not investigated. *n*-Tridecane has been identified in this species and seems to act as an aggregation pheromone at low doses and as an alarm pheromone at high doses (Ishiwatari, 1976; Lockwood and Story, 1985, 1986). This concentration-dependent pheromone seems to be produced by the dorsal abdominal glands of the nymphs. Lockwood and Story (1986, 1987) also mentioned the presence of (*E*)-4-oxo-2-decenal and tridecene in the first instars (unpublished results).

Physicochemical analyses of the heteropteran secretions have often been carried out, but very few studies mention separate analyses of the different glands' contents. However, analyses carried out on *Oncopeltus fasciatus* Dal. (Lygaeidae) (Everton et al., 1974), *Dysdercus intermedius* Dist. (Pyrrhocoridae) (Calam and Youdeowi, 1968), *D. cingulatus* Fabr. (Farine et al., 1992a), *Podisus maculiventris* Say (Pentatomidae) (Aldrich et al., 1984), and *Hotea gambiae* West. (Scutelleridae) (Gough et al., 1985) indicate differences in the contents of the glands, suggesting different functions.

On the other hand, the evolution of the secretion during postembryonic development has rarely been investigated, most of the studies dealing with the last instars.

The aim of the present study is to characterize the chemical constituents of the three DAGs in *N. viridula* and to follow their evolution during nymphal development, to underline qualitative and quantitative differences between the secretions of the five instars. Moreover, we tried to elucidate the role of the first-instars' specific compound (*E*)-4-oxo-2-decenal by testing various amounts of this compound on *N. viridula* nymphs and on a potential predator of this species, the fire-ant *Solenopsis geminata* (F.).

METHODS AND MATERIAL

Insects. We used descendants of material collected in the south of France and in Guadeloupe (French West Indies). The French strain was maintained in continuous culture in the laboratory on sunflower seeds and bean seedlings in a 16:8 hr light-dark regime. Temperature was 25 ± 2°C and relative humidity 65 ± 5%. The egg masses, laid on bean leaves or filter paper, were removed daily from the screen cages to smaller plastic boxes. The Caribbean strain was fed with sunflower seeds in a natural photoperiod (photophase ranging from 12 to 14 hr), in a room kept at 25–30°C, with a relative humidity of 90 ± 5%.

Glandular Extracts. Secretions of the dorsal abdominal glands were collected from 1-day-old nymphs of the five instars by sucking separately the three reservoir contents with a capillary glass tube and then expelled in 500 μl of *n*-hexane. In the case of DAGs 1, the micropipet was directly inserted inside the reservoir. For DAGs 2 and DAGs 3, the emission of the reservoir content was provoked by mechanical disturbance of the insect. For each instar, four bugs were extracted. Control consisted of hemolymph, sucked in the same manner (near the glands) in fifth instar. To detect eventual variations of amounts produced with the age of first-instar nymphs (N_1), whole-body extracts were made 0, 24, 36, and 60 hr after the molt, by immersing the N_1 in *n*-hexane for 10 min. To compare the two strains, whole-body extracts were made, using 20 insects (at least 24 hr old) of each instar. The different extracts were then concentrated under argon and stored at −18°C until use.

Chemical Analyses. Gas chromatography (GC) was performed on a Fractovap 2900 Carlo Erba chromatograph equipped with a split–splitless injector and fitted with an apolar fused silica capillary column (25 m × 0.32 mm ID, WCOT CPSil 8CB, Chrompack) with helium as carrier gas; temperature program: 35–90°C at 25°C/min, 1 min at 90°C, and 90–240°C at 5°C/min.

Gas chromatography–mass spectrometry (GC-MS) analyses were carried out with a Girdel 32 chromatograph coupled to a R10-10C Nermag quadrupole mass spectrometer. The chromatograph was equipped with a Ross injector and an apolar fused silica capillary column (25 m × 0.32 mm ID, WCOT CPSil 5CB, Chrompack) operated isothermally at 160°C. For identification and quan-

tification, the electron impact (EI) was carried out at 70 eV and chemical ionization (CI) was obtained with ammonia as reactant gas at 92.5 eV.

Alarm Bioassay (Figure 1). To test an eventual role of alarm pheromone, we developed a bioassay to analyze the behavior of aggregated N_1 in response to puffs of synthetic (*E*)-4-oxo-2-decenal [synthesized by Ducrot (unpublished results), according to Piancatelli et al. (1980)].

The tested N_1 were 1 day old, aggregated on empty chorions or on a piece of filter-paper. For each test, 10 μl of hexanic solutions of (*E*)-4-oxo-2-decenal of different concentrations was poured on the glass bead of an applicator. After about 10 sec to allow the solvent to evaporate, the applicator was inserted in the source chamber. The control consisted of 10 μl of *n*-hexane. One aggregate of N_1 was put in an observation chamber in which pure air flowed (0.25 cm/sec^{-1}) for 10 min. After 10 min, the three-way cock was turned and the air flowed through the source chamber before reaching the N_1. The reactions of the insects were noted for 10 min. The following doses of the studied compound were tested: 500 ng [1.2 insect equivalents (IE)], 2 μg (4.8 IE), and 100 μg (240 IE). For each dose, five replicates were made. The whole apparatus was washed between two tests. The experiments were carried out between 8:00 AM and noon.

Aggregation Bioassay. Since it is difficult to analyze the aggregation of the N_1 in response to chemicals, because they naturally aggregate and the natural products are in competition with the chemicals tested, we chose to test the effect of (*E*)-4-oxo-2-decenal on both N_1 and second-instar nymphs (N_2). In this instar, the aggregation behavior is not as marked as in N_1 but it has not totally disappeared. The N_2 do not aggregate on chorions, but they still tend to stay in groups. To quantify an eventual aggregation effect of (*E*)-4-oxo-2-decenal, we

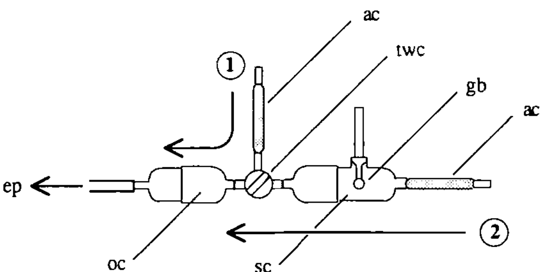

FIG. 1. Apparatus used to test the alarm activity of synthetic (*E*)-4-oxo-2-decenal on aggregates of first-instar nymphs of *Nezara viridula*. oc: observation chamber where the aggregates are disposed; ac: air cleaner; sc: source chamber; gb: glass bed of the applicator; twc: three-way cock; position 1: pure air passing through the aggregates; position 2: odorized air passing through the aggregates; ep: electric pump (aspiration).

put 10 nymphs (N_1 or N_2) in Petri dishes (1.4 cm high, 5.5 cm in diameter) in which four pieces of filter paper were placed, near the edge of the dish and in an equidistant design. One filter paper received 1 μg of (*E*)-4-oxo-2-decenal diluted in 5 μl of *n*-hexane; the three others received 5 μl of *n*-hexane. For the control, the four pieces of filter paper received *n*-hexane. For each instar tested, 12 replicates were made (12 test groups and 12 control groups). The tests were carried out under dim red light to avoid visual stimuli that could influence the locomotion of the nymphs. The position and the locomotory status of the nymphs were recorded after 15, 30, 45, and 120 min. To quantify the aggregation, we measured the surface occupied by the nymphs in the Petri dish using the formula:

$$A_t = 100\ (S_{pd} - S_t/S_{pd})$$

where A_t is the aggregation percentage at the time t, S_t the surface occupied by the nymphs at the time t, and S_{pd} the surface of the Petri dish.

The null hypotheses were, respectively, that the aggregation percentage and the number of larvae in movement in the test and the control groups were the same. It was tested by the nonparametric Mann-Whitney U test. To test the attractivity of the source, the χ^2 test was used in order to determine whether the same number of larvae were located in the control (filter paper with *n*-hexane) or in the source sector [filter-paper with 1 μg of (*E*)-4-oxo-2-decenal], in the treated Petri dishes.

Predation Test. Fragments of colonies of *S. geminata* were collected in the field by digging up a nest and placing soil containing a large number of ants into boxes whose sides were coated with Fluon. The ants were separated from the soil by allowing water to slowly drip into a box and providing them several bridges to escape into a second Fluon-coated box containing an artificial nest. We carried out two series of tests. First, we offered to the ants 20–30 living N_1 aggregating on empty chorions, and empty chorions washed with *n*-hexane with 2 μg of (*E*)-4-oxo-2-decenal in 10 μl of *n*-hexane.

For the second series of tests, the source consisted of a piece of bristol paper (1 × 1 cm) with a black point made with a pen in the middle. Two microliters of different hexane solutions of (*E*)-4-oxo-2-decenal were placed on this point. Different doses were tested: 0, 20, 50, 100, 200, 300, 400 ng, and 1 and 2 μg. For all tests, we recorded the number of ants coming in contact with the source (chorions or black point) every 6 sec for 1 min. Twelve replicates were made for each source. For statistical analyses, we used the nonparametric Mann-Whitney U test.

RESULTS

The results of quantitative gas chromatography are summarized in Figure 2. No differences were found between the French and the Caribbean strains.

DAGs 1. Comparison of chromatograms of DAG 1 extracts from all nymph

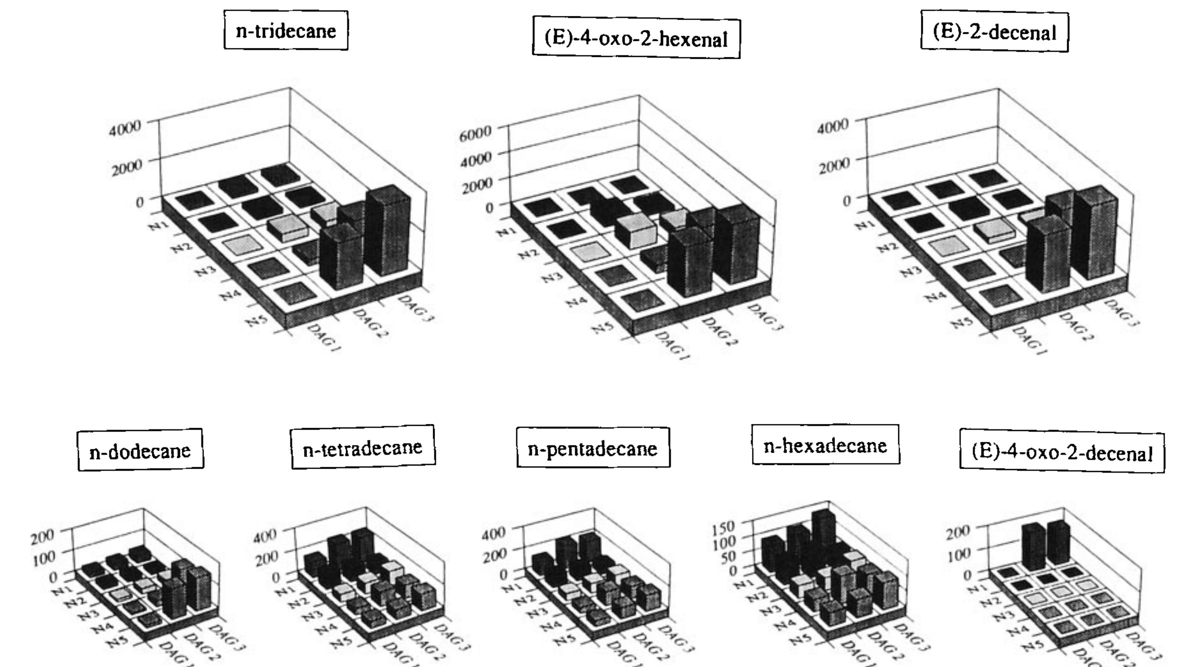

FIG. 2. Compounds extracted (nanograms) from the three dorsal abdominal glands (DAG 1, DAG 2, DAG 3) of the five instars of *Nezara viridula*.

instars revealed no significant differences. Only aliphatic hydrocarbons, the same as in the hemolymph extract of N_5, were detected. Mass spectra and GC retention times led to the identification of *n*-dodecane, *n*-tridecane, *n*-tetradecane, *n*-pentadecane and *n*-hexadecane (Figure 3a). The same ratio and similar amounts of these hydrocarbons were found in all instars.

DAGs 2. The five hydrocarbons found in the DAGs 1 were still detected in the DAGs 2. The only difference between DAG $2/N_1$ and DAG $1/N_1$ was the presence of a compound identified as (*E*)-4-oxo-2-decenal in DAG $2/N_1$. The secretions of DAG 2 in N_2, N_3, N_4, and N_5 were similar, and three compounds distinguished them from DAGs 1: (*E*)-4-oxo-2-hexenal, (*E*)-2-decenal, and *n*-tridecane (Figure 3b). The ratio of these three compounds remained the same in the different instars but the amounts detected increased with the instars, probably in relation with the size of the glands.

DAGs 3. Qualitative and quantitative compositions of the DAG $3/N_1$ were the same as DAG $2/N_1$: (*E*)-4-oxo-2-hexenal, and (*E*)-2-decenal were not detected, and (*E*)-4-oxo-2-decenal was still present (Figure 3c). DAGs 3 chromatograms of the other instars exhibited the same components as DAGs 2: *n*-dodecane, *n*-tridecane, *n*-tetradecane, *n*-pentadecane, *n*-hexadecane, (*E*)-4-oxo-2-hexenal, and (*E*)-2-decenal.

The mean percentages of each compound using whole-body extracts are represented for the five instars in Figure 4. For N_1, *n*-tridecane and (*E*)-4-oxo-2-decenal are the major compounds; in N_2, major compounds are *n*-tetradecane, *n*-pentadecane, and, to a lesser extent, *n*-tridecane and (*E*)-4-oxo-2-hexenal. In the other instars, major compounds are (*E*)-2-decenal, *n*-tridecane, and (*E*)-4-oxo-2-hexenal. We noticed that for these whole-body extracts, very low amounts of (*E*)-2-decenal were always detected. In contrast, (*E*)-2-decenal was not detected in individual reservoir collections; this difference may be due to the number of insects used and to the extraction technique.

Kinetics of Production in First Nymphal Instars. Figure 5 shows the temporal quantitative evolution of first-instar secretions after hatching. Just after the molt, they do not produce (*E*)-4-oxo-2-decenal. The amount then increases and reaches a maximum 36 hr after hatching. For *n*-tridecane, the kinetics and relative proportions are nearly the same, but just after hatching, the production is not null (about 20 ng/N_1). The minor compounds [alkanes and (*E*)-2-decenal] have approximately the same production kinetics: after a maximum at 36 hr, the quantities then decrease, probably due to the physiological changes of the following molt (occurring about 36 hr later).

Alarm Bioassay. We could not show any alarm behavior in N_1 aggregates, at any dose of (*E*)-4-oxo-2-decenal. The only reactions of the nymphs were some antennal movements when we turned the three-way cock, probably due to the difference of pressure at this moment. These movements stopped after a few

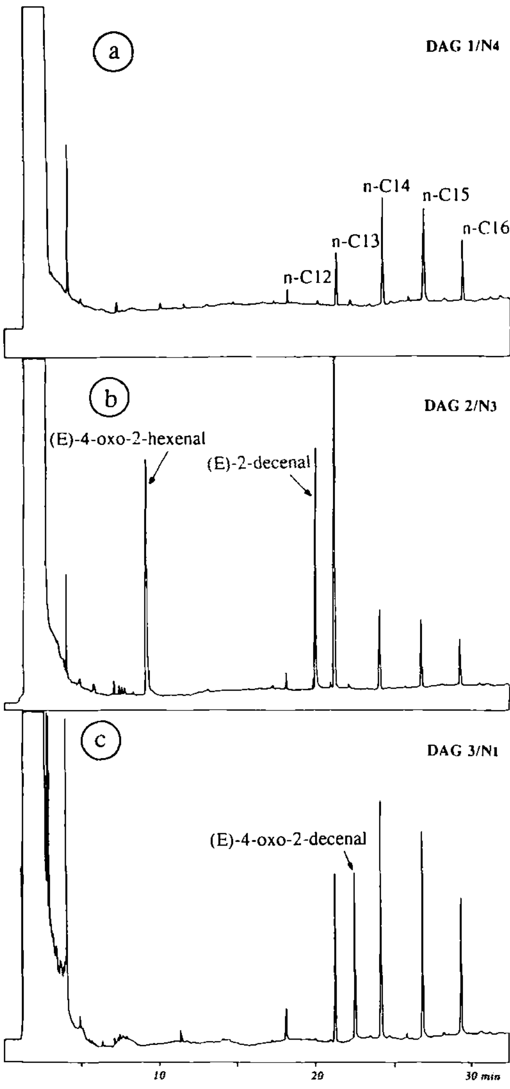

FIG. 3. Chromatograms obtained from dorsal abdominal gland reservoir collections of nymphal instars in *Nezara viridula*. Fractovap 2900 Carlo Erba chromatograph equipped with a split–splitless injector and fitted with an apolar fused silica capillary column (25 m × 0.32 mm ID, WCOT CPSil 8CB, Chrompack) with helium as carrier gas; temperature program: 35–90°C at 25°C/min, 1 min at 90°C, and 90–240°C at 5°C/min.

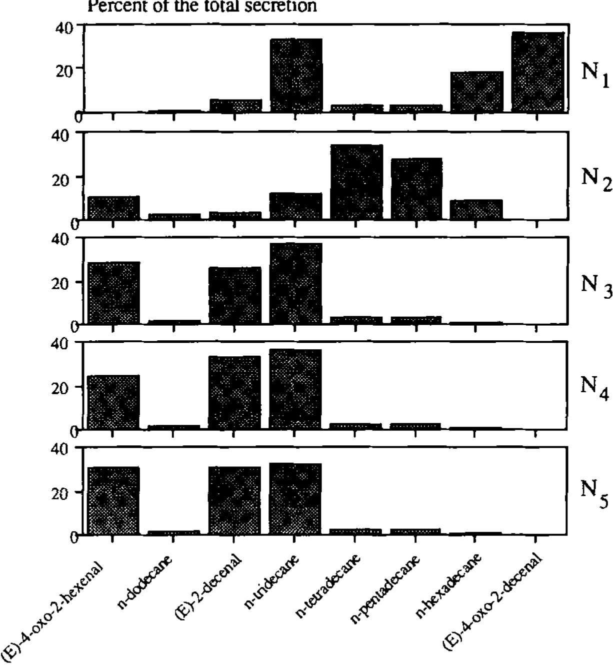

FIG. 4. Percentage of the compounds produced by the dorsal abdominal glands of the five instars of *Nezara viridula*. The nymphs were globally extracted, by washing with hexane.

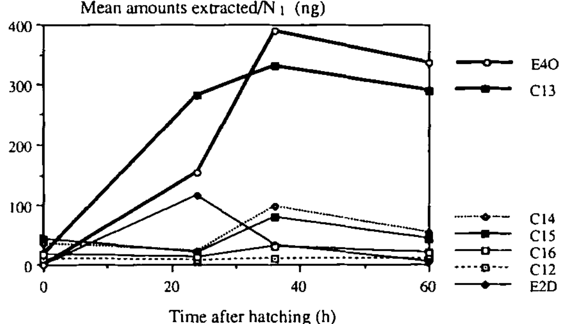

FIG. 5. Kinetics of production of the total secretion of the DAGs in the first-instar nymphs of *Nezara viridula*. For each time, 20 nymphs of the same egg masses were extracted in hexane. E2D: (*E*)-2-decenal; E4O: (*E*)-4-oxo-2-decenal; C12–C16: saturated hydrocarbons.

seconds, and the N_1 stayed immobile during the whole test. This behavioral pattern was the same for control or for (*E*)-4-oxo-2-decenal.

Aggregation Bioassay. At the beginning of the test (15 and 30 min), more N_1 are located in the test sector, but this tendency is very low (Figure 6a). The test did not show any aggregation or arrestment behavior (Figure 6b and c).

In the case of N_2 (Figure 6d), the nymphs remained near the source (mean of six to seven nymphs in the test sector) during the 120 min of the test. Figure 6e shows the mean aggregation percentage of the N_2, in response to 1 μg of (*E*)-4-oxo-2-decenal. It is significantly higher than that of the control after 15, 30, and 45 min. After 2 hr, the aggregation percentage of the control reached the same value as that of the treated series.

The synthetic (*E*)-4-oxo-2-decenal has an effect on the locomotory behavior

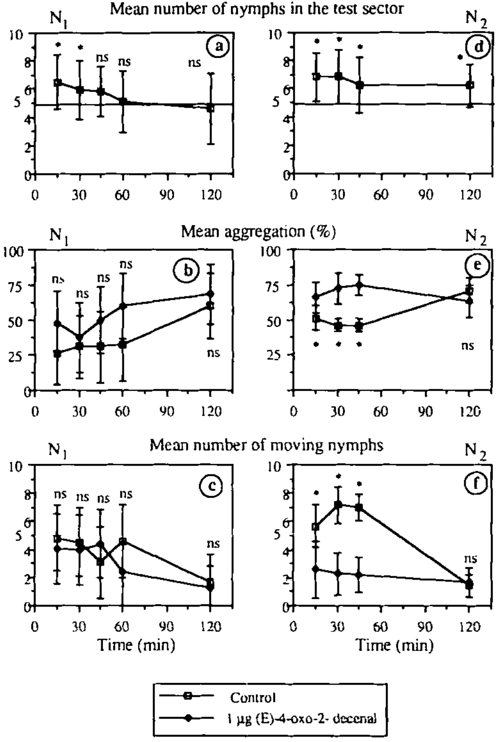

FIG. 6. Attraction, aggregation, and arrestment of first-instar nymphs (N_1) and second-instar nymphs (N_2) of *Nezara viridula* in response to 1 μg of synthetic (*E*)-4-oxo-2-decenal. For each test, 10 nymphs were placed in Petri dishes and 24 replicates made (12 for the control, 12 for the synthetic compound).

of the N_2 (Figure 6f): at least for 45 min, the number of insects in movement is higher for the control than for 1 μg of (*E*)-4-oxo-2-decenal. After 120 min, this phenomenon was no longer observed. Thus, the compound has an arrestant effect on the N_2. For the control, arrestment occurs without chemical stimulus but after at least 45 min.

Predation Tests (Figure 7). During the first 30 sec of the test, some ants came in contact with the N_1 but they quickly left the aggregate. After 1 min, the number of contacts was very low (less than 0.5 contact/6 sec) and remained at this level all during the test. The N_1 were never preyed upon by the ants when presented in aggregates. The number of contacts was higher for the control (washed chorions) than for living N_1 and washed chorions with (*E*)-4-oxo-2-decenal.

At low doses (2 and 20 ng), (*E*)-4-oxo-2-decenal had no effect on the ants. Nevertheless, doses of 50 ng (0.15–0.30 IE) and more had a repellent effect on the ants: for the control and low dose, we observed a mean value of about 38 contacts per minute and for 50 ng only 21 contacts. This mean value decreased with the dose to reach a minimum of about two contacts per minute, for 2 μg of (*E*)-4-oxo-2-decenal tested.

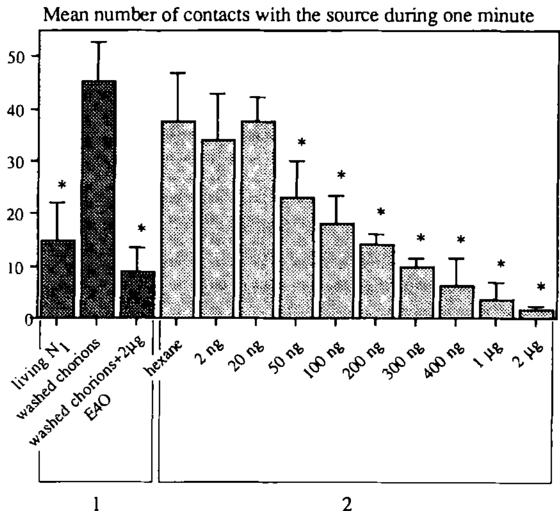

FIG. 7. Repellent activity of different sources on the ants *Solenopsis geminata*. 1: first series of treatments consisting of aggregates of N_1 on empty chorions, empty chorions washed with hexane, and empty chorions with 2 μg of synthetic (*E*)-4-oxo-2-decenal (E4O); 2: second series, carried out with pieces of bristol paper impregnated with hexanic solutions of (*E*)-4-oxo-2-decenal. Values with an asterisk are significantly different from the control at the 5% level (Mann-Whitney U test).

DISCUSSION

Our chemical analyses of the DAGs secretions in *N. viridula* revealed the presence of at least eight compounds: five alkanes (C_{12}–C_{16}), C_6 and C_{10} oxo-2-alkenals, and C_{10} alkenal. C_{12}, and C_{14}–C_{16} hydrocarbons have never been reported in the glandular secretions of *N. viridula*, while C_{13} had been detected in whole-body extracts of nymphs by Ishiwatari (1974) and Lockwood and Story (1985). This compound was always found in large amounts in our extracts (15–35% of the total secretion), but others were also detected in such proportions, particularly (*E*)-4-oxo-2-hexenal (10–30%), (*E*)-2-decenal (5–30%), and (*E*)-4-oxo-2-decenal (35%). The production kinetics of *n*-tridecane by first instars reported by Lockwood and Story (1985) is the same as ours, but the amounts that were detected were 10 times higher. On the other hand, in the study of Borges and Aldrich (1992), (*E*)-4-oxo-2-hexenal, (*E*)-4-oxo-2-octenal, (*E*)-2-decenal, *n*-tridecane, and (*E*)-4-oxo-2-decenal are reported. The qualitative and quantitative differences observed between studies are probably due to analytical differences. The extraction technique cannot explain these differences because we obtained nearly the same results with the reservoir contents and with whole-body extracts.

The semiochemicals identified in this study are more or less usual in Heteroptera DAGs. Among saturated hydrocarbons, *n*-tridecane, found in the greatest quantity in all instars of *N. viridula*, is frequent and is produced in large amounts in many Pentatomidae, in one species of Scutelleridae (Gough et al., 1985), and in various species of Pyrrhocoridae (Calam and Youdeowi, 1968; Farine et al., 1993). In addition to the role of bifunctional pheromone (alarm and aggregation) in *N. viridula* (Lockwood and Story, 1985), Calam and Youdeowi (1968) suggested that *n*-tridecane (and other saturated hydrocarbons) may act as solvents for alarm pheromones or as spreading and wetting agents in *D. intermedius*. Gunawardena and Herath (1991) demonstrated that *n*-tridecane and other medium-chain *n*-alkanes enhance the toxicity and repellency of the major toxicant in the pentatomid *Coridus janus*.

Dodecane has been identified in DAGs of Coreidae and Pyrrhocoridae (Calam and Youdeowi, 1968; Baker et al., 1972; Farine et al., 1993), but it always appears as a minor compound, as in all instars of *N. viridula*. Tetradecane and pentadecane are produced in significant amounts in *N. viridula* (about 200–300 ng/nymph), as in *P. apterus* DAG 1 (Schmuck, 1987); in this pyrrhocorid, DAG 1 of N_5 produce only simple *n*-alkanes, as in *N. viridula*. The presence of hydrocarbons in the hemolymph may be explained by the extraction technique: when piercing the insect's body, the micropipet is able to collect hydrocarbons present on the cuticle.

(*E*)-2-Decenal, which we found in all instars, was often identified in several families of Heteroptera DAGs (reviewed in Pavis, 1987). (*E*)-4-Oxo-2-hexenal,

present only in instars 2–5 in *Nezara viridula* was reported as the major compound produced by DAGs 3 in nymphs of various species of Pyrrhocoridae (reviewed in Farine et al., 1993). This gland played a role in defense mechanisms against predators and in alarm behavior in conspecifics (Farine et al., 1992b). This compound has also been reported in DAGs 2 and 3 of *Podisus maculiventris* nymphs (Aldrich et al., 1984).

Finally, we have shown that the main difference in the five instars is in the production of a major compound: (*E*)-4-oxo-2-decenal, by DAGs 2 and 3 of the first instar. It has only been detected in traces in DAGs 3 of fifth instars of *D. cingulatus* and *D. fasciatus* (Farine et al., 1993), while it appears to be a major compound in *P. apterus* (Farine et al., 1992b). In Pentatomidae, it has been reported in the genera *Thyanta*, *Euschistus*, and *Acrosternum* (Borges and Aldrich, 1992).

By its arrestant activity, (*E*)-4-oxo-2-decenal plays a role in the aggregation behavior of this species. In *N. viridula*, as in most pentatomids, the first instars aggregate upon hatching, on the egg masses; in some scutellerids, this aggregation has been shown to allow contact with symbionts from the outer surface of the egg chorions (Line et al., 1983, in Lockwood and Story, 1986). However, in *N. viridula*, Lockwood and Story (1986) reported that aggregation is not a means of inoculation of essential symbionts. After the first molt, the nymphs lose this strong tendency to stay in groups, but in *N. viridula*, the second instars often aggregate, not necessarily on the egg masses (personal observation). Lockwood and Story (1985, 1986) suggest two types of aggregation behavior in first instars: a primary aggregation on the egg shells (immediately after hatching) ensured by tactile cues, and a secondary aggregation, chemically mediated, allowing the insects to reaggregate after dispersion. This secondary aggregation would also occur on food or water resources. The production of (*E*)-4-oxo-2-decenal begins only a few hours after hatching, and this could explain the fact that N_1 are not able to reaggregate if they are artificially dispersed. The aggregation seems to depend on tactile cues just after emergence; then, both chemical and tactile cues play a role in the formation of the aggregates; (*E*)-4-oxo-2-decenal would probably concern this step.

In the control, we noticed that the aggregation percentage reaches the same value as that of the treated series after 2 hr; it seems to indicate that tactile cues alone are able to induce aggregation, but more slowly.

The aggregation activity was not modified by synthetic (*E*)-4-oxo-2-decenal in N_1, this may be due to the competition between the source offered to the N_1 and their natural secretions. The N_2 do not produce (*E*)-4-oxo-2-decenal, but they are able to respond to this compound. Electroantennographic studies carried out on the nymphs of this species demonstrated only quantitative differences in the olfactory responses of the different instars (Pavis, 1986); moreover, among the tested compounds, (*E*)-4-oxo-2-decenal evokes the highest electroantenno-

graphic responses in all instars studied (from N_3 to adults), indicating that the receptors to this compound remain present during post embryonic development (Brezot, personal communication).

At the same time, (*E*)-4-oxo-2-decenal is able to reduce the predation activity of the fire ants, the repellent activity depending on the dose emitted by the aggregates. At a physiological dose, the number of contacts of the ants with the source is four times lower than for the control. Thus, this compound specific to the N_1 is able to ensure protection towards predators, even if the nymph is isolated. In the future, it would be interesting to test the repellent activity of other compounds, particularly the aldehydes [(*E*)-2-decenal and (*E*)-4-oxo-2-hexenal] produced by the other instars, to determine if they play the same role of protection by repelling potential predators.

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INDUCTION OF PARASITOID ATTRACTING SYNOMONE IN BRUSSELS SPROUTS PLANTS BY FEEDING OF *Pieris brassicae* LARVAE: ROLE OF MECHANICAL DAMAGE AND HERBIVORE ELICITOR

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Abstract—Induction of plant defense in response to herbivory includes the emission of synomones that attract the natural enemies of herbivores. We investigated whether mechanical damage to Brussels sprouts leaves (*Brassica oleracea* var. *gemmifera*) is sufficient to obtain attraction of the parasitoid *Cotesia glomerata* or whether feeding by *Pieris brassicae* caterpillars elicits the release of synomones not produced by mechanically damaged leaves. The response of the parasitoid *Cotesia glomerata* to different types of simulated herbivory was observed. Flight-chamber dual-choice tests showed that mechanically damaged cabbage leaves were less attractive than herbivore-damaged leaves and mechanically damaged leaves treated with larval regurgitant. Chemical analysis of the headspace of undamaged, artificially damaged, caterpillar-infested, and caterpillar regurgitant-treated leaves showed that the plant responds to damage with an increased release of volatiles. Green-leaf volatiles and several terpenoids are the major components of cabbage leaf headspace. Terpenoids are emitted in analogous amounts in all treatments, including undamaged leaves. On the other hand, if the plant is infested by caterpillars or if caterpillar regurgitant is applied to damaged leaves, the emission of green-leaf volatiles is highly enhanced. Our data are in contrast with the induction of more specific synomones in other plant species, such as Lima bean and corn.

Key Words—Lepidoptera, Pieridae, Hymenoptera, Braconidae, cabbage, Brussels sprouts, behavior, tritrophic interactions, green-leaf volatiles, her-

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bivore-induced synomones, elicitor, caterpillar regurgitant, *Brassica oleracea*, *Pieris brassicae*, *Cotesia glomerata*, parasitoid, wasp.

INTRODUCTION

Herbivory leads to mechanical damage of plants and thus to an augmented emission of plant volatiles. However, the chemical blend that is emitted from mechanically damaged plants and from herbivore damaged plants can be quite different. Natural enemies of herbivores use plant volatiles to locate the herbivores, and they can discriminate between the volatiles of a mechanically damaged plant and those of an herbivore damaged plant (for reviews see Dicke et al., 1990b; Vet and Dicke, 1992; Turlings et al., 1993b; Dicke, 1994). Herbivore damaged plants may emit volatile chemicals that are not emitted by undamaged or mechanically damaged plants. This has been shown for several plant species, such as Lima bean, cucumber, and corn (Dicke et al., 1990a,b; Turlings et al., 1990, 1993b; Takabayashi et al., 1994; Dicke, 1994). Such plant volatiles increase herbivore detectability to their natural enemies. Because the volatiles are produced by the plant in response to herbivore damage and attract the herbivore's natural enemies, whose activity is favorable to the plant, they are termed herbivore-induced synomones (Vet and Dicke, 1992).

Mechanical damage may differ from herbivore damage in physical attributes, such as the amount of cellular shearing or tearing, which might result in different blends of volatiles. Since the response of plants to damage may be mediated by endogenously derived damage cues such as cell-wall fragments or cell-wall-bound enzymes, one would expect different responses to different types of damage. Comparisons of plant responses to herbivory and mechanical simulations need to be accurate not only with respect to amount of damage, but also to the spatial and temporal pattern of the damage (Baldwin, 1990). Mechanical damage of plants results in an increased emission of C_6 alcohols, aldehydes, and their esters, so-called "green odors." Several parasitoid species are known to be attracted to these green odors (Nordlund et al., 1988; Takabayashi et al., 1991a; Whitman and Eller, 1990, 1992). However, the degree of attraction of natural enemies of herbivores to volatiles emitted from artificially damaged leaves is usually much lower than to volatiles of herbivore-damaged plants (Turlings et al., 1990; Takabayashi and Dicke, 1992; Steinberg et al., 1993).

In addition to differences in temporal and spatial aspects of mechanical damage and herbivore damage, herbivory also differs from mechanical damage in that herbivores may apply or inject oral secretions onto or into the plant. When regurgitant of *Spodoptera exigua* caterpillars is applied to a mechanical wound in a corn leaf, the emitted volatiles are similar to those emitted from a caterpillar-damaged plant, but different from those emitted from a mechanically damaged plant (Turlings et al., 1990).

That plants respond to herbivory by emitting specific herbivore-induced

synomones that are not emitted by mechanically damaged plants was first demonstrated for a tritrophic system of Lima bean plants, two-spotted spider mites, and predatory mites that consume the spider mites (Dicke and Sabelis, 1988; Dicke et al., 1990a,b). A very similar phenomenon has been described for the tritrophic system of corn plants, beet army worm larvae, and their parasitoids, for which it was also shown that caterpillar regurgitant can elicit the response of the plant (Turlings et al., 1990). A few other systems have been investigated but not in as much detail as the Lima bean and corn system (see Dicke, 1994, for review). It is of interest to investigate whether all plant species respond in a comparable way to herbivory. In this paper we present data for a tritrophic system consisting of Brussels sprouts plants, caterpillars of the large cabbage white butterfly, and one of its parasitoids. We show that these plants respond to herbivory in a way similar to corn plants but that the type of response by the plant is remarkably different.

Cotesia glomerata is a gregarious larval parasitoid of several pierid species such as the cabbage white caterpillars *Pieris brassicae* (L.) and *Pieris rapae* (L.). Female *C. glomerata* discriminate among undamaged, mechanically damaged, and caterpillar-infested cabbage plants (Steinberg et al., 1992, 1993). The plant–herbivore complex (PHC), where host larvae are actively feeding, is most attractive. Yet, after removing the host larvae, the herbivore-damaged plant (HD) remains very attractive to the parasitoids for at least several hours. In contrast, the attractiveness of mechanically damaged cabbage plants quickly wanes after the infliction of the damage is stopped (Steinberg et al., 1993). Apparently herbivore damage results in a different response from the plant than mechanical damage (Steinberg et al., 1993).

In the current study, we compared the effect of true and simulated herbivore damage by *P. brassicae* to Brussels sprouts plants, in order to know whether and how herbivory influences the production of synomones by the plant. We tested two hypotheses: (1) The release of synomones by cabbage plants is activated by the mechanical disruption of leaf tissue, which needs to be properly simulated for pattern and timing, in order to be comparable to the actual herbivore damage. (2) Synomones are produced as a direct physiological response of the cabbage plant, activated when the leaf cells get into contact with possible elicitors present in the regurgitant of *P. brassicae* larvae. The study comprises behavioral observations and chemical analyses of headspace collections.

METHODS AND MATERIALS

Rearing Procedures

Plants (Brussels sprouts, *Brassica oleracea* L. var. *gemmifera*, cv. Titurel), herbivores [*Pieris brassicae* (L.), Lepidoptera: Pieridae] and parasitoids [*Cotesia glomerata* (L.), Hymenoptera: Braconidae] were reared as described by Steinberg et al. (1992).

Bioassay

Steinberg et al. (1992) showed that the strongest and most consistent response of *C. glomerata* towards volatile infochemicals from the plant–herbivore complex was exhibited by 4- to 5-day-old female parasitoids that are experienced on leaves with host-feeding damage. The same conditions and the same flight chamber set-up were adopted here to examine the response of *C. glomerata* females to cabbage leaves in a series of dual-choice tests.

The day before the experiment, females were allowed to walk and antennate for at least 20 sec on a cabbage leaf where first- to second-instar *P. brassicae* caterpillars had been eating for 24 hr, in order to acquire host-damage experience. Larvae had been removed just prior to the introduction of wasps. Experienced wasps were then transferred individually into cotton wool-stoppered glass vials (6 ml) provided with a droplet of honey. The vials were held overnight in an incubator at 15 ± 1°C and transferred from the incubator to the flight chamber 30 min prior to the experiment.

The flight chamber set-up consisted of a "tent" made of white cotton sheets inside a greenhouse compartment, at 22 ± 2°C and 60 ± 10% relative humidity. Cabbage leaves, with the petiole in a glass vial with water, were placed on a table over which two electric fans generated an airstream of 30–40 cm/sec. The leaves formed an equilateral triangle with 40-cm sides with the release point, from where the wasps were individually released. In order to minimize visual stimuli, a white screened cloth (20 mesh) was placed between the release point and the test leaves (for details see Steinberg et al., 1992).

A choice was scored when the wasp completed a flight, landing on the screen area corresponding to one of the odor sources. A "no response" was scored when the wasp landed anywhere else in the bioassay chamber. Each wasp was allowed only one flight attempt. Wasps that did not fly within 20 min from being released in the bioassay arena were discarded.

Every bioassay was conducted at least on two days, in order to overrule a day-to-day variation in the response of *C. glomerata*, which was previously correlated to barometric pressure changes within the time period of the experiments (Steinberg et al., 1992). When the overall response level in a bioassay was lower than 40%, the same wasps were immediately retested in a standard experiment (three leaves infested with about 100 first- or second-instar *P. brassicae* (plant–herbivore complex, PHC) vs. three undamaged leaves (UND), in order to make sure that the low response level was not due to a low motivation of the wasps but due to the odor sources used.

Statistics

Bioassay data were subjected to a chi-square test for goodness-of-fit (Sokal and Rohlf, 1981) to determine if the response differed from a 50:50 distribution of wasps over the two odor sources.

Herbivory Simulation by Artificial Damage

Mechanical damage was inflicted on cabbage leaves with different patterns and timing. Prior to the experiments the leaves were excised and put in a glass vial with water, in order to be used for the bioassay.

"Old" Artificial Damage (ADold). About one third of the surface of one cabbage leaf was rubbed with 180-grit carborundum powder (BDH Chemicals) on a wet cotton wool pad. This kind of damage was inflicted one day before the bioassay. Old host damage (HDold) was represented by a leaf, of a different plant, where about 100 first- or second-instar larvae had been feeding for about 20 hr. The larvae were removed with a fine brush the day before the experiment.

The attractiveness of the ADold leaf was compared to an undamaged leaf (UND) excised from a different undamaged plant just before the bioassay and, in a separate bioassay, to one herbivore-damaged leaf (HDold).

"Continuous" Artificial Damage (AD7h). In order to compare the effect of prolonged feeding activity of the larvae with the administration of mechanical damage lasting for the same period of time, one cabbage leaf was punched with a cork borer (0.2 cm diam) every 30 min during 7 hr (AD7h) the day before the experiment. In a leaf of a different cabbage plant, one hole was punched and 100 first-instar *P. brassicae* larvae were added and allowed to feed (HD7h). In this way damage of the same size was inflicted starting from the same moment on the two leaf treatments (AD7h and HD7h). The larvae were removed after 7 hr. The attractiveness of the herbivore-damaged leaf (HD7h) was compared to the attractiveness of the artificially damaged leaf (AD7h) in the flight chamber bioassay.

Herbivory Simulation Using Caterpillar Regurgitant

Collection of Regurgitant. One day before the bioassay, regurgitant was collected from third- to fifth-instar *P. brassicae* larvae. Regurgitation was induced by gently squeezing the caterpillars and rapidly collecting with a 5-μl glass capillary tube (Einmal-Mikropipetten, Blau Brand, Germany) the regurgitant droplet produced by the larvae. Per caterpillar about 1–5 μl regurgitant was collected, depending on their size. After collection, the regurgitant was immediately applied to three cabbage leaves either to artificial damage or via incubation in an aqueous solution in the amounts explained below. Leaves treated with larval regurgitant were named "REG" leaves. Control leaves had always the same type of AD or of incubation as the test leaves, but no regurgitant smeared onto the wound surface or added to the solution.

Application onto Artificial Damage. The day before the bioassay, 100 μl of larval regurgitant was collected as described and applied on three leaves with old artificial damage. Two combinations were independently tested: REG on ADold vs. ADold, and REG on ADold + fresh vs. ADold + fresh. In this last

treatment the regurgitant was applied on the wound surface of old damage and the leaf was punched with an 0.8-cm-diam. cork borer every 15 min, starting 1 hr before and continuing for the duration of the bioassay.

Incubation in Regurgitant Solution. In order to test if the regurgitant, or some elicitor contained in it, was systemically transported throughout the leaf, three intact leaves were excised and incubated overnight with their petiole in 1 ml of a 100 μl/ml aqueous solution of larval regurgitant. As a control, three intact leaves were incubated in distilled water. The two groups were used in the bioassay on the following day. Two combinations were tested: incubated leaves with fresh artificial damage (REG on ADfresh vs. ADfresh), and undamaged leaves incubated in regurgitant solution or water (REG on UND vs. UND). To obtain fresh artificial damage (ADfresh), undamaged leaves were excised just prior to the bioassay and, in order to mimic the feeding bouts of the caterpillars (Steinberg et al., 1993), one 0.8-cm-diam. hole was punched with a cork borer every 15 min, starting 1 hr before the bioassay and continuing for its duration (2–3 hr).

Collection and Analysis of Headspace Volatiles from Cabbage Plants. Eight-week-old plants with 10–12 leaves of approximately the same leaf area were used. All samples were collected in the period April–July 1993. Ten cabbage leaves were cut from one plant and placed immediately in a 5-liter glass flask, with a 10-cm-ID opening. Care was taken not to cause any additional damage to the leaves, except for cutting the petiole. The leaves were placed with their cut petiole in a 200-ml glass beaker filled with water that was located inside the flask. The beaker was covered with a perforated glass lid, in order to reduce the evaporating surface. The petioles were inserted through the holes of the lid and put in contact with water. Four plant treatments were used: undamaged leaves (UND), leaves with old artificial damage (ADold), leaves on which about 100 first-instar caterpillars/leaf had been feeding for at least 24 hr (PHC), and leaves treated with larval regurgitant (REG, 10 μl regurgitant/leaf applied on old AD, 20 hr prior to headspace collection).

An airstream was generated in the flask at 500 ml/min by air pressure. The air was cleaned at the inlet of the flask through silica gel, molecular sieves, and activated charcoal as described by Takabayashi et al. (1991b). Filters and flask were connected through 0.8-cm-diam Teflon tubing. After the introduction of the leaves, the system was purged for 3 hr, in order to remove air contaminants from the flask, and then the flow was interrupted for 1 hr to increase the quantity of volatiles produced by accumulation. A Pyrex glass tube (160 $\times$ 6 mm OD) containing 90 mg Tenax-TA was connected to the outlet of the flask through a Teflon-coated plastic fitting and the airflow was restarted to last for 1 hr. After the collection period, the adsorption tube was disconnected from the flask and closed with 1/4-in. brass Swagelok caps, using Teflon ferrules. The analysis of

the collected headspace volatiles was performed by desorption in a Thermodesorption Cold Trap Unit (Chrompack) connected to a gas chromatograph–mass spectrometer system as described in Takabayashi et al. (1991b).

RESULTS

Herbivory Simulation by Artificial Damage. Steinberg et al. (1993) demonstrated that current AD inflicted to a cabbage plant attracted *C. glomerata* females. Here we show that even a day after the AD was done, an excised cabbage leaf attracted significantly more *C. glomerata* than a control undamaged

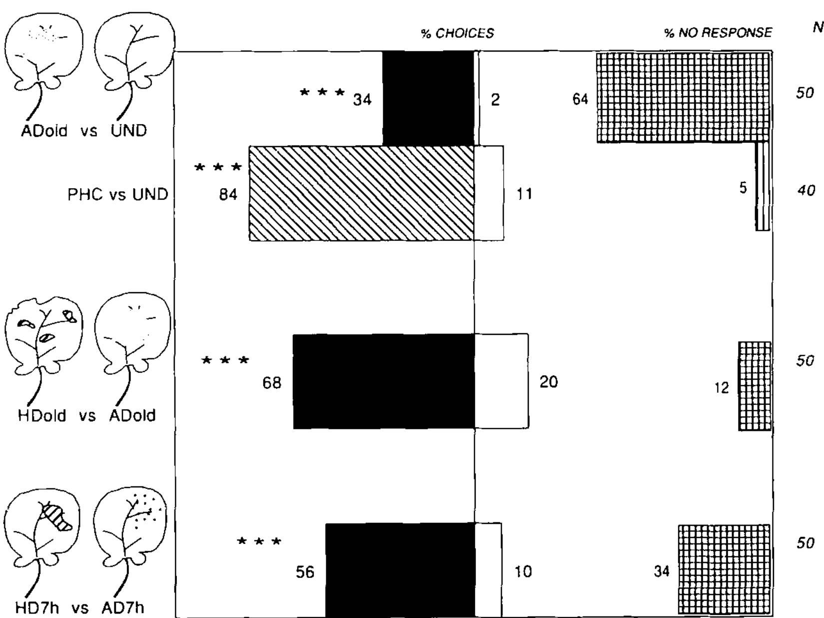


FIG. 1. Response of female *C. glomerata* to herbivory simulated by artificial damage. Drawings on the left side of the figure illustrate the types of larval or artificial damage or no damage; per pair, the left drawing refers to the left part on the choice bar, and the right drawing to the right part of the choice bar. Standard test is reported without drawings. Number of replicates (N) for every comparison are given on the right side of the figure. Numbers next to bar indicate percentage of parasitoids making a choice for one of the two odor sources, or not making a choice at all. The three percentages add up to 100%. Asterisks indicate significant differences within the choice test: *$0.01 < P \leq 0.01$, **$0.001 < P \leq 0.01$, ***$P \leq 0.001$, Chi-square test for goodness-of-fit (Sokal and Rohlf, 1981). For abbreviations of treatments see Methods and Materials.

leaf (ADold vs. UND, Figure 1). However, the overall response level was low (36% of all bioassayed females made a choice), indicating either a weak stimulation or absence of some important volatile in the blend. The same wasps, retested in the standard bioassay preferred PHC over undamaged leaves, with 95% of wasps making a choice (Figure 1), showing that atmospheric pressure fluctuation (cf. Steinberg et al., 1992) did not account for the low response level.

Host damage was significantly more attractive than artificial damage, since damage caused by *P. brassicae* larvae that had been feeding for 24 hr and then removed 20 hr before the test was more attractive than artificial damage that was inflicted 20 hr before the test (HDold vs. ADold, Figure 1). Damage from caterpillars feeding for 7 hr on the day prior to the bioassay was more attractive than AD done at regular intervals on a control leaf during the same 7 hr (HD7h vs. AD7h, Figure 1).

Herbivory Simulation Using Caterpillar Regurgitant. Female *C. glomerata* were given a choice between combinations of cabbage leaves that had been treated with caterpillar regurgitant and different types of artificial damage (Figure

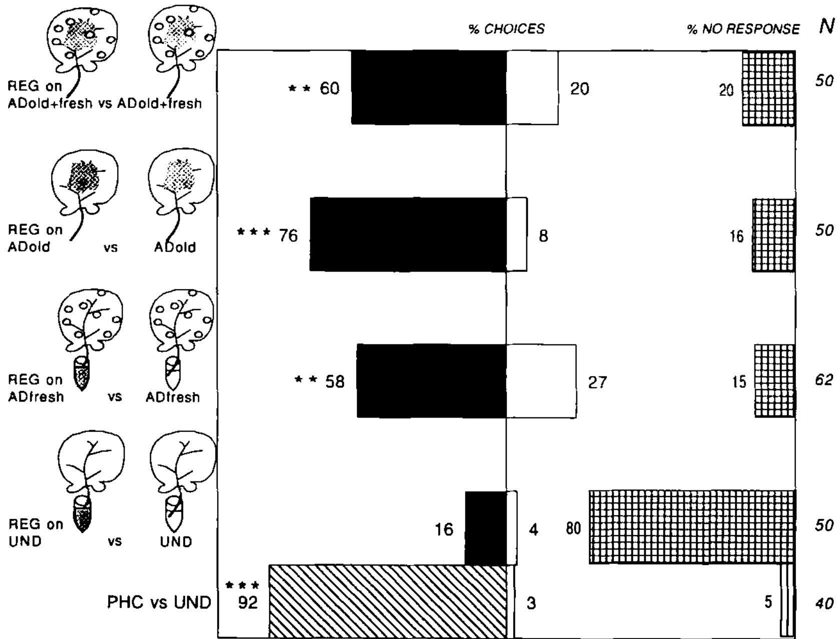


FIG. 2. Response of female *C. glomerata* to herbivory simulated by application of caterpillar regurgitant to artificial damage. For other explanations, see Figure 1 legend.

2). Wasps showed a clear preference for leaves treated with regurgitant over mechanically damaged leaves for all combinations. Only when undamaged leaves were incubated in a regurgitant solution, did the parasitoids not distinguish between the two odor sources (REG on UND vs. UND). Only 20% of the females made a choice in the bioassay, while, when they were retested in the standard bioassay, 95% of the wasps made a choice, almost exclusively in favor of the PHC.

Analysis of Headspace Volatiles of Cabbage Leaves. The composition of the volatile blends emitted by Brussels sprouts plants with different treatments (UND, ADold, PHC, REG on ADold) and the average total area of the identified peaks are given in Table 1. A very high number of chemicals was detected (73 among all treatments). The chemicals were identified as short-chain aldehydes, ketones, alcohols and esters, terpenes, fatty acids, and traces of sulfides and isothiocyanates. The variation between replicates of each treatment was considerable, with several compounds detected in only one replicate/treatment or consistently but in very small amounts.

The PHC produced the highest number of compounds (53) and amounts, since the average total peak area was more than 14-fold relative to the average total peak area of compounds produced by undamaged leaves (UND). Regurgitant-treated leaves produced a somewhat lower number of compounds (40) and in lower amounts (4.5-fold UND). Artificially damaged leaves produced fewer compounds (34) and in a lower amount (2.5-fold UND). Chromatographic profiles of headspace of plants with the four treatments are given in Figure 3.

Differences were consistently observed in the total amounts but not in the ratios of the compounds. An exception was (*Z*)-3-hexen-1-ol, which comprised 31% in PHC and 4–10% in the other three treatments. In order to evaluate the quantitative variation of single compounds across treatments (ADold, PHC, REG on ADold), we assumed that the chemicals that would show an evident increase from the amounts emitted by undamaged leaves (UND) would cause attraction of the parasitoid, although a synergistic effect with constitutive cabbage compounds could not be excluded. Therefore, the amount of a certain chemical relative to that emitted by UND leaves was calculated by dividing its average peak area in a treatment (ADold, PHC, REG on ADold) by the average peak area of the same compound in the UND treatment (lowest degree of attraction for the wasps). When a chemical was not present in undamaged leaves, a peak area value of 1 was given, in order to avoid a division by 0. This transformation was performed only for 24 compounds that were common to the treatments that proved to be most attractive during the bioassays (PHC and REG on ADold), assuming that a complete synomone blend would certainly be emitted by the leaves of these treatments. The 24 compounds comprised 85–90% of the average total quantity of identified chemicals.

The amounts relative to the amount emitted by UND leaves for compounds

TABLE 1 PERCENTAGES[a] OF TOTAL AREA OF GC PEAKS FOR COMPOUNDS DETECTED IN HEADSPACE OF CABBAGE LEAVES WITH DIFFERENT TREATMENTS[b]

	treatments			
Compounds	UND	ADold	PHC	REG on ADold
Aldehydes				
2-Butenal			0.1	
(*E*)-2-Pentenal			0.2 (0–800)	
(*Z*)-2-Pentenal			0.1 (0–250)	
Hexanal	0.3	0.6 (0–236)	0.9 (1262–1395)	0.7 (0–544)
(*E*)-2-Hexenal		0.4	5.5 (2958–18006)	1.7 (0–1324)
(*Z*)-2-Hexenal			2.1 (0–8240)	
2,4-Hexadienal			0.1	
Octanal		0.5 (0–300)	tr	0.2
Nonanal	1.1	1.5 (0–871)	tr	0.9
Decanal	2.5	4.3 (0–2135)	tr	1
Dodecanal	4.3			
Tetradecanal	2.1			
Ketones				
1-Cyclopropyl-2-propen-1-one			0.6	
3-Pentanone	5.5 (0–1370)	4.3 (0–2117)	2.6 (3556–4959)	5.9 (2195–4391)
3-Methyl-2-pentanone			tr	
1-Penten-3-one			1.1 (949–2867)	
3-Penten-2-one			0.1(0.12)	
3-Octanone				0.5 (66–555)
2,3-Pentanedione			tr	
Alcohols				
Ethanol			0.1	
2-Methyl-1-propanol	0.6	0.1	0.4	
2-Propanol				tr
1-Butanol		0.2		
3-Methyl-1-butanol			0.1	
Pentanol			0.1	
1-Pentanol			tr	

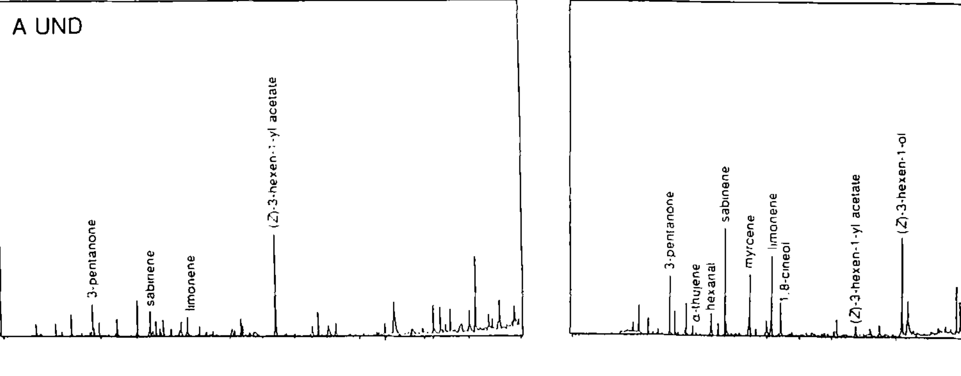
3-Pentanol	0.9	0.8 (0–636)	0.3	1.7 (0–1919)
2-Penten-1-ol				0.2
1-Penten-3-ol	0.4	2.5 (0–1612)	1.6 (2548–2696)	0.9 (0–1000)
1-Hexanol		1.1 (158–444)	1.3 (1200–2668)	0.9 (0–1038)
(*E*)-2-Hexen-1-ol			2.1 (1189–6092)	0.3
(*Z*)-2-Hexen-1-ol			tr	
(*Z*)-3-Hexen-1-ol	5.6 (213–612)	9.6 (1306–4376)	31.2 (33297–53600)	4.3 (674–4336)
1-Octen-3-ol				0.3 (0–262)
1-Nonanol				0.1
Esters				
Isobutyl acetate				0.1
1-Butyl acetate		0.5		0.9 (0–1158)
3-Methyl-3-buten-1-yl acetate		0.1	0.6 (0–2596)	
Isopentyl acetate		0.9	0.1	0.5
1-Pentyl acetate		0.2		3.7 (0–3289)
1-Hexyl acetate	2.2 (0–537)	6.5 (0–4388)	2.2 (1260–7010)	7.7 (445–5837)
(*E*)-2-Hexen-1-yl acetate		0.6 (0–382)	1.4 (669–5272)	1.4 (0–1386)
(*Z*)-3-Hexen-1-yl acetate	39.5 (1474–9266)	46.2 (201–23468)	32.8 (28972–79270)	39.6 (3196–32951)
3-Hexen-1-yl propanoate		0.5 (0–307)	0.2	1.2 (0–1097)
3-Hexen-1-yl 3-Methylbutanoate		0.5 (0–436)		0.9 (0–1011)
(*Z*)-3-Hexen-1-yl butyrate		0.2	2.3 (1145–6484)	0.4
(*Z*)-3-Hexen-1-yl isobutyrate			0.9 (0–2960)	
(*Z*)-3-Hexen-1-yl isovalerate			2.2 (1227–6026)	0.1
3-Hexen-1-yl caproate			0.1	
(*Z*)-3-Hexen-1-yl hexanoate			0.6 (0–1710)	
Heptyl acetate				0.2
Fatty acids				
Isobutyric acid			0.5	
Caproic acid	1		0.1	
Isovaleric acid			0.8	
Terpenoids				
α-Pinene	0.4	0.3 (0–245)		0.7 (243–499)
β-Pinene	0.5	0.3 (0–153)	tr	0.6 (179–325)
α-Thujene	1.6	1.5 (302–580)	0.2 (0–847)	1.3 (544–703)

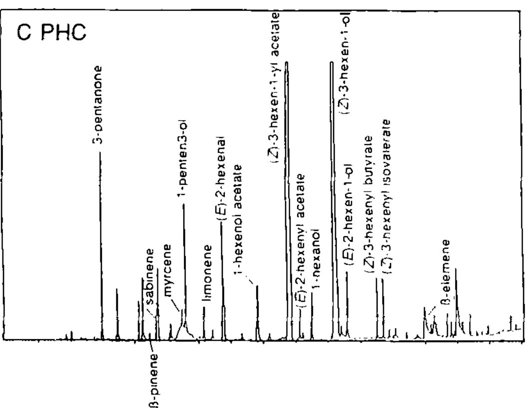
TABLE 1 CONTINUED

Compounds	treatments			
	UND	ADold	PHC	REG on ADold
Terpenoids				
Sabinene	11 (242–2100)	5.3 (1217–1757)	0.8 (600–1700)	7.2 (2349–4431)
Myrcene	4.4 (0–843)	2.6 (346–1023)	0.1	3.2 (1299–2266)
Limonene	9.1 (199–1564)	6 (1047–2587)	0.7 (711–1748)	6.7 (1720–4732)
1,8-Cineole	2.8 (114–450)	2 (331–899)	0.2 (236–422)	2.3 (645–1584)
β-Elemene	4.2 (0–910)	2.2 (0–917)	0.4 (119–1300)	1.5 (0–1132)
4,8-Dimethyl-1,3(*E*),7-nonatriene				0.2
4,8,12-Trimethyl-1,3(*E*),7(*E*)11-tridecatetraene				0.2
γ-Terpinene				tr
trans-Sabinenehydrate		0.1		0.1
α-Farnesene			0.1	
Furans				
2-Ethyl-furan			0.3	
Sulfides				
Dimethyldisulfide			0.6 (154–1741)	
Dimethyltrisulfide			0.1 (0–1600)	
Isothiocyanates				
Methyl-ITC			1.3 (1213–3451)	
Average total area	11,175	27,887	162,878	50,475
SD	(5,725)	(14,980)	(50,099)	(17,886)

[a] Percentages are calculated by dividing the average peak area of a compound in every treatment by the total average area of compounds detected in that treatment. The range of peak area found in all replicates is indicated, in parentheses, only for compounds that were found in more than one analysis. $N = 3$ for all treatments, except artificially damaged leaves (ADold) where $N = 4$

[b] Abbreviations: UND = undamaged, ADold = artificial damage, PHC = plant-herbivore complex, REG on ADold = ADold + larval regugitant, tr = chemicals found only in one analysis and in amounts smaller than 0.1%.





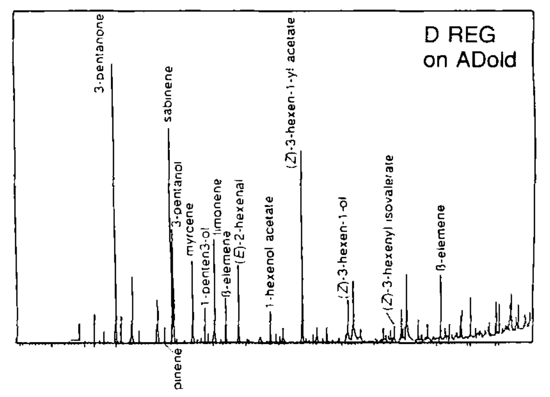


FIG. 3. Chromatographic profiles of headspace collections of Brussels sprouts plants that underwent different treatments. A = undamaged (UND). B = "old" artificial damage (ADold). C = plant–herbivore complex (PHC). D = "old" artificial damage + caterpillar regurgitant (REG on ADold).

common to the behaviorally active treatments are given in Figure 4. Clearly, all treatments (ADold, PHC, REG on ADold) show a similar trend in the increase of emission of chemicals compared to undamaged leaves. For the treatments resulting in highest behavioral attraction, PHC and REG on ADold, eight of the 24 compounds screened [(*E*)-2-hexenal, 1-hexanol, (*E*)-2-hexen-1-ol, (*E*)-2-hexen-1-yl acetate, 3-hexen-1-yl propanoate, (*Z*)-3-hexen-1-yl butyrate, (*Z*)-3-hexen-1-yl isovalerate, and octanal] showed an increase more than 20-fold UND. The PHC shows the most amplified emission, with an increase of 200–8000 times of (*E*)-2-hexenal, 1-hexanol, (*E*)-2-hexen-1-ol, (*E*)-2-hexenyl acetate, 3-hexen-1-yl propanoate, (*Z*)-3-hexenyl butyrate, and (*Z*)-3-hexenyl isovalerate. The emission of volatiles produced by REG on ADold-leaves is 50–800 times UND, and it is definitely higher than ADold. It is important to note that the ADold and REG on ADold treatments both had the same type of artificial damage, but they differ in the application of regurgitant. Artificially damaged

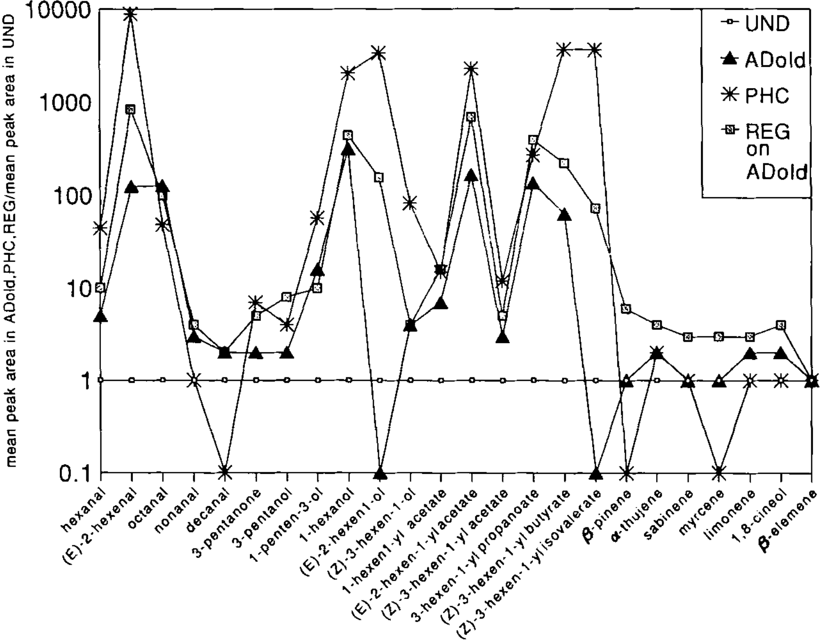


FIG. 4. Relative increase of selected compounds (*x* axis) common to behaviorally active treatments (ADold, PHC, REG on ADold), calculated in proportion of the amounts emitted by undamaged leaves (UND). Values of *y* axis (log scale) indicate the ratio between the average peak area of every compound in the ADold, PHC, and REG on ADold treatments and the average peak area of the same peak in the UND treatment.

leaves still show differences in comparison to undamaged leaves, but the emission scale is less than 10-fold UND for most compounds, exceeding a 100-fold UND increase only for (*E*)-2-hexenal, octanal, 1-hexanol, (*E*)-2-hexenyl acetate, and 3-hexenyl propanoate.

DISCUSSION

It has been shown for several tritrophic systems that an interaction is needed between the herbivore and the plant for volatile synomones to be emitted that attract predators or parasitoids of the herbivores (Sabelis et al., 1984; Dicke et al., 1990a,b; Turlings et al., 1990). Turlings et al. (1990) were the first to demonstrate that herbivore oral secretions applied into a mechanical wound can effectively mimic herbivory in this respect. Steinberg et al. (1993) showed that *C. glomerata* is strongly attracted to cabbage plants on which *P. brassicae* larvae are feeding, while infochemicals emitted by the herbivore alone or its products, such as frass, were of much less importance in parasitoid attraction. The PHC was more attractive to the parasitoids than herbivore-damaged plants from which the herbivores had been removed and mechanically damaged plants (Steinberg et al., 1993). Here we show that cabbage leaves, mechanically damaged, can produce synomones even without being in contact with *P. brassicae* larvae. Our study provides chemical data that demonstrate quantitative differences between the volatiles emitted from the PHC, from mechanically damaged plants treated with caterpillar regurgitant, and from mechanically damaged plants (treatments in decreasing order of quantity of emitted volatiles). No major qualitative differences between the blends from these treatments were recorded. The qualitative differences in minor components may be explained by these components being above the detection threshold at higher emission rates. The main components of the volatile blend released from cabbage are terpenoids and green-leaf volatiles. Terpenoids are a major class among herbivore-induced synomones that attract arthropod carnivores (reviewed by Dicke, 1994). Interestingly, the qualitative and quantitative variations of the terpenoids identified from cabbage plants of different treatments are very low. Due to an increase in the emission of other chemicals from PHC and regurgitant-treated plants, their relative contribution to the headspace of these plants is lower than in undamaged or artificially damaged plants. Their absolute quantities are hardly affected by damage. Terpenoids comprise, on average, 25–30% of the volatiles obtained from undamaged and artificially damaged leaves, while this percentage is only ca. 2.5% for volatiles from PHC. On the other hand, some green-leaf volatiles [(*E*)-2-hexenal, 1-hexanol, (*E*)-2-hexen-1-ol, (*E*)-2-hexen-1-yl acetate, 3-hexen-1-yl propanoate, (*Z*)-3-hexen-1-yl butyrate, and (*Z*)-3-hexen-1-yl isovalerate] that have not been recorded in the headspace of undamaged leaves constitute

only 2% of the blend in artificially damaged leaves but more than 13% in PHC. Green-leaf volatiles are saturated and unsaturated six-carbon alcohols, aldehydes, and derived esters formed by oxidative degradation of plant lipids, through the so-called "lipoxygenase pathway" (Hatanaka, 1993). They have been reported as volatile components of numerous plant species belonging to a variety of plant families (Visser et al., 1979). Given the dramatic increase of green-leaf volatiles in the headspace of damaged cabbage compared to undamaged plants, it is likely that these chemicals are involved in the attraction of *C. glomerata* to infested or regurgitant-treated cabbage plants.

For another tritrophic system, it has been demonstrated that herbivore-damaged leaves produce C_6 volatiles that serve as synomones for the braconid parasitoid, *Microplitis croceipes*. In a wind-tunnel bioassay female parasitoids oriented to both cowpea plants damaged by the host herbivore, *Heliotis zea*, and to individual synthetic green-leaf volatiles (Whitman and Eller, 1990). In particular green-leaf esters elicited the greatest percentage of successful orientation flights when offered to the wasps at different concentrations (Whitman and Eller, 1992). This parasitoid is also strongly attracted to plants that release only minor amounts of C_6 volatiles, but large amounts of terpenoids (McCall et al., 1993; Turlings et al., 1993a).

Further studies are needed to test the attractiveness for *C. glomerata* of an artificial mixture of the volatiles identified in cabbage and to verify qualitative and quantitative characteristics of the blend.

Chemical analyses of the volatiles emitted by plants of the same cabbage cultivar used in our study, when infested by either *P. brassicae* or *P. rapae* caterpillars showed that the blends of those two PHCs did not differ qualitatively but that only the amount of emitted volatiles per individual caterpillar differed (Blaakmeer et al., 1994). Thus, it seems that damage inflicted to this cabbage cultivar results in a blend of volatiles that is qualitatively similar but that the emitted quantities may differ with treatment. A comparison of the data of Blaakmeer et al. (1994) on headspace composition of *P. brassicae*-infested and uninfested Brussels sprouts plants with our data shows many similarities. However, apart from differences in minor components, which must be the result of sampling from quite differently sized collection vessels, some larger differences occurred. In *P. brassicae*-infested plants we find a markedly higher percentage of (*Z*)-3-hexen-1-ol and a lower percentage of terpenoids than did Blaakmeer et al. (1994). The different percentages of (*Z*)-3-hexen-1-ol may be related to differences in the amount of mechanical damage done by the different amount of caterpillars (1000 caterpillars/10 leaves vs. 50 caterpillars/10–12 leaves plant) or by the use of excised leaves vs. intact plants. In this context it is also remarkable that other compounds produced through the lipoxygenase pathway [(*E*)-2-hexenal and (*E*)-2-hexen-1-ol] are recorded in our PHC headspace and not by Blaakmeer et al. (1994).

In all our bioassays, undamaged cabbage leaves were the least attractive infochemical source for foraging *C. glomerata*. The experiments with artificial damage showed that timing and pattern of infliction are important for the induction of attractive volatiles. Steinberg et al. (1993) found that the response to artificial damage inflicted immediately prior to the bioassay wanes quickly unless the damage is repeated at regular intervals. In addition, we observed (Figure 1) that artificial damage inflicted the day before the experiment (ADold) is significantly attractive to the parasitoids when compared to undamaged leaves, although the responsiveness is low. However, the wasps always preferred herbivore damage over artificially damaged leaves, regardless of pattern and timing of infliction of both damage types. Furthermore when *P. brassicae* regurgitant was applied on ADold, the responsiveness of the wasps is high: 84% make a choice and the wasps clearly discriminate between REG on ADold and ADold (Figure 2). These observations lead to the conclusion that attractive volatiles are produced upon mechanical damage but that herbivore damage results in a different plant response in terms of quantity of emitted volatiles. In previous studies, the term herbivore-induced synomone (HIS) was used for chemicals whose production was induced by herbivory. In addition to the data of Steinberg et al. (1993), our data show that caterpillar infestation or application of regurgitant of the caterpillar *P. brassicae* on cabbage plants of the cultivar Titurel leads to effects that are different from mechanical damage. However, the induction process does not lead to the emission of different volatiles than those emitted by mechanically damaged plants but rather to higher emission rates that remain detectable during a longer period after treatment.

In other tritrophic systems the application of an exogenous (Turlings et al., 1993a) or endogenous (Dicke et al., 1993) elicitor through the petiole induces emission of HIS from undamaged leaves. Synomones in corn are released by plants incubated in an aqueous solution of regurgitant without any further damage on the leaf surface (Turlings et al., 1993a). Undamaged uninfested Lima bean leaves, incubated in water in which spider mite-infested leaves had been present for the previous seven days, became attractive to predatory mites, demonstrating that an elicitor transported out of infested leaves was taken up by uninfested undamaged leaves that subsequently initiated the release of synomones (Dicke et al., 1993). When we incubated cabbage leaves in the *P. brassicae* regurgitant solution, we could only observe an attraction by the parasitoids if the leaf surface was wounded (Figure 2). If the leaves were incubated in regurgitant but not wounded, the wasps were far less responsive and not able to discriminate between these and the control leaves. In the *P. brassicae*-cabbage interaction, the regurgitant itself or an endogenous elicitor activated by the regurgitant in the place of entrance, is transported throughout the leaf that starts producing attractive volatiles. The final reaction that leads to the production or emission of synomones requires leaf-surface wounding. It can be hypothesized

that synomones are produced inside the leaf, without being able to permeate the cell walls or the wax layer protecting the leaf. Another possibility is that the elicitor contained in the regurgitant is transported through intracellular spaces in the leaf. The cell wall breakdown, caused by mechanical damage, allows the contact between the elicitor and its receptor and, therefore, the activation of the biochemical pathway that leads to synomone emission.

Our chemical data contrast with those from studies on other PHCs (Dicke and Sabelis, 1988; Dicke et al., 1990b; Turlings et al., 1990, 1993a,b; Dicke, 1994). For several plant species, such as Lima bean, cucumber, and corn, these studies have recorded that plants that are infested with herbivores or plants that are treated with herbivore products emit volatile chemicals that are not emitted by mechanically damaged plants or by undamaged plants.

The production of induced synomones by plants in response to herbivory was first documented in 1988 (Dicke and Sabelis, 1988). Since then, it has been studied for many plant species (see Dicke, 1994, for review). By incorporating more plant species, we are discovering that the response of different plant species varies. It will be a challenge to unravel the causes for this variation.

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OLFACTORY BASIS OF CANNIBALISM IN GRASSHOPPERS (ORTHOPTERA: ACRIDIDAE): I. LABORATORY ASSESSMENT OF ATTRACTANTS

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Abstract—Two laboratory-reared and five field-collected species of grasshoppers were assayed for behavioral responses to volatile chemicals emitted from grasshopper cadavers using a two-choice olfactometer with no stimulus as a control. Necrophilic and necrophobic responses to the stimuli were dependent upon species, sex, development, starvation, crowding, and attractant. Laboratory-reared *Melanoplus differentialis* (Thomas) and field-collected *Hadrotettix trifasciatus* (Say) and *Aulocara elliotti* Thomas exhibited significant responses to the cadavers. Females of these three species showed significant movement towards the cadavers, but males were not significantly necrophilic. All tested developmental stages of *H. trifasciatus* (fourth-instar nymphs through adults) showed significant attraction to cadavers. Fed *M. differentialis* adults and *H. trifasciatus* adults and nymphs were significantly attracted to cadavers, but starved individuals were either repelled or exhibited no significant response. Although female *A. elliotti* from an uncrowded population were significantly attracted to cadavers, females from a crowded population were significantly repelled from cadavers. Contraspecific cadavers more frequently elicited a measurable response in adult *M. sanguinipes* and *H. trifasciatus* than did conspecific cadavers, and only contraspecific cadavers yielded a significant (necrophilic) response. Although *M. differentialis* was necrophilic, neither males nor females were significantly attracted to water.

Key Words—Orthoptera, Acrididae, grasshopper, olfaction, cannibalism, necrophagia, necrophilia.

INTRODUCTION

The consumption of grasshoppers (Orthoptera: Acrididae) by other grasshoppers falls within a spectrum of feeding behaviors, including predation, cannibalism,

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necrophagia, and scavenging. Predation refers to an individual consuming another living individual, and this behavior has been only rarely observed in acridids (Anderson and Wright, 1952; Chapman, 1992). Cannibalism is the consumption of living or dead members within a taxon. This behavior is common in many herbivorous species (Fox, 1975), including grasshoppers (Rizvi, 1967; Iqbal and Aziz, 1976; Majeed and Aziz, 1977; C.R.B., unpublished observations). Cannibalism has been more broadly defined to include confamilial feeding [e.g., consumption of acridids by acridids (Lockwood, 1989a)], which appears to be virtually ubiquitous among rangeland grasshoppers (Lavigne and Pfadt, 1964; Lockwood 1989a,b; O'Neill et al., 1993). Necrophagia is a broader term than cannibalism, including the consumption of any dead or decaying animal. Finally, scavenging is broader still and refers to consumption of nonliving plant or animal tissue—a common behavior in acridids (Lavigne and Pfadt, 1964; Uvarov, 1977).

Annual damage to rangeland by grasshoppers has been estimated at over $400 million (Hewitt and Onsager, 1983). Effective pest management requires a clear understanding of feeding ecology (Chapman, 1990; Lockwood, 1993). Necrophagia is of particular relevance to grasshopper management by biological control. Successful use of *Nosema locustae* Canning, the only registered, commercially available biological control agent of acridids, depends upon the horizontal transmission of the pathogen through cannibalism (Henry, 1972). The formulation of biological and chemical insecticides provides another compelling, but less direct, rationale for understanding scavenging and necrophagia.

Spores of *N. locustae* are applied on wheat bran bait, and this formulation is also the preferred method for the application of insecticides, due to the marked reduction in nontarget impacts relative to liquid formulations (USDA, 1987). Although enhanced target specificity is a widely recognized advantage of baits, this formulation has a significant liability as well. There are several pest species and stages that do not readily consume baits (Onsager et al., 1980; Bomar and Lockwood, 1991; Jech et al., 1992), and this problem precludes use of this formulation in many cases. However, the addition of an attractant to the bait might overcome this difficulty. Ideally, such an attractant would be associated with feeding (rather than mate location or other behaviors) across a wide range of pest species (Capinera, 1987; Bomar, 1993).

Understanding necrophagia provides a potential means of addressing these issues; this feeding behavior is taxonomically widespread (Lavigne and Pfadt, 1964; Lockwood, 1989b) and several grasshopper species that do not readily consume bait are necrophilic (Lockwood, 1989a,b). Circumstantial evidence suggests necrophilia is mediated by olfaction (Lockwood, 1989a), and it may therefore be possible to use semiochemicals to enhance the efficacy of bait formulations (Bomar, 1993). The first steps towards this goal were undertaken in this study. Our primary purpose was to determine if necrophilia is mediated through an olfactory response to grasshopper cadavers under controlled, labo-

ratory conditions. Secondarily, we initiated investigations of how species, sex, development, starvation, crowding, and attractant source affect grasshoppers' responses to cadaver odors.

METHODS AND MATERIALS

Subjects. Bioassays were conducted using seven species of grasshoppers. Two species were laboratory-reared: nondiapausing *Melanoplus sanguinipes* (F.) (Pickford and Randell, 1969) and *M. differentialis* (Thomas); and five species were collected from southeastern Wyoming: *M. bivittatus* (Say), *Aulocara elliotti* Thomas, *Camnula pellucida* Scudder, *Hadrotettix trifasciatus* (Say), and *Brachystola magna* (Girard). Laboratory-reared individuals were maintained following the methods of Henry (1985), where 50–100 grasshoppers were maintained in 0.1-m^3 cages at 28 ± 2°C. Fresh barley, dry wheat bran, and water were provided ad libitum. The field-collected species from each site were maintained separately. They were provided with water and fed fresh, native forage gathered at the collection sites. These individuals were kept in the laboratory for no more than one week.

Bioassay. Individual grasshoppers were assayed in a 70-cm-long × 10-cm-diam., linear two-choice olfactometer constructed of clear Lucite. Cadavers were placed at one end of the olfactometer, with no stimulus at the opposite end. The ends of the apparatus were blocked with a screen (0.028 cm^2 mesh) set into a translucent Lucite ring, which prevented the cadavers from serving as visual cues during the assay. A Whisper 1000 adjustable airflow aquarium air pump was used to create two airstreams, which entered the olfactometer from either end. Equal flow rates (355–420 mm H_2O) were achieved through both ends of the olfactometer, and air was exhausted through a 1.2-cm-diam. hole in a 4.0-cm-diam. plug at the top, center of the apparatus. Assays were conducted under uniform lighting, at 26 ± 2°C, between 1000 and 1400 hr.

Prior to testing, individuals were removed from the colony and isolated in 50-cm-long × 10-cm-diam. acetate tubes for 30 min. Individual grasshoppers were then carefully placed in the center of the olfactometer, with a randomly selected orientation. After 5 min, the grasshopper's response was classified as positive (within 20 cm of the cadavers), negative (within 20 cm of the control), or neutral (within 15 cm of the center of the olfactometer). Olfactometers were cleaned with hot, soapy water following each assay.

The neutral response region was based on a preliminary study of the distance travelled by *M. differentialis* (N = 20 males and females) in the olfactometer with no cadavers present. The neutral region corresponded to the area occupied by 85% of the tested grasshoppers after 5 min. This response category was developed because the interpretation of marginal responses can be a problem

with linear olfactometer designs, and this classification system allowed exclusion of random and exploratory movements in the assays.

We tested for the influence of surrounding conditions (e.g., presence of the observer and other subtle visual cues) on behavioral responses by placing *A. elliotti* ($N = 20$ males and 20 females) in the olfactometer with no cadavers. Neither sex was significantly attracted to either end of the apparatus.

Standard assay parameters consisted of exposing uncrowded, 1- to 4-week-old adults to two freshly thawed cadavers of *M. differentialis* adults that had been killed by freezing. Individual grasshoppers were assayed only one time in any 24-hr period. Sample sizes were variable ($N = 9$–76), depending on the availability of specimens.

Parameters. Six parameters of necrophilia are necrophobia (or repellency) were investigated: species, sex, development, starvation, crowding, and attractant. The roles of species and sex in necrophilia were examined using standard assay parameters (see Bioassay above) with all seven laboratory and field-collected species. The effect of developmental stage was examined with *H. trifasciatus*, using fourth- and fifth-instar nymphs and adults. The role of starvation was determined by comparing the responses of *M. differentialis* and *H. trifasciatus* that had been deprived of food and water for 24 hr prior to the assay to individuals that had continuous access to food and water. The latter grasshoppers are termed "fed," although the precise time between their last meal and the assay was not known [feeding gaps were probably <2 hr (Simpson, 1990; J.A.L., unpublished observations)].

The effect of crowding was assessed using two populations of *A. elliotti*. A crowded population was collected from a grasshopper outbreak in Converse County, Wyoming, composed of *C. pellucida* (87%) and *A. elliotti* (13%), with an average population density >100 grasshoppers/m^2. An uncrowded population was collected in Platte County, Wyoming from a community that was dominated by *A. elliotti* (40%) with an average density of 17 grasshoppers/m^2. Grasshoppers from both populations were maintained at normal colony densities and were assayed within 48 hr of collection.

The nature of the attractant as a factor in necrophilia was assessed by: (1) exposing fed and starved *M. differentialis* to 5 ml of distilled water and conspecific cadavers, and (2) exposing *M. sanguinipes* and *H. trifasciatus* to conspecific and contraspecific (*M. differentialis*) cadavers. Attraction to contraspecific cadavers of *M. differentialis* was also assessed in the course of standard bioassays with *A. elliotti*, *B. magna*, *C. pellucida*, and *M. bivittatus*.

Analyses. Because the statistical interpretation of neutral responses is problematical, we adopted the conservative approach of minimizing the variance due to random movements by excluding these responses from the analyses. Thus, for each assayed parameter, binomial probabilities were used to determine the statistical significance of positive and negative responses (Beyer, 1968). Although

excluding neutral responses effectively reduced sample size, there was no statistical difficulty with including small samples in binomial analysis (Siegel, 1956). The probability of a particular set of responses having occurred takes into account the sample size, such that the deviation from the expected distribution necessary to reject the null hypothesis ($p = q = 0.5$) is inversely proportional to sample size (e.g., given $\alpha = 0.05$, for $N = 10$, ≥ 9 outcomes must be positive/negative, while with $N = 20$, ≥ 15 outcomes must be positive/negative, and with $N = 100$, only ≥ 61 outcomes must be positive/negative). Thus, we analyzed all results and considered cumulative positive or negative responses for a trial to be significant when $P \leq 0.05$.

RESULTS

Species. With standard assay parameters, individuals of *M. differentialis* (Table 1), *H. trifasciatus* (Tables 2 and 3), and *A. elliotti* (Table 4) were significantly attracted to cadavers. The four other species of grasshoppers yielded inconclusive results, with neither significant attraction nor repellency (Tables 2 and 5).

Sex. In assessing the affect of sex on necrophilia with the standard assay parameters, we found female *M. differentialis* (Table 1), *H. trifasciatus* (Tables 2 and 3), and *A. elliotti* (Table 4) were significantly attracted to cadavers. Premated female *M. differentialis* appeared to provide more consistent responses toward the cadavers than older, mated females, but this variable was not rigorously evaluated. Males did not exhibit significant attraction to cadavers in six of the seven species, and significant necrophobia (repellency) was observed with male *A. elliotti* (Table 4). In pooling data from the standard assay across all

TABLE 1. NUMBER OF LABORATORY-REARED *M. differentialis* ATTRACTED TO (+) AND REPELLED BY (−) CADAVERS AND WATER AS A FUNCTION OF FEEDING REGIME.

		Feeding regime					
		Fed			Starved		
Attractant	Sex	N	+	−	N	+	−
Cadavers	M	38	14	16	25	2	2
	F	38	17[a]	6[a]	19	4	1
Water	M	18	2	1	11	2	2
	F	12	1	1	25	2	1

[a] Responses differ significantly ($P \leq 0.05$) from a binomial distribution ($p = q = 0.5$).

TABLE 2. NUMBER OF FED *M. sanguinipes* AND *H. trifasciatus* ATTRACTED TO (+) AND REPELLED BY (−) CONSPECIFIC AND CONTRASPECIFIC (*M. differentialis*) CADAVERS

		Cadaver Type					
		Conspecific			Contraspecific		
Species	Sex	N	+	−	N	+	−
M. sanguinipes	M	15	2	5	17	5	7
	F	21	5	4	24	8	12
H. trifasciatus	M	7	2	2	13	4	4
	F	35	4	1	47	16[a]	10[a]

[a]Responses differ significantly ($P \leq 0.05$) from a binomial distribution ($p = q = 0.5$).

TABLE 3. NUMBER OF *Hadrotettix trifasciatus* ATTRACTED TO (+) AND REPELLED BY (−) *M. differentialis* CADAVERS AS A FUNCTION OF DEVELOPMENTAL STAGE AND FEEDING REGIME

		Feeding regime					
		Fed			Starved		
Developmental stage	Sex	N	+	−	N	+	−
Fourth instar	M	7	1	1	4	1	1
	F	7	4[a]	0[a]	6	2	0
Fifth instar	M	10	2	2			
	F	47	11[a]	5[a]	9	0[a]	7[a]
Adult	M	13	4	4			
	F	47	16[a]	10[a]			

[a]Responses differ significantly ($P \leq 0.05$) from a binomial distribution ($p = q = 0.05$).

species (Tables 1–5), females were significantly attracted to cadavers (84 of 148 responses) and males were significantly repelled (44 of 75 responses).

Development. Fourth- and fifth-instar nymphs of *H. trifasciatus* showed attraction toward cadavers (Table 3), although low sample sizes and interacting factors suggest a qualified conclusion at this time. That is, the significant attraction across developmental stages was observed only in fed females.

Starvation. Fed grasshoppers were generally more attracted to cadavers than starved grasshoppers. In *M. differentialis*, fed females were significantly attracted to the cadavers, but starved females were not necrophilic (Table 1).

TABLE 4. NUMBER OF ADULT *Aulocara elliotti* ATTRACTED TO (+) AND REPELLED BY (−), *M. differentialis* CADAVERS AS A FUNCTION OF POPULATION DENSITY

	Density (grasshoppers/m^2)					
	>100			17		
Sex	*N*	+	−	*N*	+	−
Male	12	4	4	32	6[a]	15[a]
Female	11	1[a]	7[a]	68	27[a]	14[a]

[a]Responses differ significantly ($P \leq 0.05$) from a binomial distribution ($p = q = 0.5$).

TABLE 5. NUMBER OF THREE SPECIES OF FIELD-COLLECTED GRASSHOPPERS ATTRACTED TO (+) AND REPELLED BY (−) *M. differentialis* CADAVERS

		Response		
Species	Sex	*N*	+	−
M. bivittatus	M	2	0	0
	F	24	8	12
C. pellucida	M	4	0	1
	F	14	4	5
B. magna	M	5	2	1
	F	17	4	5

[a]Responses differ significantly ($P \leq 0.05$) from a binomial distribution ($p = q = 0.5$).

Significant attraction to cadavers was also observed only in fed *H. trifasciatus* nymphs and adults (Table 3); starved individuals either exhibited no significant response or they were necrophobic (fifth-instar nymphs) (Table 3).

Crowding. The response of *A. elliotti* to cadavers differed as a function of the density of the population from which they were collected (Table 4). The effect of crowding was further modified by the sex of the individuals. Male *A. elliotti* from the crowded population exhibited no significant response, while males from the uncrowded population were significantly necrophobic. Females from the crowded population were necrophobic, but those from the uncrowded population were significantly attracted to cadavers.

Attractant. In assessing attraction as a function of the odor source, neither fed nor starved *M. differentialis* were significantly attracted to water (Table 1). However, fed, female *M. differentialis* were significantly attracted to conspecific cadavers. Of the two species tested with respect to necrophilic responses to both

conspecific and contraspecific cadavers, only female *H. trifasciatus* exhibited a significant response—attraction to contraspecific cadavers (Table 2). In addition, contraspecific cadavers elicited a measurable response (i.e., not neutral) in 44% and 36% of the trials of *M. sanguinipes* and *H. trifasciatus*, respectively. Conspecific cadavers were less provocative, with only 31% and 18% of the trials eliciting measurable responses in *M. sanguinipes* and *H. trifasciatus*, respectively. Of the other four species exposed to contraspecific (but not conspecific) cadavers through the standard bioassay parameters, only female *A. elliotti* (Table 4) exhibited significant necrophilia.

DISCUSSION

It is evident that olfactory cues from grasshopper cadavers provide a stimulus for necrophilia, and these cues may elicit necrophobia under some circumstances. While it appears that necrophilia is the most common response of grasshoppers to cadavers, the degree to which this behavior is expressed is a function of a complex set of intrinsic and extrinsic factors, including species, sex, development, starvation, crowding, and the nature of the stimulus.

Species and Sex. It is evident that not all species are equally necrophilic, an observation that is in agreement with field studies (Lavigne and Pfadt, 1964; Lockwood, 1989a). Our laboratory studies also directly demonstrated that females are more highly attracted to the cadavers than are males, a difference that is circumstantially supported by gut content analysis of field-collected grasshoppers (Lavigne and Pfadt, 1964). Because the cadavers represent a concentration of essential nutrients required for vitellogenesis (Dadd, 1960, 1961; House, 1974; DeFoliart, 1975), reproductive females would presumably benefit disproportionately by consuming the relatively large amounts of proteins and lipids that are found in a cadaver (DeFoliart, 1975).

Necrophobia has not been previously described in acridids, perhaps because field studies have not been designed to document this behavior directly. While cadavers represent a valuable source of nutrients (House, 1974; DeFoliart, 1975), they may also be a potentially hazardous source of pathogens (Henry, 1972; Streett and McGuire, 1990) and a site of intense, physical competition (Lockwood, 1989b). In this context, the lower nutritional needs of males relative to females, combined with the risk of disease transmission by cannibalism and the possibility of injury during aggressive encounters with larger females (O'Neill et al., 1993), could account for the general lack of necrophilia and perhaps the tendency to necrophobia under some conditions in male grasshoppers. However, even if the costs of necrophagia exceed the nutritional benefits, males might be expected seek out necrophagic females in the field due to their potential for greater reproductive success (Fox, 1975; Thornhill and Alcock, 1983).

Development. Field observations have indicated that necrophilia increases with development, although differences in the number of nymphs and adults may have been due to competitive interactions at and near the cadavers (Lockwood, 1989b). Indeed, our studies suggest that late-instar nymphs and adults of *H. trifasciatus* are necrophilic. Although sample sizes were low in some cases, fed, female, fourth- and fifth-instar nymphs and adults were significantly attracted to cadavers. While this species is known for its cannibalistic behavior (Anderson and Wright, 1952; Mulkern et al., 1969), the developmental consistency of necrophilia has not been previously reported.

Necrophilia across developmental stages can be explained by the nutritional advantages of necrophagia (Dadd, 1960, 1961), but it is not evident why starved *H. trifasciatus* fifth instars were repelled from cadavers. However, it is possible that developmental processes may exacerbate the effects of starvation in two ways. First, the fifth instar is undergoing unique ontogenic changes that alter the insect's behavior and physiology [e.g., a reduction of feeding (Bomar and Lockwood, 1991)]. Secondly, depending on the proximity of a nymph to its final molt, necrophilia may have a detrimental effect by placing the vulnerable nymph in proximity of cannibals, such as *H. trifasciatus*, which have been found to attack and consume living acridids (Anderson and Wright, 1952).

Starvation. Starved grasshoppers were never significantly attracted to cadavers, although their fed counterparts frequently showed significant necrophilic responses. This suggests that starved or physically stressed individuals are not necrophilic. Such a finding appears counterintuitive and conflicts with Rizvi's (1967) hypothesis that cannibalistic feeding was primarily a function of food deprivation. However, reduced feeding behavior has been observed with other species of nutritionally deprived acridids (Simpson, 1990). Moreover, it has been shown that starved grasshoppers have reduced locomotor activity (Bernays and Simpson, 1983), which may explain why these individuals did not move to the cadavers.

Crowding. Crowding appears to play a more complex role in grasshopper cannibalism than previously thought (Fox, 1975). Necrophilia did not necessarily increase with population density, as suggested by Rizvi (1967). Rather, individuals from the two populations of *A. elliotti* exhibited remarkably different behaviors as a function of both sex and crowding. The most striking difference was in females, where individuals from a low-density population were necrophilic and those from a high-density population were necrophobic. The costs of cannibalism in high-density populations may exceed the benefits if the probability of disease transmission through necrophagia is greater in crowded populations, as may be expected with density-dependent epizootics (Brown, 1987). However, this explanation does not account for necrophobia in males from the uncrowded population. Rather, the aggressive behavior of female *A. elliotti* while engaging in cannibalism (O'Neill et al., 1993) and the sexual dimorphism

in this species [females are 2.5 times larger than males (Pfadt, 1988)] may account for movement away from cadavers by males, although a similar behavior would be expected under crowded conditions as well.

One final perspective regarding the value of cadavers to females may shed some light on the behavior of males. In uncrowded conditions, males may have difficulty finding females, so it may be advantageous to wait in close proximity of a resource that is attractive to females (i.e., a cadaver). In particular, male *A. elliotti* do not appear to be particularly efficient at finding or attracting mates (Otte, 1970). Under crowded conditions, females are more likely to be located with relative ease, thus avoiding the risks associated with close proximity to a cadaver may take precedence.

Attractants. Grasshoppers did not differ markedly in their attraction to conspecific and contraspecific cadavers. It might be expected that there should be bias against consumption of conspecifics, which could be beneficial with respect to diminishing the chances of acquiring species-specific pathogens (Harper, 1987; Streett and McGuire, 1990). However, the odor cues released from a cadaver are probably not species specific. Moreover, under field conditions, visual cues (precluded in our experiments) would quickly become obscured by ongoing cannibalism. Because neither fed nor starved *M. differentialis* were attracted to water, it appears that some other volatile component of the cadavers is responsible for necrophilic responses.

Summary. Lockwood (1989a) suggested that volatile chemicals released during decomposition may be responsible for attraction to cadavers, and we have now demonstrated this phenomenon under controlled conditions. The nature of the chemical attractant(s) has yet to be determined, but fatty acids and water constitute the majority of the volatile substances (Giral et al., 1946; Barlow, 1964; Fast, 1964). Given that water was not an attractive substance in this study, we hypothesize that fatty acids are the necrophilic stimulus. Although fatty acids are important nutrients for growth and development (Dadd, 1960, 1961), it is not necessary that the attractant be beneficial in itself. Rather, it may serve as a "token stimulus" (Matthews and Matthews, 1983) reliably indicating the presence of a valuable resource. Further research in this area should attempt to identify chemically or functionally these volatile attractants. Once identified, they may be useful in improving the efficacy of biologically and chemically treated bran baits. Our study suggests that graminivorous species that do not readily consume bait (e.g., *A. elliotti*) are necrophilic and can be attracted to chemical cues under most conditions.

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OLFACTORY BASIS OF CANNIBALISM IN GRASSHOPPERS (ORTHOPTERA: ACRIDIDAE): II. FIELD ASSESSMENT OF ATTRACTANTS

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Abstract—The responses of rangeland grasshoppers to biologically and historically derived attractants were studied in a shortgrass prairie in southeastern Wyoming in July 1990. Seven long-chain fatty acids (C_{14}-C_{20}; singly and in combination), grasshopper cadavers, molasses, fruit extracts, and chloroform (solvent control) were tested. Each attractant was applied to filter paper and placed in an arena delimited by a 0.10-m^2 aluminum ring. Grasshoppers were most attracted to linoleic and linolenic acids, with significantly more grasshoppers found in these arenas than in those of the controls or other attractants. These two fatty acids alone and in combination were more attractive at 1 grasshopper equivalent (GE) than at 5 GE. The seed bug, *Lygaeus kalmii* Stål, and five species of ants were also attracted to these two fatty acids. Molasses had significantly more grasshoppers on the filter paper than did the other attractants, but molasses had significantly fewer grasshoppers in the arena than the fatty acids. Fruit extracts were not effective at attracting grasshoppers. Water extracts of cadavers attracted significantly more grasshoppers to the bait than did chloroform extracts. Because the assayed grasshopper community was dominated by the Gomphocerinae (a subfamily that includes many pest species that do not readily consume wheat bran bait), it may be possible to use fatty acids in conjunction with insecticidal bran baits for increased control of rangeland grasshoppers.

Key Words—Olfaction, necrophily, attraction, feeding stimulant, linoleic acid, linolenic acid, fatty acids, Orthoptera, Acrididae.

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INTRODUCTION

Insects commonly seek food via olfaction, and although much is known about grasshopper responses to plant volatiles (Kafka, 1974; Blust and Hopkins, 1987; Chapman, 1990), little research has been completed on other sources of attractants. Cannibalism is a common behavior of grasshoppers in the field and in the laboratory (Lavigne and Pfadt, 1964; Rizvi, 1967; Uvarov, 1977), and this behavior may be stimulated by a lack of moisture, poor food quality, or overcrowding (Lavigne and Pfadt, 1964; Rizvi 1967; Uvarov, 1977). Cannibalism is preceded by attraction to cadavers, and Lockwood (1989a,b) hypothesized that necrophilia was mediated via olfaction because of the rapid response from live grasshoppers in the field. Bomar and Lockwood (1994) concluded that this was the case and further proposed that volatile fatty acids comprised the apneumone responsible for necrophilic behavior. Grasshoppers detect the presence of odors with four types of olfactory receptors located on their antennae: a fatty acid receptor, an amine receptor, and receptors for humidity and aridity (Kafka, 1974; Visser, 1986).

The purpose of the present study was to examine attractive olfactory cues in the field. The identification of these volatile chemicals could enhance the efficacy of commercially available bran baits treated with insecticides (e.g., carbaryl) or pathogens (e.g., *Nosema locustae* Canning). These baits are currently used to control rangeland grasshoppers, and this type of formulation has the advantage of minimizing nontarget impacts (USDA, 1987), but bran baits are limited in their efficacy because some grasshoppers (especially species within the Gomphocerinae) do not readily consume wheat bran (Onsager et al., 1980; Jech et al., 1992). However, the gomphocerines are necrophilic and cannibalistic in both the laboratory (Bomar and Lockwood, 1994) and the field (Lockwood, 1989a), although at extremely high densities males may be necrophobic.

Historically, a variety of presumptive attractants have been added to insecticidal bran baits to improve bait efficacy. These agents are considered presumptive attractants because there appear to be no data on their efficacy as attractants versus feeding stimulants, although attraction was evidently the purpose for which they were added. These attractants have included whole oranges, bananas, and lemons, and molasses (Gibson, 1915; Ford, 1922). The present study compares a variety of potential grasshopper attractants at bait stations in an attempt to identify those chemicals that induce necrophilic behavior in grasshoppers. Historically identified attractants (Gibson, 1915; Ford, 1922) were compared with biologically derived attractants, including both extracts from grasshopper cadavers and fatty acids that may be released from dead grasshoppers (Giral et al., 1944). Our goal was to identify chemicals that best mimicked the attractive properties of a dead grasshopper.

METHODS AND MATERIALS

Site Description. This study was conducted in Whalen Canyon, Platte County, Wyoming, in July 1990. The site lies at an elevation of 1300 m; it receives an annual average rainfall of 33 cm, with a mean summer temperature of 24°C (Martner, 1986). Vegetation was sampled in 20, 1-m randomly selected plots within the test area, according to the methods of Daubenmire (1959). Vegetation consisted of shortgrass prairie with 30% bare ground [see Lockwood and Bomar (1992) for details of the plant community].

The grasshopper community was assessed on the basis of density and species composition. Density was estimated using a visual square-foot method, where the number of grasshoppers was estimated every fifth step, until 30 density values were recorded (Pfadt, 1977). Community structure was evaluated by taking 200 sweep samples of the assayed area.

Bait Preparation. Estimates of the fatty acid composition of grasshoppers were made from the available literature to determine the amount of the principal fats in a grasshopper. A grasshopper was estimated to be composed of 9.0% stearic acid (18:0), 4.8% oleic acid (18:1), 4.2% arachidic acid (20:0), 2.5% palmitic acid (16:0), 1.0% linoleic acid (18:2), 1.0% linolenic acid (18:2), and 0.5% myristic acid (14:0) (Giral et al., 1944; Tietz, 1961; Barlow, 1964; Fast, 1964). It was assumed that the average rangeland grasshopper weighed 1.0 g (Bomar, 1993). Attractants were assayed in the field at doses of 0.25 g, 1.0 grasshopper equivalent (GE), and 5.0 GE, where a 1.0-GE dose was the amount of a fatty acid found in one grasshopper. These attractants were prepared by dilution with chloroform in the laboratory and stored at 2°C.

The fatty acid attractants tested included myristic acid (Kodak; lot No. 013500B), palmitic acid (Kodak; lot No. 909281E), stearic acid (Fisher; lot No. 912485), oleic acid (Fisher; lot No. 881138), linoleic acid (Fisher; lot No. 775286), linolenic acid (Kodak; lot No. 013318B), and arachidic acid (Sigma; lot No. 117F-8432). Several mixes of selected fatty acids were assayed at the relative proportions that would be expected in a grasshopper (Giral et al., 1944; Tietz, 1961; Barlow, 1964), including: oleic–linoleic acids, oleic–linolenic acids, oleic–linoleic–linolenic acids, linoleic–linolenic acids, palmitic–linoleic acids, palmitic–linolenic acids, and palmitic–linoleic–linolenic acids. We also assayed the complete synthetic mixture: stearic–oleic–arachidic–palmitic–linoleic–linolenic–myristic acids.

Surface washes and extracts of ground grasshoppers were also tested for attractiveness. Freshly decapitated female *Aulocara elliotti* Thomas collected the morning of the assay were either immersed for 30 sec in 10 ml of water or chloroform or ground with a tissue homogenizer with 10 ml of water or chloroform. After grinding, the homogenate was filtered through cheesecloth. A chloroform control was used, but a water control was not because previous

laboratory and field studies have shown that grasshoppers are not attracted to water (Lockwood, 1989a; Bomar and Lockwood, 1994).

Historically identified attractants (Gibson, 1915; Ford, 1922) included dark molasses (Breyer Rabbit; lot No. G04101C) and orange (Schilling; lot No. 6112A), lemon (Schilling; lot No. 6127A), and banana (Schilling; lot No. 5481B) ethanol extracts, assayed using 0.25 g of each material.

Assay. Five assays were conducted in July 1990. The first assay consisted of comparing single fatty acids at 0.25-g doses. In the second assay, single fatty acids and the mixtures containing oleic acid (1 GE) were assessed [based on anticipated behavioral activity of oleic acid (Wilson et al., 1958)]. The third assay consisted of comparing single fatty acids and mixtures with palmitic acid (1 and 5 GE) (based on the results of the first assay, which suggested that palmitic acid was behaviorally active). In the fourth assay, cadaver extracts were tested along with linoleic–linolenic acid mixes (1 and 5 GE) (based on results of the second and third assays, which indicated these fatty acids were the most attractive). The final assay included historical attractants and linoleic and linolenic acids (1 GE) (based on the results of the previous assays, which showed these fatty acids were the most attractive).

Each assay began at 1000 hr. During the assays, temperatures ranged from 24 to 32°C, winds ranged from 3 to 10 km/hr, and weather conditions were clear to partly cloudy. Attractants were placed on a 5-cm^2 filter paper disk and then placed in the center of a 0.10-m^2 aluminum ring. The rings were placed approximately 5 m apart, in a single transect perpendicular to the prevailing wind to prevent mixing of the odor plumes. A blank piece of filter paper was used as a control, and a decapitated female *A. elliotti*, collected the morning of the assay, was used as a standard. There were four or five replicates of each attractant, control, and standard.

During each assay, each ring was observed at four consecutive 30-min intervals. Grasshoppers were counted as the observer approached the ring, noting if grasshoppers were either in the arena or on the bait; these response categories were considered to be mutually exclusive. Total attraction was the sum of the grasshoppers that were on the bait and in the arena. After each observation, grasshoppers were flushed from the arena. During the assay, no attempt was made to identify grasshoppers that were attracted to the baits.

Statistics. Observations were pooled across times within an assay but not between assays. Analysis of variance (GLM routine; SAS, 1985) was used to compare differences between the attractants, with post-ANOVA analysis using Tukey's mean separation. Differences were considered significant when $P < 0.05$.

RESULTS

Grasshopper density ranged from 15 to 25 grasshoppers/m^2 throughout the assay period. The grasshopper community was dominated by adults and included 62.0% Gomphocerinae, 30.2% Melanoplinae, and 7.8% Oedipodinae (Table

1). The dominant species were *Ageneotettix deorum* (Scudder), *Cordillacris crenulata* (Bruner), and *Melanoplus occidentalis* (Thomas).

Single fatty acids assayed at 0.25 g resulted in no significant difference in the number of grasshoppers on the bait, in the arena, or in total, as compared to the control (Table 2). However, there were significantly more grasshoppers in the arena and in total at the cadaver than at the control.

There were significantly more grasshoppers on the cadaver than on any

TABLE 1. ADULT SPECIES COMPOSITION AT STUDY SITE IN WHALEN CANYON, PLATTE COUNTY, WYOMING (N = 116)

Subfamily	Species	Percent of community
Melanoplinae		30.2
	Melanoplus occidentalis (Thomas)	17.2
	Melanoplus spp.	4.4
	Hesperotettix viridis (Scudder)	8.6
Gomphocerinae		62.0
	Ageneotettix deorum (Scudder)	23.2
	Cordillacris crenulata (Bruner)	20.7
	Aulocara elliotti Thomas	14.6
	other spp.	3.5
Oedipodinae		7.8
	Trachyrhachys kiowa Thomas	6.9
	Hadrotettix trifasciatus (Say)	0.9

TABLE 2. MEAN NUMBER OF GRASSHOPPERS OBSERVED AT BAIT STATIONS (N = 4–5) FOR SINGLE FATTY ACIDS AT 0.25 g

Attractant	On bait[a]	In arena[b]	Total (bait + arena)
Myristic	0.05 a[c]	1.40 ab	1.465 abc
Palmitic	0.20 a	2.40 ab	2.60 ab
Stearic	0.00 a	1.55 ab	1.55 abc
Oleic	0.15 a	1.35 ab	1.50 abc
Linoleic	0.50 a	1.30 ab	1.35 bc
Linolenic	0.10 a	1.90 ab	2.00 abc
Arachidic	0.00 a	1.20 ab	1.20 c
Cadaver	0.30 a	2.45 a	2.75 a
Control	0.10 a	1.15 b	1.25 bc

[a] The bait consisted of a 5-cm^2 filter paper to which the attractant was applied.
[b] The arena consisted of a 0.10-m^2 area marked by an aluminum ring.
[c] Means within a column followed by different letters differ significantly ($P \leq 0.05$) according to Tukey's mean separation test.

fatty acid bait (1 GE) or the control (Table 3). The cadaver, linoleic acid (1 GE), and linolenic acid (1 GE) had significantly greater numbers of grasshoppers in the arena than did the control. The cadaver, linolenic acid (1 GE), linoleic acid (1 GE), and oleic–linolenic acid (1 GE) also had significantly more total grasshoppers than the control.

Assessment of 1 GE and 5 GE doses of single and mixed attractants (Table 4) revealed no significant differences between any attractant and the control with respect to the mean number of grasshoppers on the bait. There were significantly more grasshoppers in the arena of linoleic acid (1 GE) and palmitic–linolenic acids (1 GE) than in the cadaver or control arenas. There were significantly more grasshoppers in the arena of linoleic acid (1 GE), palmitic–linolenic acids (1 GE), linolenic acid (1 GE), palmitic–linoleic acids (1 GE), palmitic–linoleic–linolenic acids (1 GE), and linolenic acid (5 GE) than were in the control arena. Only palmitic acid (5 GE) had fewer grasshoppers in the arena than the control. Linolenic acid (1 GE), palmitic–linoleic acids (1 GE), and palmitic–linoleic–linolenic acids (1 GE) had significantly more total grasshoppers than the control but not the cadaver. Linoleic acid (1 GE) and palmitic–linolenic acids (1 GE) had significantly more total grasshoppers than did the other attractants (except the three aforementioned), the cadaver, and the control. The complete mixture of fatty acids (1 GE) resulted in more grasshoppers on the bait but fewer grasshoppers in the arena that the partial mixes, but the complete mixture did not attract significantly more grasshoppers than the control.

TABLE 3. MEAN NUMBER OF GRASSHOPPERS OBSERVED AT BAIT STATIONS (N = 5) WITH SINGLE AND MIXED FATTY ACIDS AT 1 GRASSHOPPER EQUIVALENT (1 GE)

Attractant	On bait[a]	In arena[b]	Total (bait + arena)
Palmitic	0.25 b[c]	3.00 ab	3.25 ab
Linoleic	0.15 b	3.30 a	3.45 a
Linolenic	0.15 b	3.35 a	3.50 a
Oleic–linoleic	0.15 b	3.05 ab	3.20 ab
Oleic–linolenic	0.25 b	3.15 ab	3.40 a
Oleic–linoleic–linolenic	0.10 b	3.15 ab	3.25 ab
Complete[d]	0.35 b	1.90 b	2.25 b
Cadaver	0.95 a	3.50 a	4.45 a
Control	0.25 b	1.55 b	1.80 b

[a] The bait consisted of a 5-cm^2 filter paper to which the attractant was applied.
[b] The arena consisted of a 0.10-m^2 area marked by an aluminum ring.
[c] Means within a column followed by different letters differ significantly ($P \leq 0.05$) according to Tukey's mean separation test.
[d] The complete mixture was composed of stearic–oleic–arachidic–palmitic–linoleic–linolenic–myristic acids.

TABLE 4. MEAN NUMBER OF GRASSHOPPERS OBSERVED AT BAIT STATIONS ($N = 5$) WITH SINGLE AND MIXED FATTY ACIDS AT TWO DOSES

Attractant	Dose	On bait[a]	In arena[b]	Total (bait + arena)
Palmitic	1 GE	0.10 a[c]	1.20 bc	1.30 cde
Linoleic	1 GE	0.50 a	3.70 a	4.20 a
Linolenic	1 GE	0.10 a	3.55 ab	3.65 ab
Palmitic-linoleic	1 GE	0.10 a	2.95 ab	3.05 abc
Palmitic-linolenic	1 GE	0.30 a	3.70 a	4.00 a
Palmitic-linoleic-linolenic	1 GE	0.05 a	2.80 ab	2.85 ab
Palmitic	5 GE	0.05 a	0.45 c	0.50 e
Linoleic	5 GE	0.00 a	2.05 bc	2.05 bcde
Linolenic	5 GE	0.05 a	2.35 ab	2.40 bcd
Palmitic-linoleic	5 GE	0.15 a	1.90 bc	2.05 bcde
Palmitic-linolenic	5 GE	0.00 a	1.75 bc	1.75 bcde
Palmitic-linoleic-linolenic	5 GE	0.15 a	1.80 bc	1.95 bcde
Cadaver	1 GE	0.20 a	1.45 bc	1.45 bcde
Control	0	0.00 a	0.70 c	0.70 de

[a] The bait consisted of a 5-cm^2 filter paper to which the attractant was applied.
[b] The arena consisted of a 0.10-m^2 area marked by an aluminum ring.
[c] Means within a column followed by different letters differ significantly ($P \leq 0.05$) according to Tukey's mean separation test.

Assessment of naturally occurring attractants and attractive fatty acids revealed that only the water extract attracted significantly more grasshoppers onto the bait than the control (Table 5). There were also significantly more grasshoppers on the water extract and water wash than on the linoleic–linolenic acid mixes (1 GE and 5 GE). Conversely, significantly more grasshoppers were in the arenas of the linoleic–linolenic acid mixes (1 GE and 5 GE) than the washes, extracts, cadavers, and controls. The linoleic–linolenic acid mixes (1 GE and 5 GE) attracted significantly more total grasshoppers than the control, while the water extract and wash were not significantly different from the cadaver or control. The chloroform washes and extracts attracted fewer total grasshoppers than those made with water.

In comparing historically based attractants to attractive fatty acids, molasses had significantly more grasshoppers on the bait than any other attractant (Table 6). Linoleic acid (1 GE) and linolenic acid (1 GE), on the other hand, had significantly more grasshoppers in the arena than did any of the historically identified attractants, the cadavers, or the controls. Linoleic acid (1 GE) and linolenic acid (1 GE) also had significantly more total grasshoppers than the controls, cadavers, and all other attractants, except for molasses.

TABLE 5. MEAN NUMBER OF GRASSHOPPERS OBSERVED AT BAIT STATIONS ($N = 5$) WITH CADAVER EXTRACTS AND SELECTED FATTY ACIDS

Attractant	On bait[a]	In arena[b]	Total (bait + arena)
Water wash	1.06 ab[c]	1.50 b	2.56 abc
Water extract	1.31 a	1.38 b	2.68 abc
Chloroform wash	0.31 bc	1.81 b	2.13 bc
Chloroform extract	0.31 bc	1.50 b	1.81 c
Linoleic–Linolenic			
1 GE	0.00 c	4.13 a	4.13 a
5 GE	0.00 c	3.94 a	3.94 a
Chloroform	0.20 bc	1.20 b	1.40 c
Cadaver	0.68 abc	1.81 b	2.50 abc
Control	0.06 c	1.19 b	1.25 c

[a] The bait consisted of a 5-cm^2 filter paper to which the attractant was applied.
[b] The arena consisted of a 0.10-m^2 area marked by an aluminum ring.
[c] Means within a column followed by different letters differ significantly ($P \leq 0.05$) according to Tukey's mean separation test.

TABLE 6. MEAN NUMBER OF GRASSHOPPERS OBSERVED AT BAIT STATIONS ($N = 5$) WITH HISTORICALLY IDENTIFIED GRASSHOPPER ATTRACTANTS AND SELECTED FATTY ACIDS

Attractant	Dose	On bait[a]	In arena[b]	Total (bait + arena)
molasses	0.25 g	2.10 a[c]	1.15 b	3.25 ab
orange	0.25 g	0.55 b	0.85 b	1.40 c
banana	0.25 g	0.60 b	1.20 b	1.80 bc
lemon	0.26 g	0.35 b	1.15 b	1.50 bc
linoleic	1 GE	0.50 b	3.70 a	4.20 a
linolenic	1 GE	0.10 b	3.55 a	3.65 a
cadaver	1 GE	0.20 b	1.45 b	1.65 bc
control	0	0.00 b	0.70 b	0.70 c

[a] The bait consisted of a 5-cm^2 filter paper to which the attractant was applied.
[b] The arena consisted of a 0.10-m^2 area marked by an aluminum ring.
[c] Means within a column followed by different letters differ significantly ($P \leq 0.05$) according to Tukey's mean separation test.

Other insects observed on the baits included ants (Hymenoptera: Formicidae) and the small milkweed bug, *Lygaeus kalmii* Stål (Hemiptera: Lygaeidae). The ants included >75 *Monorium minimum* (Buckley) on linoleic acid (1 GE), two *Formica pallidefulfa nitidiventris* Emery on linolenic acid (1 GE), two *Lasius alienus* (Foerster) on molasses, one *Pogonomyrmex occidentalis* (Cresson) on the palmitic–linoleic–linolenic acid (1 GE) mixture, and one individual from the *Formica rufa* group on a grasshopper cadaver. Other ants were observed, but not collected or identified, on grasshopper cadavers, palmitic acid (1 GE), palmitic–linolenic acids (1 GE), oleic–linolenic acids (1 GE), linoleic acids (1 GE), and linolenic acid (1 GE). Individual *L. kalmii* were observed on palmitic–linolenic acid (1 GE), oleic–linoleic acids (1 GE), linoleic acid (1 GE), and linolenic acid (5 GE).

DISCUSSION

This study showed that certain decompositional chemicals are attractive to grasshoppers, and some are more attractive than grasshopper cadavers, washes, and extracts. The fatty acids that attracted grasshoppers most consistently were linoleic and linolenic acids. Three mixtures that included either linoleic or linolenic acids also had significantly more grasshoppers in the arena than did the cadavers, and in only one assay were more grasshoppers on a cadaver than on a fatty acid bait. As limiting nutrients, linoleic and linolenic acids are required for growth and sexual development (Dadd, 1960, 1961; House, 1974), and they are the only fatty acids that grasshoppers cannot synthesize (Dadd, 1960, 1961; House, 1974); they must be acquired through feeding. Linoleic acid is found in leaves, and linolenic acid is found in seeds, but concentrations are low relative to nutritional requirements (Salsbury and Ross, 1969). However, by finding and consuming dead grasshoppers, necrophiles can secure a substantial reservoir of these limiting nutrients. Necrophagia is common in grasshoppers (Lockwood, 1989a,b; Bomar and Lockwood, 1994). This behavior is most frequently observed during periods of environmental stress (e.g., drought) and at other times when normal levels of limiting nutrients are unavailable (Fox, 1977).

Historically used compounds appear to be generally ineffective as attractants, particularly compared to fatty acids. Indeed, the fruit extracts were not demonstrably effective, suggesting that when fruits were used in the past, grasshoppers were not attracted to the associated odors. More likely, grasshopper feeding was induced by the high sugar content of fruit. Molasses was far better than any other attractant with respect to having grasshoppers on the bait, with 10 times more grasshoppers than the cadaver. However, molasses did not attract many grasshoppers into the arena, having significantly fewer grasshoppers than both linoleic and linolenic acids.

This contrast in behavior stimulated by molasses and fatty acids demonstrates an important difference between the types of responses exhibited by grasshoppers. Rather than attracting grasshoppers to the bait, molasses arrested those grasshoppers that found the bait. The high sugar content in the molasses presumably served as a feeding stimulant (Chapman, 1990). Fatty acids, such as linoleic and linolenic acids, attracted grasshoppers to the area of the bait, but these compounds did not stimulate feeding on the filter paper. This finding suggests that fatty acids may function as a token stimulus (Matthews and Matthews, 1978; Bomar and Lockwood, 1994).

The discovery that ants were attracted to the fatty acids also provides some insights regarding necrophilia. Ants were found in association with 14 bait stations, including fatty acids (10), grasshopper cadavers (3), and molasses (1). Of the 10 fatty acid stations where ants were found, nine contained either linoleic or linolenic acid, and only one contained oleic acid, the "funeral pheromone" of ants (Wilson et al., 1958). Because ants were attracted to only one of the 20 oleic acid bait stations, it appears that this fatty acid is not attractive outside of the colony. Rather, ants were attracted to the baits as scavengers, and they were apparently able to distinguish between fatty acids associated with decomposing food sources and those emitted from dead nestmates.

Four milkweed bugs were also observed at bait stations containing either linoleic or linolenic acid, but no milkweed bugs were observed on grasshopper cadavers. Two previous studies suggest that the milkweed bugs may be attracted to the fatty acids because they mimicked dead grasshoppers. First, Root (1986) described these bugs as scavengers, consuming a variety of dead insects during the period when its preferred food, milkweed seeds, was unavailable. While rearing this insect in captivity, Root (1986) supplemented their diet of water and seeds with frozen grasshoppers. Secondly, milkweed bugs were collected on a glacier that contained numerous decomposing grasshopper remains (Lockwood et al., 1993).

The olfactory basis for cannibalism is now apparent, and fatty acids clearly play a vital role in necrophilia. Application of these fatty acids to bran baits could be useful in improving control of rangeland grasshoppers. Although the species of grasshoppers that were attracted to the baits were not identified, the community structure was heavily biased to the Gomphocerinae, a graminivorous subfamily of grasshoppers, which is not normally considered to consume bran bait (Onsager et al., 1980; Jech et al., 1992). Previous work has also shown that species within this subfamily are attracted to cadavers (Lockwood, 1989a; O'Neill et al., 1993; Bomar and Lockwood, 1994). This subfamily does not readily discover wheat bran when applied in the field because these grasshoppers do not forage on the ground. However, the gomphocerines will readily consume bran when it is discovered [e.g., if it is placed on plants (Jech and Foster, 1992)]. Therefore, if an insecticidal bran bait (e.g., carbaryl) included a fatty

acid (e.g., linoleic or linolenic acid), there is an increased potential for control by virtue of attracting grasshoppers to the bait. Finally, in this light, the addition of molasses or other feeding stimulants may be expected to provide less substantial benefits than attractants.

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OLFACTORY BASIS OF CANNIBALISM IN GRASSHOPPERS (ORTHOPTERA: ACRIDIDAE): III. USE OF ATTRACTANTS ON CARBARYL WHEAT BRAN BAIT

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Abstract—Two known necrogenic attractants, linoleic acid (18:2) and linolenic acid (18:3), were added to carbaryl bran bait to enhance control of rangeland grasshoppers in southeastern Wyoming. The primary goal was to increase control of species of Gomphocerinae, which normally do not consume bran bait. Each attractant was applied at 1, 5, and 10 grasshopper equivalents (GE) (e.g., a 1-GE treatment had the amount of fatty acid per unit weight of wheat bran that would be found in one grasshopper). Controls included carbaryl bran with no attractant and no treatment. Bran was applied at a rate of 1 kg/ha to 1-ha blocks on June 6, 1992, with four replicates per treatment and control. Plots were sampled for grasshoppers and nontarget organisms one day prior to and one, two, and three days after treatment. The addition of linoleic acid (10 GE) resulted in significantly lower total grasshopper densities than carbaryl bran alone. None of the attractants significantly improved control of all Gomphocerinae due to inconsistent effects among species. Relative to carbaryl bait alone, all doses of both fatty acids significantly improved control of *Amphitornus coloradus* (Thomas). However, the attractants did not change the level of control of *Cordillacris occipitalis* or *Aulocara elliotti*, and linolenic acid (5 GE) and linoleic acid (1 GE) resulted in significantly poorer control of *Ageneotettix deorum* (Scudder) than carbaryl bait alone. Nontarget arthropods were largely unaffected by the attractants, except for the spiders, which were significantly reduced in all linolenic acid treatments.

Key Words—Grasshopper, Orthoptera, Acrididae, attractants, carbaryl, linoleic, linolenic, fatty acids.

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INTRODUCTION

Insecticidal bran baits have been used for over a century to control grasshoppers (Riley et al., 1880). This formulation was commonly used in grasshopper outbreaks during the 1920s across the Great Plains (Gibson, 1915; Ford, 1922). Bran baits provide an ecologically sound and economically viable alternative to broad-spectrum liquid insecticides (e.g., malathion). Because baits are selective towards grasshoppers, this formulation is less damaging to nontarget species (USDA, 1987). While bran baits are selective, they are usually not as efficacious as the liquid insecticides. In particular, several species of one subfamily of grasshoppers (Gomphocerinae) do not readily consume wheat bran when applied in the field (Onsager et al., 1980). The Gomphocerinae prefer to feed on grasses while resting on the vegetation (Pfadt, 1988). The two other grasshopper subfamilies that include major pest species (Melanoplinae and Oedipodinae) are more polyphagous, consuming a variety of grasses and forbs, and commonly scavenging on the ground (Mulkern et al., 1969).

Grasshoppers are attracted and stimulated to feed by a variety of chemicals released from food sources (Blust and Hopkins, 1987; Chapman, 1990). Grasshoppers have also been shown to be attracted to the cadavers of other grasshoppers (Lockwood, 1989a,b; O'Neill et al., 1993), and it is now known that they locate the cadavers via olfactory cues (Bomar and Lockwood, 1994a,b). Moreover, grasshoppers have been shown to be attracted to volatile fatty acids that are released by grasshopper cadavers (Bomar and Lockwood, 1994b). Most importantly, the gomphocerines have been found to be necrophilous and necrophagous (Lockwood 1989b; Bomar and Lockwood, 1994a,b; O'Neill et al., 1993). Past attempts to enhance control of rangeland grasshoppers with baits have used orange and lemon pulp (Gibson, 1915; Ford, 1922), but these substances appear to stimulate feeding rather than act as attractants (Bomar and Lockwood, 1994b). Based on available biological and management data, we hypothesized that the addition of fatty acids to an insecticide-treated wheat bran may improve the efficacy of bran baits.

The purpose of this study was to determine if it were possible to improve control of rangeland grasshoppers, specifically species of Gomphocerinae, with the addition of fatty acids to bran baits. Bomar and Lockwood (1994b) found that linoleic and linolenic acids were most attractive to grasshoppers in a field study. As such, these fatty acids were combined with carbaryl wheat bran, the standard insecticidal bran bait used for grasshopper control (USDA, 1987).

METHODS AND MATERIALS

Site Description. The study site was native rangeland approximately 30 km north of Guernsey, Platte Country, Wyoming. The slope was <5% and ground cover was dominated by grasses [50% needle and thread, *Stipa comata* Trin. &

Rupr., 30% blue grama, *Bouteloua gracilis* (H.B.K)], with 5% forbs, and 15% bare ground. Annual precipitation at the study site is 33.0 cm, with an average of 6.2 cm in June. Annual daily mean temperature is 9.1°C, with 19.4°C in June.

Bait Preparation and Application. To apply the appropriate amount of linoleic (Fisher; lot No. 775286) or linolenic (Kodak; lot No. 1173062062) acid per unit of wheat bran, it was necessary to estimate the amount of fatty acid in grasshoppers. Based on available data (Giral et al., 1944; Tietz, 1961; Barlow, 1964; Fast, 1964), it was estimated that linolenic and linoleic acids each comprise 1.0% of a grasshopper. The carbaryl wheat bran (Sidwell Enterprises, Parker, Colorado 80134) was formulated with the fatty acids using a mortar mixer and a Hudson sprayer. Three doses of each fatty acid (linoleic and linolenic) were prepared and applied to the wheat bran to yield 1, 5, and 10 GE, where a 1-GE dose was 10 g of fatty acid per kilogram of bran (i.e., 1% by weight). Two controls were used: carbaryl bran with no attractant and no treatment.

Wheat bran was applied at 1 kg/ha using a truck-mounted Peacock applicator with a 5-hp motor. Each treatment was applied to four 1-ha replicate plots, such that there were 32, adjacent 1-ha plots (100 m × 100 m) in a grid layout treated with a completely randomized block design. Each plot was sampled one day prior to, and one, two, and three days after treatment. At the time of treatment on June 6, winds were <3 km/hr and temperatures ranged from 15 to 25°C.

Density and Diversity. Grasshopper density was estimated by counting grasshoppers in 40, visualized, 1-ft^2 samples (Pfadt, 1977), along four transects located at least 20 m inside the plot boundaries. Sweep-net samples consisted of 50 high-fast and 50 low-slow sweeps taken daily in each plot. Samples were evaluated with respect to grasshopper species composition and developmental stage and abundance of nontarget arthropod orders. The proportion of grasshopper species found in the sweep-net samples was multiplied by the density estimate to determine the density of each grasshopper species in each plot. Developmental stages were determined for each species of grasshopper, and populations were characterized with respect to the total grasshopper community, the target stage, and the target taxon. The target stage was defined as third- and fourth-instar nymphs of all species, those grasshoppers which are most likely to consume wheat bran (Bomar and Lockwood, 1991). The target taxon was Gomphocerinae within the target stage; this is a graminivorous subfamily not known to consume baits readily. All nontarget arthropods were identified to order, but the densities of only the six most common nontarget orders were adequate for quantitative analysis.

Statistical Analyses. Treatments were analyzed by first applying square-root transformations of the grasshopper densities to control the variance (Steel

and Torrie, 1980). Percent control of grasshoppers in each plot was then calculated with a correction for natural mortality by Abbott's formula. The treatment effects were then assessed using analysis of variance, with time as a covariant (SAS, 1985) for total grasshopper community, the target stage, the target taxon, and the five most abundant species. In all cases, significant F tests ($P < 0.05$) allowed us to use linear contrasts to compare each of the attractants to carbaryl bait alone (SAS, 1985). Differences were considered significant when $P \leq 0.05$.

The treatment effects on nontarget arthropods were assessed by analysis of variance of square-root transformed densities, with time as a main effect in a factorial design. Fisher's protected least-significant difference test allowed us to compare all treatments, which was relevant in assessing the responses of nontarget insects.

RESULTS

Target Effects. The treated population was dominated by the target stage; third and fourth instars comprised 73.7% of the population. The target taxon represented 62% of the total population. Four species of Gomphocerinae were abundant: *Ageneotettix deorum* (Scudder) (37% of the population), *Cordillacris occipitalis* (Thomas) (13%), *Amphitornus coloradus* (Thomas) (9%), and *Aulocara elliotti* Thomas (3%). Nymphal Oedipodinae and *Melanoplus* spp. accounted for 34% of the population, and the remaining 4% of the species were adults of *M. occidentalis* (Thomas), *Psoloessa delicatula* Scudder, *Opeia obscura* (Thomas), and *C. crenulata* (Bruner). Prior to bait application there were no significant differences between plots with respect to the densities of total grasshoppers, target stage, and target taxon. The total grasshopper densities ranged from 15 to 18 grasshoppers/m^2.

The adjusted mortalities for all treatments revealed that only linoleic acid (10 GE) significantly increased total grasshopper mortality, as compared to carbaryl bait alone (Table 1). This treatment also increased mortality of the target stage by fivefold, as compared to the unaltered carbaryl bait, but this difference was not significant. None of the fatty acid amendments significantly increased mortality of the target taxon, compared to carbaryl bait alone. This lack of enhanced control within the Gomphocerinae appears to have been the result of disparate results between species within this subfamily.

All of the fatty acid amendments, at all doses, increased the mortality of *A. coloradus*, compared to carbaryl bait (Table 2). However, none of the fatty acids significantly increased the mortality of either *A. elliotti* or *C. occipitalis*. The only significant decreases in mortality with the amended baits were seen in the response of *A. deorum* to the 5-GE dose of linolenic acid and the 1-GE dose

TABLE 1. PERCENT MORTALITY OF RANGELAND GRASSHOPPERS TREATED WITH 2% CARBARYL BRAN BAIT AND BAIT AMENDED WITH FATTY ACIDS

		Percent adjusted control[a] ($\bar{X}$ of days 1, 2, and 3)		
Additive	Dose	All grasshoppers	Target stage	Target taxon
None	0	12.3	6.5	33.3
Linolenic acid	1	23.0	18.6	36.8
	5	11.8	1.6	28.0
	10	−9.2	−19.7	−1.8
Linoleic acid	1	9.1	−9.0	25.5
	5	10.5	−0.2	16.7
	10	40.9*	32.1	41.7

[a] Mortalities were adjusted based on untreated controls, according to Abbott's formula. An asterisk indicates that the mean differs significantly ($P \leq 0.05$) from the mean of the unamended carbaryl treatment.

TABLE 2. PERCENT MORTALITY OF RANGELAND GRASSHOPPER PEST SPECIES TREATED WITH 2% CARBARYL BRAN BAIT AND BAIT AMENDED WITH FATTY ACIDS

		Percent adjusted control[a] ($\bar{X}$ of days 1, 2, and 3)				
Additive	Dose	*Melanoplus* spp.	*Aulocara elliotti*	*Ageneotettix deorum*	*Amphitornus coloradus*	*Cordillacris occipitalis*
None	0	−41.4	77.8	15.8	−112.9	59.0
Linolenic acid	1	−31.0	33.3	39.6	69.0*	−3.0
	5	−30.1	94.4	−31.2*	45.2*	5.5
	10	−58.7	−50.0	−15.9	55.4*	42.6
Linoleic acid	1	−65.3	−66.7	−51.7*	69.2*	42.2
	5	−26.8	55.6	0.4	61.5*	0.3
	10	17.5	88.9	7.4	74.7*	59.1

[a] Mortalities were adjusted based on untreated controls, according to Abbott's formula, except for *A. elliotti*. This species was not present in sufficient numbers in the untreated controls, so percent control was based on pretreatment densities in control plots. An asterisk indicates that the mean differs significantly ($P \leq 0.05$) from the mean of the unamended carbaryl treatment.

of linoleic acid. Although the 10-GE dose of linoleic acid markedly increased mortality of *Melanoplus* spp. (from −41 to 18% control), this difference was not significant.

Nontarget Effects. Carbaryl bran bait caused no significant reductions or increases in the nontarget arthropod densities, and the densities of Homoptera

and Hymenoptera were not significantly altered by any of the treatments relative to pretreatment counts.

Linoleic acid was associated with a significant increase in Diptera (1 GE) and a significant decrease in Hemiptera (5 GE), at three days after treatment relative to pretreatment densities. The 10-GE dose of linoleic acid caused no significant changes in the densities of nontarget arthropods.

All doses of linolenic acid significantly reduced Araneae at three days after treatment relative to pretreatment densities. The 1-GE dose was associated with a significantly fewer Hemiptera at one and three days after treatment. The 10-GE dose resulted in a significant reduction in Coleoptera at two and three days after treatment and a significant increase in Diptera at one and three days after treatment relative to pretreatment densities.

DISCUSSION

Our biorational approach to the development of bait attractants for rangeland grasshoppers (Bomar and Lockwood, 1994a,b) can be considered a qualified success, tempered by several logistical and ecological considerations. We have demonstrated that it is possible to use basic behavioral and ecological information on grasshopper feeding (Lavigne and Pfadt, 1964; Lockwood, 1989a,b) to develop agents that can enhance the efficacy of insecticidal baits for grasshopper control. It is evident from this field assay that increases in grasshopper control can be gained by adding necrogenic attractants (fatty acids) to standard carbaryl bait.

Significant increases in control were observed in both general and specific cases. First, the 10-GE dose of linoleic acid increased the mortality of total grasshoppers. Increased mortality was observed in both gomphocerine and melanopline species, although the increases were statistically significant only in the latter case. In this case, the mortality of *A. coloradus* was increased with all fatty acid amendments. This species is known to be poorly controlled with standard carbaryl bait; Onsager et al. (1980) reported only a 4% reduction in this species.

The nontarget arthropods, for the most part, were reduced only slightly by the addition of attractants, compared to reductions caused by carbaryl bran bait alone. While no significant decreases were observed in our carbaryl treatment, George et al. (1992) observed significant decreases in the Coleoptera and Araneae at two days after treatment using pitfall traps. They also observed that significantly fewer ants were collected at two days after treatment using pitfall and sticky traps. In our study, the most affected nontarget arthropods were the Araneae, which were reduced by all three doses of linolenic acid, and this fatty acid accounted for all but one of the significant reductions of nontarget arthro-

pods. Given that spiders sometimes consume dead insects (Gertsch, 1979), they may have been attracted to the insecticidal bran bait by necrophilia.

Despite the encouraging results of this preliminary field test, there are several reasons for caution with respect to extrapolation of our data. First, the fatty acid amendments only resulted in the enhanced control of one species. The control of other species of gomphocerines, which are also difficult to manage with insecticidal baits, was not improved, and two of the amendments (5 GE linolenic acid and 1 GE linoleic acid) significantly decreased mortality of *A. deorum* compared to standard bait. Thus, the goal of generalized attractant for the gomphocerines was not achieved.

Second, the carbaryl bait alone and with fatty acids generally failed to control melanoplines, which is somewhat unusual (Onsager, 1980; Jech et al., 1992). However, this lack of control may have been the consequence of the spatially limited nature of the study, which leads to the next consideration.

The third reason for cautious interpretation was the scale of the treatment plots. Movement of grasshoppers between the 1-ha plots almost certainly confounded the outcome of our assay. For example, grasshopper densities in two of the control plots decreased by $\geq 50\%$ at one day after treatment, while the other two control plots maintained a constant density. In addition, the increased density of *Melanoplus* spp. in some treatments, including the carbaryl bait plots, may have been a function of movement as densities changed with the effects of treatment. That is, melanoplines may have moved into treated plots as densities of gomphocerine species declined. Thus, large-scale replication of this experiment will help to clarify the rate of attraction and efficacy of these fatty acid attractants when added to carbaryl bran. Larger blocks (e.g., ≥ 10 ha) would reduce the variance by limiting the rate of interplot movement. In addition, fewer treatments, perhaps even a single dose of one attractant, would decrease the potential of confounding the volatile stimuli.

Fourth, although there were fairly high total grasshopper densities at the site (15–18 grasshoppers/m^2), the density of any particular species never exceeded 5/m^2 after treatment. At these densities, the sampling error is large (e.g., we were unable to detect a significant difference between a 41% increase in density with carbaryl bait alone and an 18% decrease with the addition of 10 GE linoleic acid), and only with higher densities or more homogeneous grasshopper assemblages will it be possible to fully elucidate species-level differences.

Finally, the economic limitations of attractants must be considered. Production of an attractant-based bran formulation would be prohibitively expensive with technical-grade fatty acids. For example, linoleic acid at 10 GE would increase the price of a carbaryl bran treatment from \$7.48/kg (\$3.40/lb; USDA, 1987) to over \$9.68/lb. A novel alternative would be to use a vegetable oil (e.g., soybean, sunflower, or safflower oil), several of which contain 50–78%

linoleic acid (Noller, 1966). These oils would increase the carbaryl bran treatments by only $0.25–0.50/lb. Use of these commercially produced oils may also benefit the application efficacy. They are considered drying oils (Noller, 1966), and may prevent the bran from clumping, a difficulty encountered with the higher doses of linoleic and linoleic acids in our experiments.

The application of grasshopper behavioral ecology to the development of bait attractants appears to have continuing promise for improving the efficacy of insecticidal baits while retaining the environmental advantages of this formulation. Our work to date has allowed qualified success in this regard and has served to highlight the major ecological and logistical impediments. In ecological terms, it may yet be possible to discover a generalized attractant, but our studies suggest that species-specific responses to apneumones may be problematical. With respect to logistics, behavioral assays in the laboratory and field (Bomar and Lockwood 1994a,b) allow considerable control over external variables and may be a viable approach to early screening. However, bait tests under field conditions will almost certainly have greater variability. Finally, the paradoxical requirement of large-scale field tests that reduce variation due to grasshopper movement but increase variation due to the spatial heterogeneity of grasshopper populations and rangeland conditions will continue to challenge the development and testing of grasshopper control methods.

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ALLELOPATHIC EFFECTS OF PHENOLIC MIXTURES ON RESPIRATION OF TWO SPRUCE MYCORRHIZAL FUNGI

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Abstract—Investigations were conducted to determine whether the allelopathic effect (inhibition of oxygen consumption) of mull- and mor-type humic solutions on ectomycorrhizal fungi was due to the solutions' phenolic contents. In the first experiment, two concentrations (10^{-3} M and 10^{-7} M) of binary equimolar phenolic mixtures were tested on the oxygen consumption of *Laccaria laccata* and *Cenococcum graniforme*. The high concentrations of most of the mixtures induced an increase of fungal respiration, whereas the 10^{-7} M treatments all reduced the rate of respiration. In the second experiment, the effects on respiration of the phenolic mixture reproducing mull- and mor-type humic solutions were compared with the effects of natural humic solutions. The resulting data suggest that allelopathic effect of humic solutions can at least in part be attributed to their phenolic contents.

Key Words—Allelopathy, *Cenococcum graniforme*, humus, *Laccaria laccata*, mixture, oxygen, mycorrhizal fungi, phenol, *Picea abies*, respiration.

INTRODUCTION

Fungi in forests soils are strongly affected by water-soluble substances coming from the litter of needles and leaves of forest trees and herbaceous plants (Melin, 1946; Persidsky et al., 1965; Dix, 1974). Previous results indicated that water, and its soluble substances, from mull- and mor-type humus (termed "humic

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solution'' for short) of subalpine spruce [*Picea abies* (L.) Karst.] forests decreased the respiration of two spruce ectomycorrhizal fungi: *Laccaria laccata* and *Cenococcum graniforme* (Pellissier, 1990). Catechol, *p*-hydroxyacetophenone, *p*-hydroxybenzoic acid, and protocatechuic acid, four phenols present in these humic solutions, significantly reduced respiration of these two fungi (Pellissier, 1993). This inhibition was observed at concentrations ranging from 10^{-3} M to 10^{-7} M, and the naturally occurring humic solutions contained 10^{-5} M phenolic compounds.

The objectives of the study reported here were: (1) to evaluate the effects of combinations of phenolic compounds on the rate of *L. laccata* and *C. graniforme* respiration and (2) to ascertain whether the allelopathic effect of the humic solutions observed previously on fungal respiration could be attributed to their phenolic composition.

METHODS AND MATERIALS

General Procedure. Previous experiments demonstrated that single phenolic acid solutions reduced the oxygen consumption of *L. laccata* and *C. graniforme* over a wide range of concentrations (Pellissier, 1993). Therefore, the two experiments described here were conducted to determine whether mixtures of these phenolic acids cause such inhibition. In addition to the testing of phenolic acid mixtures, the phenolic acid composition of the mull and mor humic solutions were duplicated exactly and compared to the effects of natural humic solutions on mycorrhizal fungi respiration.

Fungal Material. We measured the respiration of *Cenococcum graniforme* (Sow). Ferd. and Winge, and *Laccaria laccata* (Scop. ex Fr.) Berk and Br. The fungi were grown in a liquid medium. Erlenmeyer flasks containing 100 ml of modified Melin-Norkrans nutrient solution (Norkrans, 1949) were inoculated with a 5-mm-diameter agar disk cut from a fungal colony. The flasks were then placed in a dark incubator at +24°C for five weeks before experimentation.

Phenolic Mixtures. All possible combinations of aqueous solutions (demineralized water) of two specific compounds (Sigma Chemical Co.) were prepared with: *p*-hydroxyacetophenone, *p*-hydroxybenzoic acid, catechol (1,2-benzenediol), and protocatechuic acid (3,4-dihydroxybenzoic acid). Concentrations tested were 10^{-3} M and 10^{-7} of these mixtures (for example: [catechol + protocatechuic acid] 10^{-3} M = one test; [catechol + protocatechuic acid] 10^{-7} M = a second test, etc.).

In a second experiment we used humic solutions and phenolic acid mixtures representing humic solutions: (1) mull = [1.09 *p*-hydroxyacetophenone + 0.11 catechol + 0.03 *p*-hydroxybenzoic acid + traces of protocatechuic acid] × 10^{-5} M, (2) mor = [5.83 *p*-hydroxyacetophenone + 0.35 catechol + 0.28 *p*-

hydroxybenzoic acid] $\times 10^{-5}$ M. Solutions were not buffered, as natural humic solutions are not and pH values were comparable, around 4.

Oxygen Electrode. Oxygen utilization of fungal mycelium was measured polarographically with a Hansatech instrument (King's Lynn, Norfolk, England), which has a Clark-type oxygen electrode. The fungal sample (1 $\pm$ 0.2 g fresh weight.) was set in the measuring cell containing 1 ml of demineralized, oxygen-saturated water (250 nmol/ml at 25°C). After several minutes, oxygen consumption becomes stable at a level corresponding to the basal respiration rate of the fungal sample. At that point, 1 ml of filter-sterilized (Millipore, 0.22 μm) phenolic mixture or humic solution was injected into the measuring cell. The oxygen consumption of the mycelium was then observed for a total of 60 min.

Each experiment was repeated seven times. To demonstrate that no physicochemical reaction was responsible for the reduction of oxygen dissolved in the water, experiments without fungi were performed. One milliliter of the test mixture was added to 1 ml of demineralized, oxygen-saturated water. The oxygen concentration in the measuring cell remained unchanged during the subsequent 60 min. Therefore, it was assumed that no oxygen-reducing chemical reactions took place. We ignored the oxygen-consumption of the probe because it was negligible (about 0.5 nmol/min). At the end of O_2 measurements, the sample was dried at 105°C for 48 hr to obtain dry weight. Respiration values are expressed as nanomolar O_2 consumption per gram dry weight per minute. The effects of phenolic mixtures and natural and simulated humic solutions are expressed as percent of basal O_2 consumption.

Statistical Analysis. Comparisons between respiration before and after injection were analyzed using the Student's t test for two paired groups with two-tailed probability ($\alpha < 0.05$). Comparisons between effects of natural humic solutions and reconstituted humic solutions were analyzed using the Student's t test for two unpaired groups with two-tailed probability ($\alpha < 0.05$).

RESULTS

The basal rates of oxygen consumption of *L. laccata* and *C. graniforme* were, respectively, 3134 $\pm$ 757 and 2790 $\pm$ 711 nM O_2/g dry weight/min.

Expect for the binary mixtures protocatechuic/*p*-hydroxybenzoic on *L. laccata* and acetophenone/*p*-hydroxybenzoic acid on both fungi, the 10^{-3} M phenolic mixtures caused a spectacular increase of oxygen consumption of *L. laccata* (Figure 1) and *C. graniforme* (Figure 2). All the 10^{-7} M phenolic mixtures, however, induced a significant decrease in oxygen consumption for both species.

Phenolic mixtures simulating mull- and mor-type humic solutions were responsible for a significant reduction of *L. laccata*'s and *C. graniforme*'s res-

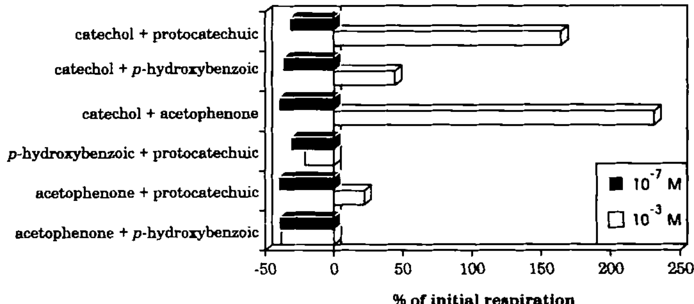

FIG. 1. Effects of equimolar phenolic acid mixtures on respiration of *Laccaria laccata* expressed as percent of initial consumption of oxygen. Each bar is the mean of seven replicates. For the purpose of better presenting the results, standard deviations, which ranged from ± 4 to ± 23, are not shown. All the results are significant at $\alpha < 0.05$, according to Student's t test for two paired groups.

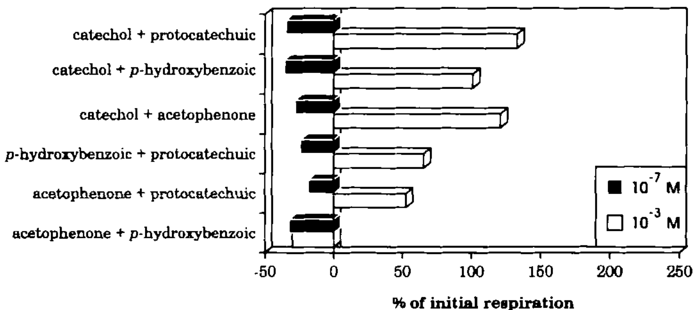

FIG. 2. Effects of equimolar phenolic mixtures on respiration of *Cenococcum graniforme* expressed as percent of initial consumption of oxygen. Each bar is the mean of seven replicates. For the purpose of better presenting the results, standard deviations, which ranged from ± 3 to ± 25, are not shown. All the results are significant at $\alpha < 0.05$, according to Student's t test for two paired groups.

piration (Figure 3). The observed levels of inhibition were essentially identical with those observed with 10^{-7} M phenolic acids mixtures. Furthermore, natural and simulated mor humic solutions had an identical significant deleterious effect on *L. laccata*'s respiration. The same was true for natural and simulated mull humic solutions on *C. graniforme*. These data suggest that phenols present in mor- and mull-type humic solutions may be responsible for the inhibited respiration of the two fungal species.

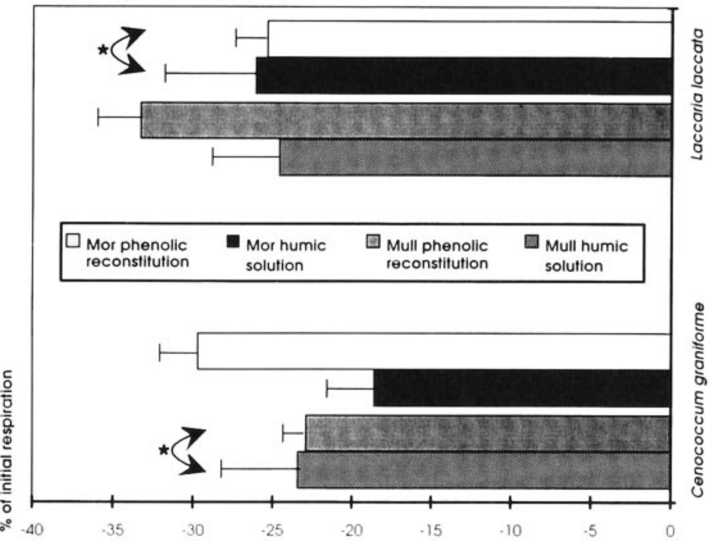

FIG. 3. Effects of natural and simulated phenolic acids in humic solutions on respiration of *Laccaria laccata* and *Cenococcum graniforme* expressed as percent of initial consumption of oxygen. Each bar is the mean of seven replicates ± standard deviation.* No significant difference between natural and reconstituted humic solutions.

DISCUSSION

Humus forms the natural substrate of fungi and water-soluble substances produced or leached from humus can affect the metabolism of mycorrhizal fungi in the soil (Rice, 1984; Pellissier, 1993). The studies conducted here were short term, on the order of 1 hr, in which immediate responses were measured. Of course, such an approach is far from the extreme chemical complexity of the soil environment. Nevertheless, such a method can, by its simplicity, provide indications of the allelochemical potential of common phenolic acid compounds found in humic solutions.

A previous study demonstrated that respiration of *C. graniforme* and *L. laccata* was inhibited by the two kinds of humic solutions from subalpine spruce forests (mull- and mor-type). The same work demonstrated also that four phenolic acids present in these humus forms could individually significantly inhibit the oxygen consumption of the two fungi (Pellissier 1993).

Joint action of phenolic compounds has been demonstrated on various models: germination (Einhellig, 1987), seedlings growth (Gerig et al., 1989; Gerig and Blum, 1991), and mycorrhizal fungi growth (Olsen et al., 1971). Concentrations of the phenolic mixtures tested produced opposite responses. A

decrease of oxygen consumption was observed for 10^{-7} M mixtures and an increase of O_2 consumption was observed for 10^{-3} M mixtures. Due to the important and immediate increase of respiration rate they provoked, these high concentration mixtures could act as uncouplers, particularly when catechol was present in the mixture. Nevertheless, additional investigations are necessary to elucidate the mechanisms of action in the cell. The allelopathic power of catechol was also particularly important in fungus growth bioassays (Boufalis et al., 1994).

Several workers have attempted to simulate chemical combinations from field situations (Glass, 1976; Rice et al., 1981). They observed that the inhibitory effects of mixtures were greater than the individual compounds tested separately. The simulated mor phenolic solution on *C. graniforme* and the simulated mull phenolic solution on *L. laccata* had inhibitory effects greater than observed with natural humic solutions. On the other hand, the simulated solution and mull on *C. graniforme* and the simulated solution and mor on *L. laccata* had effects that were not significantly different. As suggested by Blum et al. (1985), "the nature of the response depends on the magnitude of inhibition associated with each compound, the compounds in the mixture and the factor measured" (in Einhellig, 1987). These results suggest that at least part of the allelopathic effects of the humic solutions can be attributed to their phenolic contents.

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MALE SEX PHEROMONE OF COCKROACH *Eurycotis floridana* (WALKER) (BLATTIDAE, POLYZOSTERIINAE): ROLE AND COMPOSITION OF TERGITES 2 AND 8 SECRETIONS

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Abstract—In *Eurycotis floridana*, the male calling behavior is associated with the exposition of epidermal glands located under tergites 2, 7, and 8. 4-Hydroxy-5-methyl-3(2H)-furanone and 4-hydroxy-2,5-dimethyl-3(2H)-furanone were recently identified as the specific components of tergite 7 secretion. Methylene chloride extracts of tergite 7 and its major compound 4-hydroxy-5-methyl-3(2H)-furanone attract the conspecific females at a distance. Methylene chloride extracts of tergite 8 are also attractive at a distance to the females, whereas extracts of tergite 2 had no effect on males and females. Our GC investigations showed the absence of specific compounds in tergite 2 secretions. The GC-MS analyses revealed that the male secretion of the gland under tergite 8 is mainly a mixture of (2*R**, 3*R**)-butanediol, 1-dodecanol and benzyl 2-hydroxybenzoate. These compounds were tested at different concentrations on their own, or as a mixture. Only (2*R**,3*R**)-butanediol and 1-dodecanol were attractive for the females. Their functions, as components of the male sex pheromone, in addition with the two derivatives of the furanone are discussed.

Key Words—Cockroach, Dictyoptera, Blattidae, *Eurycotis floridana*, (2*R**,3*R**)-butanediol, dodecanol, benzyl-2-hydroxybenzoate, sex pheromone, sexual behavior.

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INTRODUCTION

Among many species of cockroaches, sexual behavior is very stereotyped (Roth and Willis, 1952, 1954; Barth, 1968a; Sreng, 1983; Abed, 1992). This behavior is dependent on several chemical signals that are emitted by specialized abdominal exocrine glands. Typical sexual behavior can be summarized as follows: a sex pheromone emitted by one sex attracts from a distance individuals of the opposite sex. After antennal recognition, the male exposes his tergal glands (wings raised, abdomen lowered). As the female comes to feed on the exposed tergites of the male, she is brought into a favorable position for copulation. The existence of sex pheromones attractive at a distance has been clearly demonstrated in many species of cockroaches (Roth and Willis, 1952; Barth, 1968a; Wendelken and Barth, 1971; Moore and Barth, 1976; Takegawa and Takahashi, 1989; Hales and Breed, 1983; Sreng, 1990; Sirugue et al., 1992; Abed et al., 1993a–c; Liang and Schal, 1993a), but the glandular sources of the sex pheromone have been unambiguously located in only a few species (Sreng, 1983, 1990; Schal et al., 1992; Sirugue, 1992; Sirugue et al., 1992; Abed et al., 1993a–c; Liang and Schal, 1993b).

The large wingless cockroach *Eurycotis floridana* lives in the southern part of the United States and has been reported from Georgia, Florida, and Mississippi, occurring in outdoor sheltered areas such as stumps and under the bark of dead trees (Brenner and Pierce, 1991). The sexual behavior of *E. floridana* was first studied by Roth and Willis (1954) and by Barth (1968b), who showed that it is the male that initiates the mating sequence. The male positions itself near the female and repeatedly shakes its body from side to side. Numerous behavioral observations of *E. floridana* revealed that a sexually mature male displays a characteristic posture, which first promotes the exposure of the anterior part of tergites 2 and 8. The anterior part of tergite 7 is only exposed from time to time. The attracted female then mounts the male's back and genital connection subsequently occurs. In our laboratory, histological studies have shown that the exposed anterior parts of tergites 2, 7, and 8 are the glandular areas (David-Henriet, 1993), not the intersegmental membrane as previously described (Barth, 1968b). Recently, we identified the major compounds of the secretion of the seventh tergite, 4-hydroxy-5-methyl-3(2H)-furanone (HMF) (about 8.5 μg per sexually mature male) and 4-hydroxy-2,5-dimethyl-3(2H)-furanone (furaneol, registered trade mark of Firmenich SA, Geneva, Switzerland) (about 240 ng per sexually mature male) (Farine et al., 1993). These two components have a characteristic odor of caramel. Additionally, we proved that the male secretions of tergite 7 were attractive at a distance to the female, the same results being obtained by using various amounts of the major component, HMF.

In this paper, we report on the chemical analysis of tergite 2 and tergite 8

glandular secretions. Using a Y-maze olfactometer, the biological function of the whole secretion of the tergites 2 and 8, of the mixed secretions of the tergites 7 and 8, and of each of the identified compounds (separately or in combination) in tergite 8 was investigated and then discussed.

METHODS AND MATERIALS

Insects. Colonies of *E. floridana* were reared in glass aquariums (60 × 40 × 20 cm) maintained at 28°C and 80% relative humidity under a 12:12 hr dark–light period. The insects had free access to dry dog food and water-soaked cotton pads. Males and females were isolated from the colonies after the adult ecdysis and kept separately in plastic boxes (12 × 8 × 5 cm) until behavioral bioassays.

Preparation of Extracts. The insects, at least 15 days old, were caught during the scotophase and cooled for a few minutes at −20°C before dissection. Tergites 2 and 8 and their associated epidermal glands were dissected from each male with microscissors and forceps. The glands were removed from the adjacent tissues and allowed to soak for 1 hr at room temperature in a vial of distilled methylene chloride (CH_2Cl_2) (about 100 μl/gland). Then, the tergites were removed from the solvent. One hundred forty males were dissected and the extracts from tergites 8 and 2 pooled separately for bioassays. In Results, we will refer to these extracts as 140T8 and 140T2 extracts. The extracts were concentrated to 200 μl by the Kuderna-Danish method (Marquardt and Luce, 1961) and kept at −20°C until used.

As the amounts and relative percentages of each compound in glandular insect secretions always vary widely between individuals, quantitative analyses were made on five males. Five hundred nanograms of 2-phenylethanol were added to each sample as an internal standard before dipping the dissected glands. Then, the samples were concentrated to few microliters under a gentle flow of nitrogen before analysis. The response factor of each major compound was established at 0.1, 0.5, 1, and 5 μg and compared to that of 500 ng of 2-phenylethanol. Each standard analysis was replicated three times. The mean proportions of the integrated areas between the internal standard and each of the three identified compounds were used for the quantitative analysis.

To investigate the presence of specific cuticular hydrocarbons or of apolar compounds of high molecular weight on the glandular surface of tergites 2, 7 and 8 and in the gland secretions, pentane extracts were used for analyses. Just before the GC analysis, one tergite was dissected as previously described and extracted in 500 μl of redistilled pentane at room temperature for 20 min. Then, the extract was evaporated to dryness under a gentle nitrogen stream and resuspended in 20 μl of pentane. A 2-μl aliquot was analyzed by GC. Each analysis

was replicated using three different extracts. As all the GC profiles were very similar, we did not attempt chemical identification on these extracts.

Chemical Analysis. The CH_2Cl_2 extracts were analyzed by GC and GC-MS. GC analyses were performed by a Packard 437 A instrument fitted with a flame-ionization detector. A CP Wax 58 CB (30 m × 0.25 mm ID, 0.22-μm film thickness, Chrompack) and a DB 5 (30 m × 0.32 mm ID, 1-μm film thickness, J and W Scientific) fused silica capillary columns were used for analyses. One microliter of each sample was injected via a split–splitless injection system, operating with a split flow of 25 ml/min and a septum purge of 3 ml/min. The split and purge ports were closed during injection and then opened 30 sec after injection. The column was held isothermally at 40°C for 2 min, programmed at 20°C/min for 1 min, and then at 2°C/min to 240°C, using hydrogen as the carrier gas (50 cm/sec velocity at room temperature). The injector and detector temperatures were 250 and 270°C, respectively. The integrated areas of the compounds are based on the millivolt output from the detector, using a Shimadzu CR 4 A computer (Kyoto, Japan).

GC-MS was carried using a Nermag R 10-10 C quadrupole mass spectrometer coupled with a Girdel 31 gas chromatograph. The column used for GC analyses was a CP Wax 58 CB (30 m × 0.25 mm ID, 0.22-μm film thickness, Chrompack), and the GC conditions were the same as those just described. The column was connected directly to the ion source of the spectrometer through a heated transfer line maintained at 260°C. Electron impact (EI) mass spectra were obtained at 70 eV with a 0.8-sec cycle, the instrument scanning from 25 to 300 amu with a source temperature of 150°C.

Compounds were identified by comparing their spectra to those of the library of the Laboratoire de Recherches sur les Arômes (I.N.R.A., Dijon, France). Furaneol, (2*R**,3*R**)-butanediol, 1-dodecanol (dodecanol), benzyl 2-hydroxybenzoate (B-2-H), and palmitic, stearic, oleic, and linoleic acids were purchased from Interchim (Montluçon, France). HMF was provided by Haarmann and Reimer (Nanterre, France). Correct identification of tergite 8 compounds was checked by analyzing the synthetic compounds under the same conditions on DB 5 and CP Wax 58 CB columns.

The analyses of the pentane extracts were performed using a Chrompack CP-9000 instrument fitted with a flame-ionization detector and a split–splitless injector (30 sec split at 25 ml/min). The column was a CP Sil 5 fused silica capillary column (25 m × 0.25 mm ID, 0.12-μm film thickness, Chrompack). Helium was used as carrier gas (35 cm/sec velocity at 120°C). The injector and detector temperatures were 260 and 285°C, respectively. The oven temperature was programmed from 120 to 140°C (10°C/min), then from 140 to 300°C (4°C/min). The signal was recorded and integrated on a Shimadzu CR 4 A computer.

Bioassays (Y-Maze Olfactometer). Each olfactory stimulus was tested on

30 sexually mature virgin males (15 days old), and on 30 sexually mature virgin females (30 days old). All the tests were conducted under red light of low intensity after the second hour of the scotophase. The olfactometer was a glass tube (2 cm ID) with a 40-cm-long common arm, dividing into two 30-cm-long choice arms (the angle between the two choice arms was 70°). All individuals were placed in an adaptation box (6 × 9 × 5 cm) for at least 2 hr before each test. The boxes had a screen door that could be raised to allow the insect to enter the common arm at the starting point. The samples tested were impregnated V-folded filter papers (1 × 2 cm), the sample being changed after each tested insect. Samples were placed alternately at the end of one choice arm. An airstream (3.2 cm/min) conveyed the chemical stimuli through the arms to the common arm and to the "starting box" containing the insect to be tested. Virgin adults were given the choice between the odor stimuli (test) and the air. Each insect was tested once for 5 min, and if it did not respond within this period, the test was stopped. The number of insects that entered the air or the test arms was recorded. The olfactometer was washed with CH_2Cl_2 after each test.

Different chemical stimuli were tested after solvent evaporation: 1 μl of CH_2Cl_2 (control); whole CH_2Cl_2 extracts from tergite 2 (140T2) and from tergite 8 (140T8), combinations of extracts from tergites 7 and 8 (140T7,T8), corresponding to 10^{-1}–10^{-6} male equivalents (ME); 1000, 500, 100, 10, 1, 0.1, 0.01, and 0.001 ng of (2*R**,3*R**)-butanediol, dodecanol, and B-2-H; mixtures of the three identified compounds in tergite 8 (mixture 1) and mixtures of (2*R**,3*R**)-butanediol and dodecanol (mixture 2) corresponding to 10^{-1}–10^{-6} ME. The quantity of each compound in the mixtures has been determined relative to their ratio in their corresponding whole extract.

Olfactometer data were compared by using a Monte Carlo test procedure (Vaillant and Derridj, 1992) based upon χ^2 at level $P = 0.05$.

RESULTS

Chemical Identification. A typical GC trace derived from a male tergite 8 extract (Figure 1a) reveals the presence of three major specific compounds (peaks 1, 2, and 3). Some of the trace compounds detected were issued from the previous tergite 7 secretion, but most of them, as (*E*)-2-hexenal and (*E*)-2-hexenol, came from the sternal glandular defensive secretion (Farine, unpublished results), which continually oozes as there is no sphincter muscle at the opening of the gland (Stay, 1957). All compounds eluting after a retention time of 80 min were always present in fat body extracts from nymphs and adults of both sexes. They were identified as palmitic (peak 4), stearic (peak 5), oleic (peak 6), and linoleic (peak 7) acids.

Compound 1 was identified as (2*R**,3*R**)-butanediol (molecular weight,

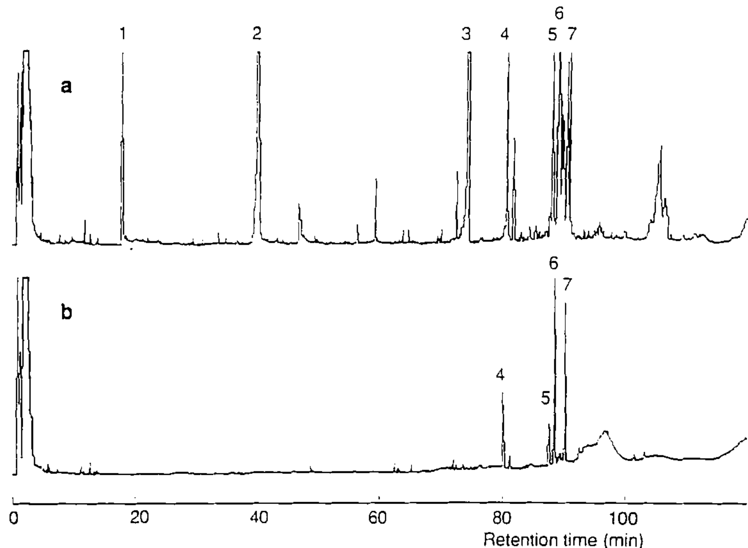

FIG. 1. Typical GC traces of CH_2Cl_2 glandular extracts from male tergites 8(a) and 2(b) of *E. floridana*. Peak 1: (2*R**,3*R**)-butanediol; 2, 1-dodecanol; 3, benzyl 2-hydroxy-benzoate; 4, palmitic acid; 5, stearic acid; 6, oleic acid; 7, linoleic acid. Experimental conditions are given in Methods and Materials.

90). The mass spectrum and coinjection with the reference sample on polar and apolar columns confirmed that the synthetic and the natural compound were identical. Quantitative analyses revealed a mean of 560 ng of (2*R**,3*R**)-butanediol per sexually mature male, the amount varying from 290 ng to 1.2 μg among the five males analyzed. From 140T8 extract, we estimated the amount of (2*R**,3*R**)-butanediol to be 370 ng/ME.

Compound 2 was identified as 1-dodecanol (molecular weight, 186) by comparison of retention time, coinjection, and GC-MS of a reference sample with the natural substance. Quantitative analyses revealed a mean of 1.23 μg of dodecanol per sexually mature male, the amount varying from 454 ng to 2.7 μg among the five males analyzed. We estimated the amount of dodecanol to be 1.5 μg/ME from the 140T8 extract.

Compound 3 was identified as benzyl 2-hydroxybenzoate (molecular weight, 228). The mass spectrum and coinjection with the reference sample confirmed its chemical nature. Quantitative analyses showed a mean of 570 ng of B-2-H per sexually mature male, the amount varying from 280 to 1064 ng among the five males analyzed. From the 140T8 extract, we estimated the amount of B-2-H to be about 660 ng per male.

GC and GC-MS analyses of CH_2Cl_2 extracts of tergite 2 showed the absence of volatile compounds, whereas palmitic, stearic, oleic, and linoleic acids were detected (Figure 1b). When analyzing the various pentane extracts, neither qualitative nor quantitative significant differences were observed between the GC profiles of tergites 2 (Figure 2), 7, and 8.

Bioassays. The biological activity of 140T2 extract used at different amounts is shown in Table 1. With a blank (control test), males and females responded in the same way, although the anemotactic response of the females that entered

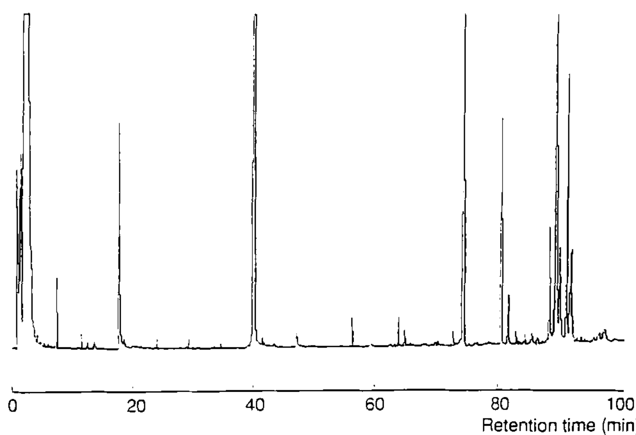

FIG. 2. Typical GC traces of pentane glandular extract from male tergite 2 of *E. floridana*. Experimental conditions are given in Methods and Materials.

TABLE 1. Y OLFACTOMETER: NUMBER OF MALES AND FEMALES OF *E. floridana* ($N = 30$) ATTRACTED TO VARIOUS AMOUNTS OF MALE TERGITE 2 EXTRACTS

Amount (ME)	Males			Females		
	Air	Test		Air	Test	
10^{-1}	9	6	NS[a]	17	13	NS
10^{-2}	8	7	NS	15	15	NS
10^{-3}	12	15	NS	10	14	NS
10^{-4}	14	10	NS	11	13	NS
10^{-5}	13	11	NS	11	15	NS
10^{-6}	12	12	NS	14	10	NS
Control	7	10	NS	11	9	NS

[a] NS = not significant

the common arm was a little higher (67%) than the male response (57%). Only about 30% of adults of both sexes entered the test arm. Our results revealed that the tested females were not significantly more attracted than the males by the 140T2 extracts. All the insects that entered the olfactometer were unable to discriminate between the air and the test arms. The only noticeable difference was the behavioral reactions of males and females when highest amounts (10^{-1} and 10^{-2} ME) of tergal secretions were tested: 100% of the females entered the olfactometer, while only 43–50% were attracted to the extracts. Fifty percent of males entered the olfactometer, while about 20% of them visited the test arm.

The biological activity of the secretion of the 140T8 extract, of mixture 1 [(2*R**,3*R**)-butanediol/dodecanol/B-2-H], and of mixture 2 [(2*R**-3*R**)-butanediol/dodecanol)] are shown in Tables 2, 3, and 4. The tested females that entered the test arm of the olfactometer were significantly more attracted than the males by the 140T8 extract; 73–86% of the females vs. 33–50% of the males, being attracted to 10^{-1}–10^{-6} tergite ME (Table 2). The females usually ran directly and quickly to the test arm, their antennae vibrating at a rapid rate, whereas the males walked slowly down the common arm, stopping and licking their antennae at the intersection of the two arms.

Using various amounts of the three major compounds identified (mixture 1), it appeared that 50–90% of the females vs. 13–30% of the males, entered the test arm of the olfactometer (Table 3). All the attracted females were significantly attracted to 10^{-1}–10^{-6} standard ME, whereas the males sometimes were significantly repulsed by the mixture. The behavioral responses of males and females were comparable when various amounts of (2*R**,3*R**)-butanediol and dodecanol (mixture 2) were tested (Table 4). Thirty to 50% of the adults of both sexes entered the test arm of the olfactometer. However, although the

TABLE 2. Y OLFACTOMETER: NUMBER OF MALES AND FEMALES OF *E. floridana* ($N = 30$) ATTRACTED TO VARIOUS AMOUNTS OF MALE TERGITE 8 EXTRACTS

Amount (ME)	Males			Females		
	Air	Test		Air	Test	
10^{-1}	12	15	NS[a]	5	25	S
10^{-2}	11	10	NS	6	24	S
10^{-3}	15	15	NS	4	26	S
10^{-4}	13	14	NS	5	25	S
10^{-5}	16	11	NS	8	22	S
10^{-6}	9	15	NS	7	23	S

[a] S = significant; NS = not significant

TABLE 3. Y OLFACTOMETER: NUMBER OF MALES AND FEMALES OF *E. floridana* (N = 30) ATTRACTED TO VARIOUS AMOUNTS OF ($2R^*$, $3R^*$)-BUTANEDIOL, 1-DODECANOL, AND BENZYL 2-HYDROXYBENZOATE (MIXTURE 1)

Amount (ME)	Males			Females		
	Air	Test		Air	Test	
10^{-1}	18	9	NS[a]	6	18	S
10^{-2}	23	7	S	2	22	S
10^{-3}	5	4	NS	3	27	S
10^{-4}	4	5	NS	4	26	S
10^{-5}	15	9	NS	5	22	S
10^{-6}	19	5	S	6	15	S

[a] S = significant; NS = not significant.

TABLE 4. Y OLFACTOMETER: NUMBER OF MALES AND FEMALES OF *E. floridana* (N = 30) ATTRACTED TO VARIOUS AMOUNTS OF ($2R^*$, $3R^*$)-BUTANEDIOL AND 1-DODECANOL (MIXTURE 2)

Amount (ME)	Males			Females		
	Air	Test		Air	Test	
10^{-1}	17	13	NS[a]	19	11	NS
10^{-2}	16	11	NS	17	10	NS
10^{-3}	12	15	NS	17	13	NS
10^{-4}	13	11	NS	18	12	NS
10^{-5}	15	9	NS	14	16	NS
10^{-6}	15	15	NS	11	16	NS

[a] NS = not significant.

behavioral tests were not significant, results showed that both sexes were quite repelled by various amounts of mixture 2.

Using various amounts of 140T7, T8 extracts 63–93% of the females vs. 7–27% of the males entered the test arm (Table 5). All the females were significantly attracted to 10^{-1}–10^{-6} standard ME, whereas the tested males were repulsed by the mixture.

Using pure compounds (Table 6), 43–83% of the females vs. 13–30% of the males entered the test arm of the olfactometer when various amounts of ($2R^*$,$3R^*$)-butanediol were tested separately in various amounts. Similar results were obtained when dodecanol was used. As observed in males, the majority

TABLE 5. Y OLFACTOMETER: NUMBER OF MALES AND FEMALES OF *E. floridana* ($N = 30$) ATTRACTED TO VARIOUS AMOUNTS OF MALE TERGITES 7 AND 8 EXTRACTS

Amount (ME)	Males			Females		
	Air	Test		Air	Test	
10^{-1}	13	2	S[a]	3	27	S
10^{-2}	17	7	S	6	24	S
10^{-3}	12	3	S	2	28	S
10^{-4}	18	6	S	5	25	S
10^{-5}	13	8	NS	5	22	S
10^{-6}	11	7	NS	8	19	S

[a] S = significant; NS = not significant.

TABLE 6. Y OLFACTOMETER: NUMBER OF MALES AND FEMALES OF *E. floridana* ($N = 30$) ATTRACTED TO VARIOUS AMOUNTS OF ($2R^*$, $3R^*$)-BUTANEDIOL, 1-DODECANOL AND BENZYL 2-HYDROXYBENZOATE

Amount	(2R*, 3R*)-Butanediol			Dodecanol			Benzyl 2-hydroxybenzoate		
	Air	Test		Air	Test		Air	Test	
Males									
1μg	22	5	S[a]	18	9	NS	18	9	NS
500 ng	20	4	S	17	7	S	16	8	NS
100ng	21	6	S	24	6	S	15	12	NS
10 ng	25	5	S	15	9	NS	19	5	S
1 ng	18	9	NS	22	8	S	18	7	NS
0.1 ng	19	5	S	22	8	S	16	5	S
0.01 ng	16	8	NS	24	6	S	15	12	NS
0.001 ng	15	9	NS	23	7	S	17	10	NS
Females									
1 μg	5	25	S	6	24	S	21	3	S
500 ng	6	24	S	11	19	NS	19	5	S
100 ng	8	22	S	6	21	S	19	11	NS
10 ng	11	19	NS	8	22	S	20	7	S
1 ng	12	18	NS	6	18	S	21	9	S
0.1 ng	11	16	NS	12	18	NS	22	5	S
0.01 ng	12	15	NS	14	13	NS	17	10	NS
0.001 ng	11	13	NS	12	15	NS	18	9	NS

[a] S = significant; NS = not significant.

of the concentrations of B-2-H tested were significantly repellent for the females. The males were always more or less significantly repelled when using various amounts of the three major identified compounds. The females were significantly attracted when 100 ng to 1 μg of (2*R**,3*R**)-butanediol were used, the insects having some difficulty in discriminating lower amounts. Similar results have been obtained when various amounts of dodecanol were tested, females responding significantly when 1 ng to 1 μg of this compound was used.

DISCUSSION

In many species of cockroaches, the female releases a sexual pheromone that attracts conspecific males at a distance (Barth, 1968a; Schal and Bell, 1985), but their glandular origin and chemical nature remain largely unknown (reviewed in Schal and Smith, 1990). In the American cockroach, *Periplaneta americana*, the pheromone consists of several periplanones (Persoons et al., 1990), and one of them, periplanone-B, was recently localized in atrial glands (Abed et al., 1993c). In the Japanese cockroach, *Periplaneta japonica*, Takegawa and Takahashi (1989) identified periplanone-J, while the sex pheromone of the brown cockroach, *Periplaneta brunnea*, was tentatively identified as periplanene-Br (Ho et al., 1992). In the German cockroach, *Blattella germanica* (Abed et al., 1993b; Liang and Schal, 1993a,b) and in *Blaberus craniifer* (Abed et al., 1993b), the pheromone is produced by pygidial glands. In *Supella longipalpa*, calling females release, through tergal glands, a volatile sex pheromone that was recently isolated and confirmed by synthesis (Smith and Schal, 1990; Charlton et al., 1993).

The chemical identification of volatile sex pheromones has been reported in male cockroaches of only two species belonging to the subfamily of the Oxyhaloinae: *Nauphoeta cinerea* (Takahashi and Fukui, 1980, 1983; Sreng, 1990; Sirugue et al., 1992) and *Leucophea maderae* (Sirugue, 1992). In *N. cinerea*, the pheromone that attracts the females from a distance is a mixture of methyl-2-thiazolidine and 4-ethylguaiacol, whereas another compound, hydroxy-3-butanone-2, acts at close range and keeps the female in the vicinity of the male (Sirugue et al., 1992). The volatile sex pheromone attractive at a distance of the closely related species *L. maderae* is hydroxy-3-butanone-2. (*E*)-2-Octenoic acid an senecioic acid act at short range as an arrestant (Sirugue, 1992). In *B. orientalis*, Sreng (1993) mentioned on the existence of a female sex pheromone, but Abed et al. (1993a) clearly demonstrated that it is the male that adopts a calling posture, emitting a sex pheromone. GC-MS analysis showed that only one unidentified compound was specific to the male secretion.

Among the many studied species of Oxyhaloinae, the male sex pheromone attracting the females from a distance is produced by well-developed sternal

glands, whereas the aphrodisiac secretion is secreted by tergal glands (Brossut and Sreng, 1985). The number of the glands varies according to species (Sreng, 1984, 1993). In *B. orientalis*, the male tergal glands that produce the sex pheromone attractive at a distance are located on the anterior part of tergites 1–8, while the posterior part of the same tergites produce the aphrodisiac secretions (Abed, 1992; Abed et al., 1993a). In *E. floridana*, David-Henriet (1993) showed that it is the male, and not the female as mentioned by Sreng (1993), that produces a sex pheromone attractive at a distance. Behavioral observations revealed that the glandular areas situated on the anterior part of tergites 2, 7, and 8 were responsible for the production of the pheromone. Morphological observations also revealed the presence of glandular areas on the anterior part of tergites 2 in nymphs and females and on the anterior part of the tergite 7 in females. As observed in male secretions, our GC investigations (unpublished results) have never revealed the existence of specific compounds in glandular extracts of tergites 2 and 7 in nymphs and females. All the GC traces look like those obtained with CH_2Cl_2 or pentane male extracts of tergite 2. Additionally, behavioral observations using various amounts of nymphs and female extracts never revealed any attractiveness to the nymphs or adults of either sex. However, histological sections, by the presence of numerous secretory vesicles into the cytoplasm, proved unambiguously that the glandular cells are active (David-Henriet, 1993). The nonexistence of male specific compounds in the extracts of tergites 2 does not imply the nonexistence of high-molecular-weight compounds (i.e., proteins), undetected by the classical GC techniques. This hypothesis is confirmed by the fact that, in *E. floridana*, nymphs, males, and females expose their tergal glands during their well-developed agonistic behavior (Diboine and Farine, unpublished results). These observations might explain the existence of low-volatility products acting at close range in tergites 2 and 7 in nymphs and adults of both sexes.

This is the first time that dodecanol has been identified in cockroach secretions, whereas it was identified as a component of the female sex pheromone in various lepidopteran species (reviewed in Inscoe, 1982), and as a minor constituent in glandular exudates of Dufour's glands of Formicidae (Bergström and Löfqvist, 1968, 1970). As far as we know, (2*R**,3*R**)-butanediol and its isomer (2*S**,3*S**)-butanediol have only been identified as minor constituents in male sternal and tergal secretions of *N. cinerea* and *L. maderae* (Sirugue, 1992; Sirugue et al., 1993). Although these compounds appeared specific to the males, no attractiveness at a distance of the conspecific females was demonstrated when tested at 1, 10, 100, and 1000 ng. Benzyl 2-hydroxybenzoate has never been previously reported in insect secretions. This compound was mentioned as occurring in trace amounts as a volatile constituent from peach fruit and leaves (Horvat and Chapmann, 1990) and bud exudate of *Populus euramericana* (Greenaway et al., 1987). Concerning the fatty acids identified in all the glan-

dular secretions, our GC investigations are in accordance to those of Bade (1964). Ninety-six percent of the total fatty acids found in *E. floridana* is a combination of oleic (56%), linoleic (18%), palmitic (18%), and stearic (4%) acids.

Recently, we demonstrated that the male secretion of tergite 7 is attractive from a distance to the females (Farine et al., 1993). The major compound 4-hydroxy-5-methyl-3(2H)-furanone (HMF) is clearly attractive to the females, while the role of the minor compound furaneol remains ambiguous. In male tergite 8 secretion, our data showed the presence of three major compounds, (2*R**,3*R**)-butanediol, dodecanol, and benzyl 2-hydroxybenzoate. We demonstrated the attractiveness of the glandular extract of tergite 8 to the conspecific females and its varying degree of repellency to the males. We also observed that the addition of tergite 7 secretions has no effect on the attractiveness to the females, while it enhanced the repellency to the males. When tested on their own, the three identified compounds are repulsive to the males. (2*R**,3*R**)-Butanediol and dodecanol are significantly attractive to the females when 100 ng to 1 μg and 1 ng to 1 μg are tested, respectively. The role of B-2-H vis-à-vis the females was ambiguous when tested on its own. Whatever the amounts tested, this compound was always repulsive to the females, whereas behavioral tests using mixtures 1 and 2 showed that the three major compounds were necessary to elicit an attraction response.

At the present time, numerous questions concerning chemical communication in cockroaches sexual behavior remain unanswerable. Within the Blattidae, the only identified sex pheromones take part of females of the genus *Periplaneta* (Takegawa and Takahashi, 1989; Persoons et al., 1990; Ho et al., 1992; Abed et al., 1993c). The existence of a male sex pheromone is now proved in *B. orientalis* but, in this species, behavioral observations revealed that the female atrial glands are certainly the site of production of a sex pheromone that must contribute to sexual isolation (Abed et al., 1993a). Our data have proved unambiguously that the males of *E. floridana* attract their conspecific females at a distance. Additionally, we can assume that the male sex pheromone is a mixture of 4-hydroxy-5-methyl-2(3H)-furanone (produced by the seventh tergite) (Farine et al., 1993) and of (2*R**,3*R**)-butanediol, 1-dodecanol, and benzyl 2-hydroxybenzoate (produced by the eighth tergite). Although 4-hydroxy-5-methyl-2(3H)-furanone, 1-dodecanol, and (2*R**,3*R**)-butanediol are very attractive for the females and repulsive for the males when tested alone, benzyl 2-hydroxybenzoate is repulsive for adults of both sexes. However, the females of *E. floridana* are only attracted and display full sexual behavior in response to combined tergites 7 and 8 secretions. In order to decipher the final role of each male tergal gland during the sex behavioral sequences, complementary experiments need to be done.

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CUTICULAR LIPID PROFILES OF QUEENS, WORKERS, AND MALES OF SOCIAL WASP *Polistes metricus* SAY ARE COLONY-SPECIFIC

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Abstract—The cuticular lipids of *Polistes metricus* queens, workers and males from seven laboratory-maintained colonies were extracted and analyzed by combined gas chromatography–mass spectrometry. Males had higher proportions of alkenes (20.5%) in their cuticular lipids than did queens (2.3%) or workers (7.7%). Discriminant analyses of the cuticular lipid profiles of the adult wasps showed that males group separately from females. Additional analyses showed that queens group with their respective workers by colony and that queens group even more closely with males by colony. The most distinct groupings occurred with workers only by colony and with males only by colony. Stepwise discriminant analyses showed that each type of grouping was dependent upon a different combination of cuticular lipids.

Key Words—*Polistes metricus*, Hymenoptera, Vespidae, cuticular hydrocarbons, discriminant analysis, nestmate recognition, social wasps.

INTRODUCTION

Cuticular hydrocarbons have been shown to serve a variety of functions in insects. They provide a hydrophobic barrier to desiccation, and they act as sex pheromones in some insects (Blomquist and Dillwith, 1985; Hadley, 1985; Lockey, 1988; Howard, 1993). Recently, cuticular hydrocarbons have also been identified as chemical cues utilized by social insects in nestmate recognition.

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Heterospecific insects that integrate into social insect colonies were found to have the same cuticular hydrocarbons as their hosts (Howard et al., 1980; Vander Meer and Wojcik, 1982; Howard et al., 1990). Since these social parasites are able to avoid being recognized as aliens, the similarity in cuticular hydrocarbon composition between host and parasite suggested that these chemicals may serve as the components involved in kin recognition.

Newly emerged workers and gynes of *Polistes* wasps demonstrate nestmate recognition only if they have had exposure to their nest from which they learn, and possibly acquire, a characteristic colony odor (Ross and Gamboa, 1981; Pfenning et al., 1983; Gamboa et al., 1986a,b). Hydrocarbons on the surface of a social wasp nest were found to have the same composition as the cuticular hydrocarbons of the individuals from the colony (Espelie and Hermann, 1988, 1990; Espelie et al., 1990; Lorenzi, 1992; Singer et al., 1992a). *Polistes* workers that had been able to discriminate between their own nest and a foreign nest were no longer able to identify their nest if the surface hydrocarbons were removed (Espelie et al., 1990). When the hydrocarbons were replaced, the wasps were able to recognize their nest again. Furthermore, newly emerged *Polistes* workers allowed to emerge on nests with hydrocarbons removed were unable to recognize their nestmates when given a choice between a nestmate and a nonnestmate (Singer and Espelie, 1992). Newly emerged workers allowed to emerge on nests with hydrocarbons showed a significantly higher ability to distinguish their nestmates from nonnestmates. *Polistes* workers that were returned to their natal nest were more likely to recognize their nest and more likely to be accepted by their nestmates if they had previously been exposed to their nest paper hydrocarbons (Layton and Espelie, 1994).

All of these studies indicate that hydrocarbons are necessary cues for kin recognition in social wasps. The purpose of this study was to examine the cuticular hydrocarbon composition of colonies of *Polistes metricus* reared in the laboratory. Different methods of discriminant analysis were applied in order to determine which hydrocarbons were most important in separating the wasps by colony. To date, cuticular hydrocarbon analyses of social wasps have concentrated on adult females: workers, queens, and gynes. In this study, we characterize the cuticular hydrocarbon patterns of males of known colony membership and compare these patterns to those of the queens and workers utilizing stepwise and canonical discriminant analyses.

METHODS AND MATERIALS

Colony Collection. Early spring nests with a single queen were collected from sites in Clarke County, Georgia, in April and May 1992 and taken to the laboratory. The queens were marked on the thorax with a dot of gold Testor's

enamel paint. Each nest was glued to a thin wooden strip attached to the top of a Plexiglas chamber (20 × 20 × 20 cm). Pieces of Whatman No. 1 filter paper were provided for nest construction (Matthews and Ross, 1985; Downing and Jeanne, 1988; Singer et al., 1992b). Wasps were fed a diet of 20% honey water and caterpillars (*Spodoptera frugiperda*, *S. exigua*, or *Galleria mellonella*) daily until the colonies had several workers and at least three males. The wasps were separated from their nests and frozen at −20°C.

Chemical Analysis. Wasps were extracted individually in 2 ml redistilled hexane at room temperature for 60 sec (Blomquist et al., 1987). The hexane extracts were concentrated to dryness under a stream of nitrogen. The samples were resuspended in hexane (20 μl), and 1-μl aliquots were analyzed by combined gas chromatography–mass spectrometry (GC-MS). A Hewlett Packard 5890A gas chromatograph was equipped with a 25-m cross-linked methyl silicone HP-1 (Hewlett Packard) capillary column with helium as the carrier gas. The oven temperature was kept at 55°C for 3 min after injection (splitless), and then the temperature was increased at a rate of 15°C/min to a final temperature of 305°C, which was held for 30 min. The column was connected to a Hewlett Packard 5970 mass selective detector and mass spectra were recorded at 1.3-sec intervals at 70 eV. Data were analyzed with a Hewlett Packard Chem Station and individual components were characterized by their mass spectra and determination of their equivalent chain lengths (Nelson, 1978; Blomquist et al., 1987; Yang et al., 1992). Quantitation was based upon peak areas of total ion chromatogram integrations, which were corrected for response factors by utilizing a standard for each class of cuticular lipid component (Espelie and Bernays, 1989; Yang et al., 1993).

Statistical Analysis. Results from the GC-MS analysis of the cuticular lipids from each wasp were loaded according to percent composition onto a 65 × 28 matrix (Lotus 123, Lotus Development Corp., Cambridge, Massachusetts). These data were transferred to a flat ASCII file for analysis. Three different methods of discriminant analysis were used. The first method was canonical discriminant analysis. This analysis takes a classification variable (gender or colony) and several quantitative variables (percent composition for the cuticular lipids from each wasp) and derives canonical variables that summarize between-class variation. This was done on a Sun Microsystems 4/280 computer running SunOS 4.1.1 (UNIX) using the 2.3 version of the S-plus statistical analysis software package (Statistical Sciences, Inc., 1989). This analysis generated graphs that indicated relative between-class variation for the following groups: all wasps by colony, workers and queens by colony with males grouped by gender, males and queens by colony, males only by colony, workers and queens by colony, and workers only by colony.

The second and third methods of analysis used the 2.2 version of the SAS statistical analysis software package for personal computers (SAS Institute, Inc.,

1985). The second method, a stepwise discriminant analysis, was used to determine which cuticular lipid components (quantitative variables) were the most important in discriminating different groups of wasps (classification variables). This method uses forward selection, backward elimination, and stepwise selection to find a subset of the variables that best reveals differences between groups. The third method was a classificatory discriminant analysis that indicated how many of the wasps were correctly classified by group using the subset of lipid components generated by the stepwise discriminant analysis.

RESULTS

Combined GC-MS analysis of the cuticular lipids of *Polistes metricus* adults resulted in the identification of 25 compounds (Table 1). The major components of the cuticular lipids were *n*-pentacosane; *n*-heptacosane; 9-, 11-, 13-, and 15-methylhentriacontane; 13,17- and 15,19-dimethyltritriacontane; and 11,15- and 13,17-dimethylpentatriacontane. Alkenes ranging in chain length from C_{25} to C_{35} and two aldehydes, heneicosanal and tricosanal, were also identified in the cuticular lipids of these wasps (Table 1). *P. metricus* individuals from the same colony had similar cuticular hydrocarbon patterns. For example, the queen, a worker, and a male from colony 4 had higher proportions of *n*-tricosane (peak 1) and dimethylhentriacontane (peak 17) than wasps from other colonies, while wasps from colony 5 had higher percentages of the methyl- and dimethylpentatriacontanes (peaks 23 and 24) (Figure 1).

The cuticular lipid compositions of queens and workers were distinct from those of the males. Male *P. metricus* had a higher percentage of alkenes (20.5%) than did the queens (2.3%) or workers (7.7%) (Table 2). Conversely, queens and workers had higher proportions of methyl-branched alkanes (23.9 and 17.9%, respectively) than did the males (9.9%). However, *n*-alkanes were the most prevalent cuticular lipid components for the queens, workers, and males.

Canonical discriminant analysis was done using the percent composition of all 25 compounds for the 7 queens, 32 workers, and 26 males from the seven colonies in order to determine relative separation for different classifications of wasps. Using colony as the only classification variable, between-class separation was distinct for colony 4 and colony 1 relative to the other five colonies (Figure 2A). However, there was poor separation between colonies 3 and 5, and between colonies 2, 6, and 7. For each colony, the queen (circled numbers) and the males (underlined numbers) clustered with the workers (isolated numbers) of their respective colonies. In order to determine if male cuticular lipid patterns allow for separate classification from the female wasps, a canonical discriminant analysis was run using gender as the classification variable for males and colony as the classification variable for queens and workers (Figure 2B). Although

TABLE 1. MEAN PERCENT COMPOSITION OF CUTICULAR LIPIDS FOR QUEENS, WORKERS, AND MALES FROM SEVEN *Polistes metricus* COLONIES

Peak[a]	ECL[b]	Component[c]	Queens[d]	Workers[e]	Males[e]
1	23.0	*n*-Tricosane	2.8 (0.5–8.9)	2.0 (1.1–3.6)	2.1 (1.7–2.9)
2		Heneicosanal	0.02 (0.0–0.1)	0.02 (0.0–0.1)	0.2 (0.0–0.6)
3	24.9	Pentacosene	0.4 (0.0–1.1)	1.2 (0.1–1.8)	1.2 (0.0–1.6)
4	25.0	*n*-Pentacosane	16.0 (8.9–23.1)	19.0 (18.2–20.1)	18.4 (11.9–22.5)
5		Tricosanal	0.8 (0.0–1.9)	1.5 (0.0–3.2)	2.0 (0.5–5.0)
6	26.0	*n*-Hexacosane	1.7 (1.0–2.0)	1.8 (1.5–2.2)	1.9 (1.0–2.6)
7	27.0	*n*-Heptacosane	14.2 (12.2–16.3)	12.4 (11.1–15.4)	10.7 (8.4–13.0)
8	27.3	9-, 11-, and 13-Methylheptacosane	1.9 (0.3–3.5)	1.5 (0.2–2.7)	0.6 (0.1–0.9)
9	27.7	3-Methylheptacosane	0.2 (0.0–0.6)	0.4 (0.1–1.0)	0.3 (0.1–0.4)
10	28.0	*n*-Octacosane	0.8 (0.5–1.1)	0.5 (0.2–0.6)	0.5 (0.3–0.6)
11	28.9	Nonacosene	0.2 (0.0–0.5)	0.2 (0.04–0.4)	0.6 (0.4–0.8)
12	29.0	*n*-Nonacosane	5.6 (3.8–7.8)	3.0 (2.4–4.1)	3.0 (2.4–3.6)
13	29.3	9-, 11-, 13-, and 15-Methylnonacosane	5.0 (3.6–6.5)	2.9 (2.0–3.5)	1.3 (0.9–2.4)
14	30.9	Hentriacontene	0.6 (0.0–1.8)	2.0 (1.5–3.0)	5.9 (4.8–7.0)
15	31.0	*n*-Hentriacontane	1.0 (0.7–2.0)	0.8 (0.5–1.1)	1.1 (0.8–1.2)
16	31.3	9-, 11-, 13-, and 15-Methylhentriacontane	8.7 (6.2–11.2)	6.0 (3.7–11.2)	3.7 (2.4–8.7)
17	31.6	9,13-, 11,15-, and 13,17-Dimethylhentriacontane	1.8 (0.0–3.9)	3.3 (0.1–11.2)	3.6 (0.1–20.2)
18	32.9	Tritriacontene	1.3 (0.0–3.1)	4.0 (2.0–5.8)	11.6 (7.7–14.2)
19	33.0	*n*-Tritriacontane	0.5 (0.0–1.4)	0.6 (0.0–1.5)	0.5 (0.0–1.1)
20	33.3	9-, 11-, 13-, 15-, and 17-Methyltritriacontane	5.3 (0.0–9.0)	4.0 (1.7–5.3)	2.5 (1.9–3.1)
21	33.6	13,17- and 15,19-Dimethyltritriacontane	6.1 (2.9–8.4)	6.3 (2.0–8.8)	5.0 (4.5–6.1)
22	34.9	Pentatriacontene	0.1 (0.0–0.5)	0.4 (0.0–0.6)	1.2 (0.3–1.7)
23	35.3	11-, 13-, 15-, and 17-Methylpentatriacontane	2.7 (0.0–6.3)	3.2 (0.8–5.2)	1.4 (0.1–2.5)
24	35.6	11,15- and 13,17-Dimethylpentatriacontane	8.3 (1.3–12.4)	10.6 (0.0–13.2)	10.0 (0.5–12.9)
25	35.9	13,17,21-Trimethylpentatriacontane	0.4 (0.0–1.6)	1.0 (0.0–2.2)	1.8 (0.1–3.4)

[a] Peak numbers correspond to those in Figure 1.
[b] Equivalent chain length.
[c] Components are only listed if their presence was confirmed by mass spectral analysis.
[d] Values in parentheses are the lowest and highest for the seven queens.
[e] Values in parentheses are the lowest and highest colony averages.

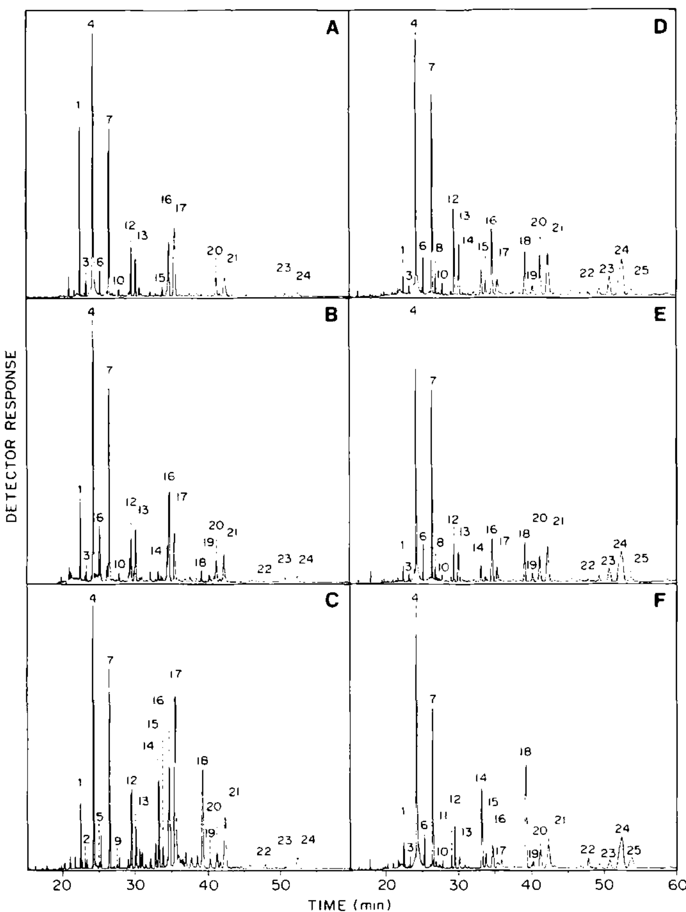

FIG. 1. Total ion chromatograms of the cuticular lipids of an individual *Polistes metricus*: (A) queen (B) worker, and (C) male from colony 4; and (D) queen, (E) worker, and (F) male from colony 5. Numbered components are identified in Table 1.

between-class separation for the queens and workers of individual colonies did not improve, males did group together relative to the queens and workers.

In order to identify the components responsible for separation of the males, a stepwise discriminant analysis was done. Six of the cuticular lipid components, including pentacosene and hentriacontene, can account for separation between male and female wasps (Table 3). When the proportions of these six compounds were given as the variables in the SAS classificatory discriminant analysis, 100%

TABLE 2. MEAN PERCENT COMPOSITION BY CLASS OF CUTICULAR LIPID COMPONENTS OF QUEENS, WORKERS, AND MALES FROM SEVEN *Polistes metricus* COLONIES

Class of component	Queens (N = 7)	Workers (N = 32)	Males (N = 26)
n-Alkanes	42.7	40.1	38.0
Alkenes	2.3	7.7	20.5
Methylalkanes	23.9	17.9	9.9
Dimethylalkanes	16.2	20.2	18.6
Trimethylalkanes	0.4	1.0	1.8
Aldehydes	0.8	1.5	2.1

of the *P. metricus* males and 100% of the females were correctly classified according to gender.

Further canonical discriminant analysis was done using colony as the classification variable for a subset of the data containing only workers and queens from the seven colonies. In this analysis, the queens clustered with their respective workers according to colony (Figure 3A), although there was not a clear separation between individuals in colonies 3 and 5. Stepwise discriminant analysis showed that 12 of the 25 cuticular lipid components were needed in order to classify the queens and workers by colony. These 12 compounds included four *n*-alkanes and both of the aldehydes (Table 3). SAS classificatory discriminant analysis using these 12 components resulted in correct classification of all wasps, except one individual: a worker from colony 5 that was classified as a wasp from colony 6.

When the queens were removed from the canonical analysis and the *P. metricus* workers were analyzed alone, there was good separation between colonies (Figure 3B). Workers from colonies 2, 3, 5, and 6 were relatively close to one another, but they clustered well according to colony. Stepwise discriminant analysis indicated that this separation was due to six compounds, three of which were methyl-branched hydrocarbons (Table 3). Classificatory discriminant analysis using these six components classified 100% of the workers correctly by colony.

A canonical discriminant analysis was run excluding all workers to determine what kind of clustering occurred using colony as the classification variable for *P. metricus* queens and males (Figure 4A). This analysis indicated that males and their respective queens separated clearly by colony. Some of the males (indicated by the isolated numbers) were so similar within a colony that they were superimposed on the plot. Stepwise discriminant analysis showed that nine of the cuticular lipid components accounted for this separation (Table 3). Seven of these compounds were the same as those needed to separate the queens and

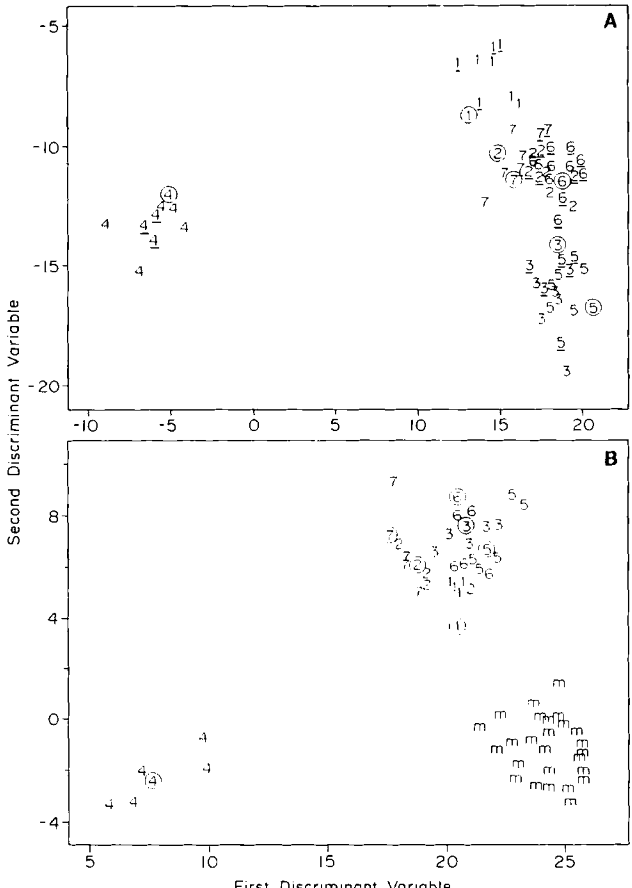

FIG. 2. Plot of the first two discriminant variables from canonical discriminant analyses of the cuticular lipids from seven colonies (1–7) of *Polistes metricus* adults: (A) queens (circled), workers, and males (underlined) grouped according to colony; and (B) queens (circled) and workers grouped according to colony and males (m) grouped according to gender.

workers by colony. Classificatory discriminant analysis using these nine components classified 100% of the males and queens correctly by colony.

Canonical discriminant analysis done on male *P. metricus* wasps only showed distinct separation by colony (Figure 4B). Within each colony the males were so similar that they plotted directly on top of one another. There was also much better relative separation between the seven colonies in this analysis than in any of the previous canonical plots. In the stepwise discriminant analysis, six

TABLE 3. DISCRIMINATING COMPONENTS FOR MALES, QUEENS, AND WORKERS OF *Polistes metricus* USING STEPWISE DISCRIMINANT ANALYSIS

Peak	Component[a]	Males from females	Queens, workers by colony	Workers only by colony	Queens, males by colony	Males only by colony
1	n-C_{23}	*	*		*	
2	Heneicosanal	*	*		*	*
3	$C_{25:1}$	*	*			*
4	n-C_{25}				*	
5	Tricosanal		*	*		
6	n-C_{26}		*			*
7	n-C_{27}			*		*
8	Methyl-C_{27}			*		*
9	3-Methyl-C_{27}		*		*	
10	n-C_{28}		*		*	
11	$C_{29:1}$					
12	n-C_{29}					
13	Methyl-C_{29}					
14	$C_{31:1}$	*				
15	n-C_{31}	*				
16	Methyl-C_{31}					
17	Dimethyl-C_{31}					
18	$C_{33:1}$					
19	n-C_{33}		*	*	*	*
20	Methyl-C_{33}					
21	Dimethyl-C_{33}		*	*	*	
22	$C_{35:1}$		*			
23	Methyl-C_{35}				*	
24	Dimethyl-C_{35}		*	*	*	
25	Trimethyl-C_{35}	*	*			
Total components for discrimination		6	12	6	9	6

[a] Full names of components are listed in Table 1.

components accounted for the separation of males by colony, three of which were n-alkanes (Table 3). Classificatory discriminant analysis using these six components classified 100% of the males correctly by colony.

DISCUSSION

The results of the discriminant analyses of the cuticular lipid compositions of the *P. metricus* queens, workers, and males suggest three important points: (1) the cuticular hydrocarbon profiles of queens and workers within colonies can

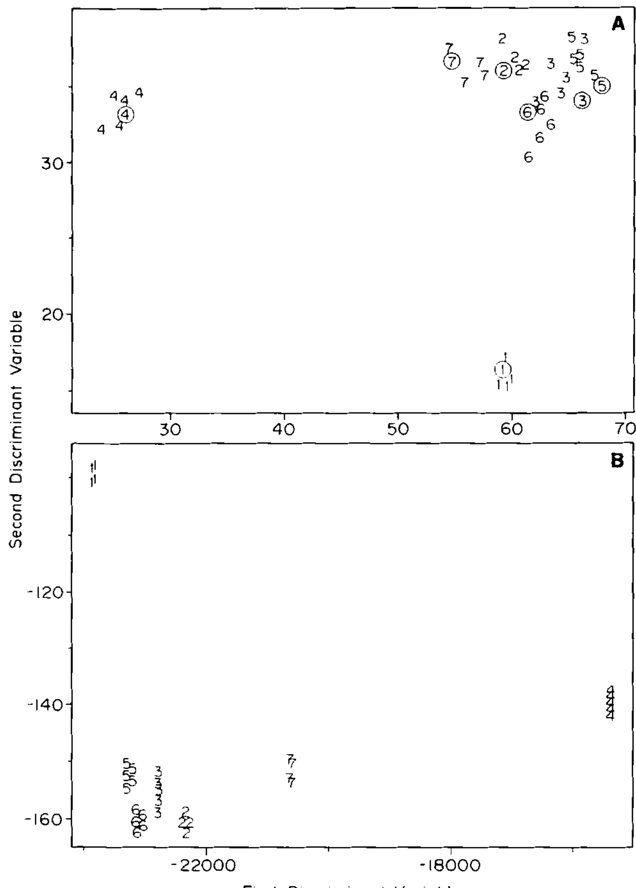

FIG. 3. Plot of the first two discriminant variables from canonical discriminant analyses of the cuticular lipids from seven colonies (1–7) of *Polistes metricus* queens and workers: (A) queens (circled) and workers grouped according to colony; and (B) workers grouped according to colony.

be used to distinguish them from queens and workers of other colonies; (2) male *P. metricus* cuticular hydrocarbon compositions distinguish them from female *P. metricus*; and (3) the cuticular hydrocarbon compositions of males from a given colony can be used to distinguish these wasps from males of other colonies.

Our findings that *P. metricus* queens and workers cluster according to colony in discriminant analyses based on the cuticular lipid profiles (Figure 2A)

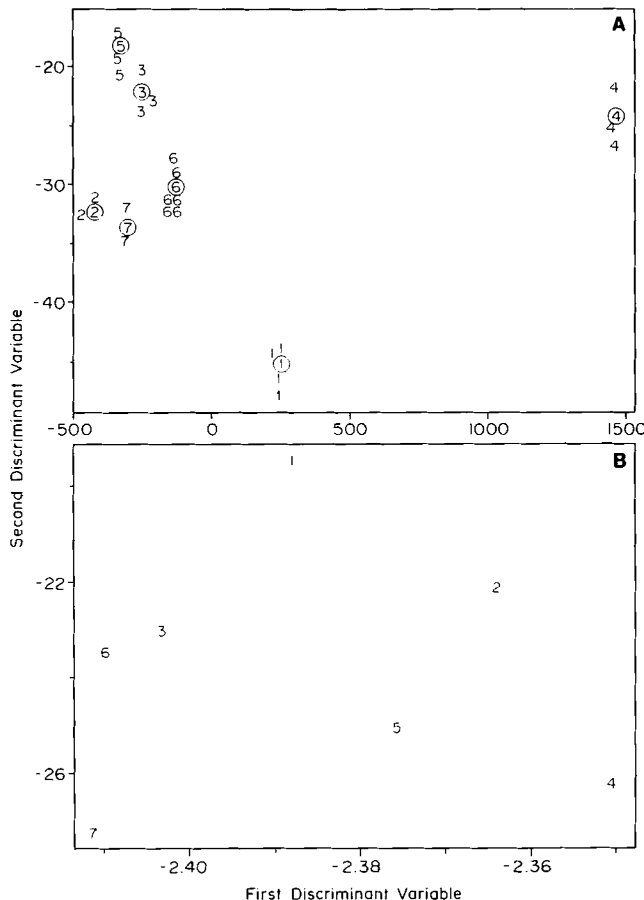

FIG. 4. Plot of the first two discriminant variables from canonical discriminant analyses of the cuticular lipids from seven colonies (1-7) of *Polistes metricus* queens and males: (A) queens (circled) and males grouped according to colony; and (B) males grouped according to colony.

supports previous results with *P. metricus* (Espelie et al., 1990), *P. dominulus* (Bonavita-Cougourdon et al., 1991), *P. exclamans* (Singer et al., 1992a), *P. fuscatus* (Espelie et al., 1994), and *Dolichovespula maculata* (Butts et al., 1993). In each of these previous studies, methyl-branched hydrocarbons were important components in the analyses that discriminated wasps according to their colonies. For instance, the three cuticular hydrocarbons that were most important in assigning *P. fuscatus* wasps to their respective colonies were 13-

and 15-methylhentriacontane, 11,15- and 13,17-dimethylhentriacontane, and 13-, 15-, and 17-methyltritriacontane (Espelie et al., 1994). Five of the methyl-branched cuticular hydrocarbons of *P. metricus* were shown by stepwise discriminant analysis to be important in the separation by colony of queens and workers and workers only (Table 3). Two of these hydrocarbons, 13,17- and 15-19-dimethyltritriacontane and 11,15- and 13,17-dimethylpentatriacontane, were necessary for both of these separations and are very similar in structure to the compounds that were most heavily weighted in the analysis of *P. fuscatus* wasps. The stereochemistry provided by a midchain or terminal methyl moiety may result in methyl-branched hydrocarbons being important for the ability of social wasps to recognize their nestmates. However, there is currently very little information available regarding the importance of the stereochemistry of long-chain methyl-branched hydrocarbons for insect chemical communication (Howard, 1993).

Although the cuticular hydrocarbon compositions of the *P. metricus* workers and queens that were obtained in this study (Figure 1) were very similar to those previously reported for *P. metricus* females (Espelie et al., 1990), the aldehydes, heneicosanal and tricosanal, that were found in the cuticular lipids of *P. metricus* (Table 1) have not previously been identified in the cuticular lipids of social wasps. Long-chain aldehydes (C_{20}–C_{32}) are relatively rare in insect cuticular lipids, and when they are present, the more dominant components usually have an even number of carbons (Blomquist and Dillwith, 1985; Buckner, 1993).

A novel aspect of this study involved the identification and analysis of the cuticular lipid components of *P. metricus* males. The GC-MS profiles of the male wasps are distinct from those of queens and workers due to larger amounts of the alkenes, hentriacontene and tritriacontene (Figure 1, peaks 14 and 18), and lower amounts of methyl-branched hydrocarbons (Table 2). Stepwise analysis selected six components that were necessary to distinguish males from females. These components were hentriacontene, *n*-hentriacontane, 13,17,21-trimethylpentatriacontane, heneicosanal, *n*-tricosane, and pentacosene. The amount of tritriacontene was significantly lower in the males of colony 4 than it was in the males from the other six colonies. When the males from colony 4 were excluded from the analysis, tritriacontene was selected by stepwise analysis as an important component for the discrimination between male and female wasps. Both the male and female wasps in colony 4 had large amounts of 9,13-, 11,15-, and 13,17-dimethylhentriacontane (peak 17, Figure 1), and this difference in cuticular lipid composition may explain the distinct separation of these wasps from those of other colonies in the discriminant analyses (Figures 2–4).

Alkenes have previously been reported to be minor components in the cuticular lipids of *Polistes* queens, workers, and gynes. Of the several species that have been examined, *P. fuscatus* had the highest proportion (4%) of alkenes

(Espelie et al., 1994). Males of *P. major major* and *P. major castaneicolor* have high proportions of alkenes in their cuticular hydrocarbons (25 and 48%, respectively), while the workers have low percentages of alkenes (8 and 0%, respectively) (Layton, Singer and Espelie, in preparation). The large difference in alkene composition of male and female *Polistes* suggests that these compounds may play a role as contact sex pheromones, as has been shown for other insects (Blomquist et al., 1987; Howard, 1993). It is possible that the alkenes are not part of the cuticular lipids of these wasps, but that they are glandular products produced only (or primarily) by males. The low percentage of alkenes previously reported for *Polistes* females may be due to the fact that the wasps that were analyzed were collected from colonies that did not have adult males. The alkenes found on the *P. metricus* females in this study may have been acquired from their male nestmates.

An interesting finding of this study was that the cuticular lipid profiles of *P. metricus* males of one colony are distinct from those of males of other colonies (Figure 4B). These differences may be very important for the mating strategy of *Polistes*. Ryan and Gamboa (1986) showed that *P. fuscatus* wasps were able to recognize nestmates of the opposite gender and that females, which had not previously been exposed to males, were more likely to mate with non-nestmates than with nestmates. It may be that one group of cuticular compounds allows wasps to recognize female nestmates (e.g., the six components in Table 3 that separate *P. metricus* workers by colony), while a different group of these cuticular lipids is used for recognition of males. However, it would be premature to draw conclusions about the importance of specific cuticular compounds based solely on the mathematical modeling of the discriminant analyses presented here. Future work will involve behavior tests to determine the relative importance of these different cuticular lipids for the recognition of males and females by *Polistes* wasps.

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SEX PHEROMONE-MEDIATED FLIGHT AND LANDING BEHAVIORS OF THE EUROPEAN CORN BORER, *Ostrinia nubilalis* (Hübner).

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Abstract—The pheromone-mediated flight and landing behaviors of male *Ostrinia nubilalis* were studied in a wind tunnel. The pheromone source was placed in the middle of an 18 × 18-cm horizontal surface, and a smaller surface placed 4, 18, or 36 cm downwind. The smaller surface did not appear to affect significantly the flight tracks or position of landing of males on the upwind surface, and it allowed the positions and altitudes of males as they passed over the downwind surface to be estimated. The flight altitude and position of males as they passed over the downwind surface related to where males landed on the upwind surface. Regardless of the downwind position of the downwind surface, most males flew over its center (i.e., in line with the source) and landed in line with the source on the upwind surface. When a small 2.5 × 10-cm vertical object was placed on the upwind surface, just upwind and to one side of the source, males flew over the downwind surface in positions skewed toward the vertical object and in broader distributions than for the comparable situation without an object; males landed on the upwind surface on positions skewed toward, or on, the object and with a broader distribution (laterally). Flight altitude also corresponded with landing position. Thus, when there was no vertical object, most males flew just above the downwind surface and landed on the downwind edge of the upwind surface. In contrast, with the vertical object, males flew significantly higher and tended to land past the downwind edge of the upwind surface. With a taller object (20 cm), males flew even higher, past the downwind edge and most landed on the vertical object. These data show the close relationship between

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flight and landing behaviors of male *O. nubilalis* and suggest that flight maneuvers that determine track and altitude largely govern where a male lands.

Key Words—Pheromone, orientation, visual cues, plume structure, *Ostrinia nubilalis* Lepidoptera, Pyralidae.

INTRODUCTION

Studies on several species of moths have shown that males of these species fly in a sex pheromone plume using two behavioral mechanisms, optomotor anemotaxis (the use of visual information for heading and groundspeed to steer a resultant track upwind), and an internal program of self-steered counterturns. The combination of these two behavioral mechanisms during pheromone-mediated flight is thought to shape the commonly observed zigzag-shaped flight tracks of male moths flying upwind toward a pheromone source (Baker, 1989).

As a male moth approaches a sex pheromone source, he flies with relatively small lateral displacement (i.e., small intertrack reversal distances, in the sense of Kuenen and Baker, 1982) and relatively (compared to flight farther downwind) slow net upwind velocity, followed by apparently controlled landing on the source or surface that the source is on (see for example, Foster and Harris, 1992). In an early study on male *Pectinophora gossypiella* (Saunders), Farkas et al. (1974) concluded that pheromone-mediated landing was elicited by a combination of pheromonal concentration and visual stimuli. Recent work on the pheromone-mediated landing behavior of male *Epiphyas postvittana* (Walker) has shown that visual stimuli of a surface on which the pheromone source is located influences the position on the surface where the male lands (Foster and Harris, 1992).

The European corn borer, *Ostrinia nubilalis* (Hübner), is a major pest of corn and other crops in Europe and North America. It has been the subject of much study on sex pheromones, first, because of its pest status and a desire to find better ways of controlling it, and, second, because two biotypes or so-called "strains" that use distinct sex pheromone blends have been identified within the described entity. One strain uses a blend of (*Z*)-11-tetradecenyl acetate (*Z*11–14:OAc) and (*E*)-11-tetradecenyl acetate (*E*11–14:OAc) in a ratio of roughly 97:3, respectively (so-called Z strain) (Klun et al., 1973), while the other uses the same chemicals in the opposite ratio of 3:97 (so-called E strain) (Kochansky et al., 1975). These two strains hybridize readily in the laboratory, and consequently have been the subject of a number of studies investigating the genetic basis of pheromone production, perception, and response (e.g., Klun and Maini, 1979; Roelofs et al., 1987; Klun and Huettel, 1988; Löfstedt et al., 1989).

As part of our investigations into the pheromone-mediated behavior of

moths, we have studied the landing behavior of *O. nubilalis*. In this paper we report on this work and particularly the relationship between where a male lands and its preceding flight track.

METHODS AND MATERIALS

Insects. Male *O. nubilalis* of the Z strain were supplied as pupae by Dr. Aupinel, INRA, Laboratoire de Lutte Biologique, Le Magneraud, France, and stored at 21 ± 1°C under a L16:8D photoperiod. Adults were collected daily and placed in plastic containers along with a 10% sugar solution absorbed onto cotton wool, under the same light and temperature conditions as the pupae. The moths were used in the wind tunnel experiments when two to five days old.

Wind-Tunnel Protocol. Males were flown in a 1.5-m-long × 0.8-m-wide × 0.45-m high wind tunnel based on the design of Miller and Roelofs (1978). The wind was pushed through the tunnel by a 30-cm-diameter desktop fan, at a velocity of 25 cm/sec. The tunnel was illuminated by four high-frequency fluorescent light tubes (Philips, Netherlands, model TLD 80 HF). The tubes were housed in two boxes with red-painted plastic covers. The light level inside the tunnel was dimmed to approximately 0.5 lux.

Male *O. nubilalis* were released, individually, into the tunnel between the third and fifth hours of the scotophase. The pheromone source consisted of a 97 μg:3 μg mixture of *Z*11-14:OAc and *E*11-14:OAc loaded on to a rubber septum (A.H. Thomas Co., Philadelphia, Pennsylvania). The chemicals were available from the laboratory. The ratio of the two components in the mixture was confirmed by capillary gas chromatography. In each experiment, the pheromone source was placed in the middle of an 18 × 18-cm horizontal, white cardboard (3 mm thick) surface. The surface was divided into a grid of 2 × 2-cm cells. The cell coordinates along the axis of the wind (hereafter referred to as rows) were labeled from A (being the most downwind) to I (the most upwind). The cell coordinates horizontally perpendicular to the axis of the wind (hereafter referred to as columns) were labeled from 1 (being to the right as one looked upwind in the tunnel) to 9 (to the left). Thus, according to this labeling scheme, the source was located in cell E5. The horizontal surface was placed atop a 10-cm-high, four-legged wire stand located in the middle, at the upwind end, of the tunnel.

Seven wind-tunnel experiments were conducted. At least 30 males were flown to each treatment for each of the experiments in which the landing positions of males were determined.

Experiment 1. The responses of males to two treatments were compared: (1) an 18 × 18-cm horizontal surface, and (2) the same surface with a 2.5-cm-wide × 10-cm-high vertical object placed at position G7 (i.e., upwind, and to

the side, of the pheromone source). The vertical object was constructed of the same white cardboard as the surface, and it was positioned on the surface so that the two sides of greatest area were facing upwind and downwind. The initial landing position and the time from initiation of flight until the male landed or flew past the downwind edge of the surface were recorded for both treatments. It was usually clear if a male landed on the surface, as he usually began walking toward the source or paused briefly (<0.5 sec) before walking toward the source or taking flight again. However, in a few cases it was not so apparent whether the male had landed. In these cases, landing was considered as the extension of the legs and contact of the tarsi with the surface for greater than approximately 0.25 sec.

Experiments 2 and 3. In addition to the standard 18 × 18-cm horizontal surface on which the source was located, an 18-cm-wide × 4 cm-long (length along the direction of the wind) surface was positioned 4, 18 (both in experiment 2), or 36 cm (experiment 3) downwind of, and in line with, the larger surface (refer also to Figures 2 and 3 below for the experimental layout). Males were flown to these surfaces either with or without a 2.5 × 10-cm vertical object at position G7 on the upwind (larger) surface (i.e., totals of four and two treatments, respectively, for the two experiments). In both experiments, the initial landing position on the upwind (larger) surface was recorded, as was the column number (divided as for the larger surface) that the male initially flew over (or landed) on the downwind surface. If the male flew to one side of the downwind surface (as frequently happened when vertical objects were employed on the upwind surface), then the column position was noted as one of the extreme positions, 1 or 9 (in practice, this only occurred to the side on which the object was located; i.e., in these cases the column position was noted as 9). For experiment 2, the altitude that the male flew over the downwind surface was estimated as either <2 cm or ≥2 cm above the surface. In experiment 3, three categories of flight altitude past the downwind surface were estimated: within (either over or under) 2 cm of the surface, ≥2 cm above the surface, and ≥5 cm above the surface. A small, upright, marked wire on one edge of the surface aided these estimations.

Additionally, in experiment 3, black and white video recordings (using a COHU CCD 4722-5000 camera with a 8 mm, F 1.3 lens) were made overlooking the tunnel floor (i.e., a plan view) of males flying to (1) the two-surface experimental design (i.e., a smaller surface 36 cm downwind of a larger surface), and (2) an 18 × 18-cm horizontal surface. The position of the moth in every second frame of the video recording (i.e., every 2/25 sec), from 10 cm downwind of the downwind edge to 1 cm past the upwind edge of the smaller surface (or over the same region for the treatment without the smaller surface), was transcribed onto clear Mylar placed over a 22-cm black and white video screen. The flight tracks of 10 males to each of the two treatments were recorded.

Experiment 4. The landing positions on a 18 × 18-cm horizontal surface were recorded for two treatments: (1) the surface with a 2.5 × 10-cm vertical object, and another surface with a 2.5 × 20-cm vertical object. In both treatments the object was positioned at cell G7. The objects were divided into 5-cm regions up their height (i.e., the 10-cm object had two regions and the 20-cm object had four regions). If a male landed on an object, the region in which it landed was noted (numbered, with the bottom region, 1, and the top region either 2 or 4).

Experiment 5. A black and white video camera, positioned on one side of the tunnel at the same height as the pheromone source (i.e., 10 cm above the floor of the tunnel), was used to record the changes in altitude of males from 35 cm downwind of the edge of the surface. The flight tracks of six males to each of (1) a horizontal surface, (2) a horizontal surface with a 10-cm vertical object at cell G7, and (3) a horizontal surface with a 20-cm vertical object at cell G7, were recorded. The position of males every two frames was transcribed onto Mylar as in experiment 3.

Experiment 6. The positions that males landed on a horizontal surface with either a black or a white 2.5 × 10-cm vertical object at position G7 were recorded as for the previous experiments. The black object was prepared by wrapping matt black electrical tape around the cardboard.

Experiment 7. The behavioral responses of males to three treatments were compared: (1) a horizontal surface, (2) a surface with a 2.5 × 20-cm-high white vertical object on the floor of the tunnel (i.e., 10 cm above the surface) and just upwind of the surface in row 7 (i.e., just upwind of cell I7), and (3) a similar array as for (2) except the 20-cm-high object was 10 cm upwind of the surface, in line with column 7 (refer figure 7, for the experimental layout). The landing positions of the males, whether males flew beyond (upwind of) the source, and whether males flew beyond the upwind edge of the surface, were recorded.

Visualization of Pheromone Plume. The direction and apparent structure of the pheromone plume was visualized using smoking incense. The incense was placed on the surface and the smoke plume recorded onto video tape for later comparison.

Statistical Analyses. Analyses of landing positions on the upwind surface in the various experiments were conducted as previously described (Foster and Harris, 1992). Briefly, the total number of insects that landed in each row or column were summed, and the respective distributions of insects in rows or columns for each treatment were compared by χ^2 tests. Distributions of males landing on or passing over the columns of the downwind surface (in experiments 2 and 3) were also compared by χ^2 tests.

For the video data of experiment 3, each frame was divided into columns whose axis was horizontally perpendicular to the wind direction (i.e., each column was aligned with the wind direction, and ran the same direction as the

columns on the surfaces). The amount of time a male spent in each column was determined, and the distributions of the times across the columns, for the two treatments (i.e., with or without a smaller, downwind surface), were compared by a χ^2 test.

Mean times of flight in experiments 1 and 4 were compared by a one-way ANOVA. For the altitude data in experiment 4, the region from the downwind edge of the surface to 35 cm downwind was split into five 7-cm-long zones: zone 1 being from the edge to <7 cm downwind, zone 2 from 7 to <14 cm, and so on. The mean altitudes and SEM for the three treatments were computed in each zone. Mean altitudes were considered different if they differed by >2 SEM. Unless otherwise stated, all differences are reported to $P < 0.05$.

RESULTS

In all experiments, high percentages of males ($>85\%$) flew upwind and landed on the (upwind) surface. There were no significant differences in the proportions of males that landed on the upwind surface between any of the treatments for any experiment. For this reason, these data are not reported or discussed further. The following data concern only the males that landed on the upwind surface (n.b., in experiments 2 and 3, all males that landed on the downwind surface, took flight again, and landed on the upwind surface).

Experiment 1. In response to the pheromone source on the horizontal surface, a high proportion (28/39, 71.8%) of *O. nubilalis* males landed on the downwind row of the surface (Figure 1A); most of these males landed in line (i.e., in the same column) with the pheromone source (e.g., 66.7% of all the males landed in cell A5). Ten (25.6%) of the males landed on the pheromone source. When the vertical object was present on the surface, few (5/38; 13.2%) males landed at the downwind row (Fig. 1b). Instead, the greatest proportions of the males landed either on the pheromone source (18/38; 47.4%) or on the vertical object (12/38; 31.6%). Consequently, the distributions of landing positions in both rows and columns were significantly different ($P < 0.001$) between the two treatments.

The times of flight from takeoff to landing, or passing the upwind edge of the surface, were recorded for 12 males to each of the treatments. The time of flight for males to the surface with the vertical object (mean of 7.9 ± 1.7 sec) was significantly less than that for males to the horizontal surface (mean of 13.1 ± 1.7 sec).

Experiment 2. Ten of 28 (35.7%) males landed on the downwind surface when it was 4-cm away from the upwind surface with no vertical object. Nine of these males landed in column 5 of the downwind surface (i.e., in line with the source), while the other landed in column 6 (Figure 2A). In contrast, only

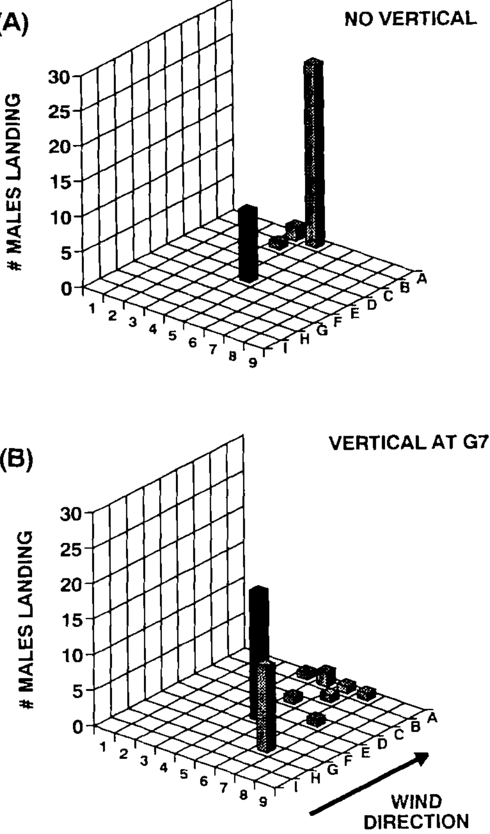

FIG. 1. Distribution of landing positions of male *Ostrinia nubilalis* on (A) an 18 × 18-cm horizontal surface, and (B) an 18 × 18-cm horizontal surface with a 2.5 × 10 cm (width × height) vertical object at position G7. The position of the pheromone is indicated by the cell with the all-black column. Males that landed on the object (for B) have been plotted in the cell position in which the object was positioned (i.e., G7).

one of 26 (3.8%) of the males landed on the downwind surface when the two surfaces were 18 cm apart (with no vertical object). This male also landed in column 5 (Figure 2C). The proportions of males that landed on the downwind surface were significantly different between the two treatments ($P < 0.005$). When the vertical object was present on the upwind surface, none of 27 and one of 29; (3.4%) males landed on the downwind surface when it was, respectively, 4 and 18 cm away from the upwind surface (Figure 2B, D). The male that landed on the downwind surface did so on column 9.

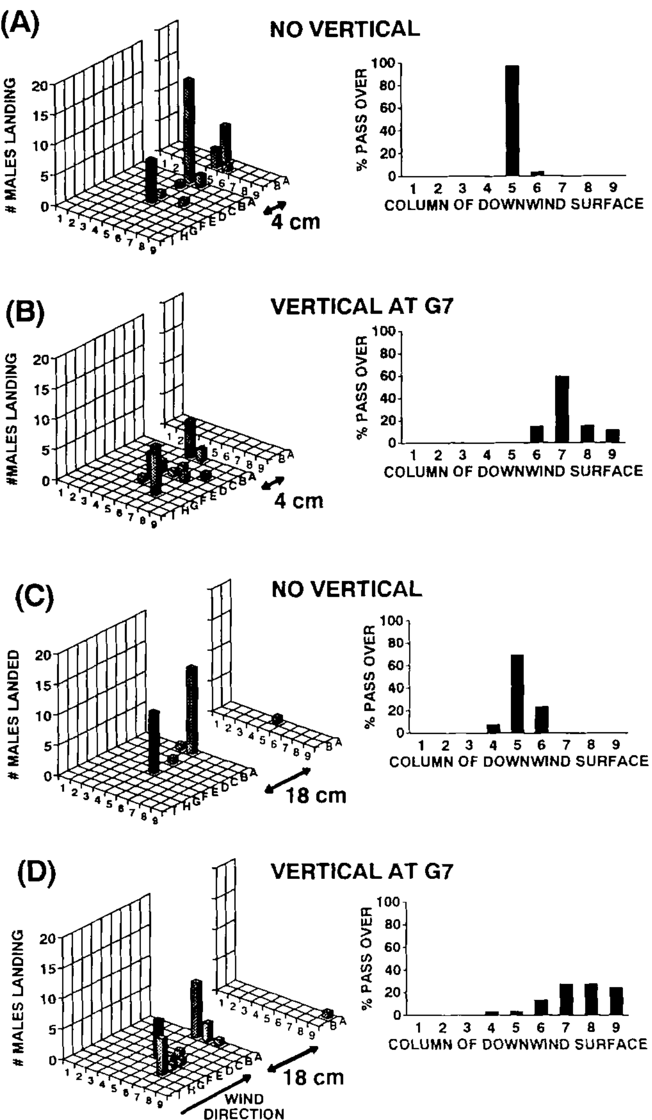

FIG. 2. Distribution of landing positions of male *Ostrinia nubilalis* on an 18 × 18-cm horizontal surface (upwind) and an 18 × 4-cm surface (downwind), and the distribution of column positions that males either landed on or passed over the downwind edge of the downwind surface when (A) the two surfaces were separated by 4 cm, (B) the two surfaces were separated by 4 cm and a 2.5 × 10-cm vertical object was positioned on the upwind surface at cell G7, (C) the two surfaces were separated by 18 cm, and (D) the two surfaces were separated by 18 cm and a 2.5 × 10-cm vertical object was positioned on the upwind surface at cell G7. The position of the pheromone is indicated by the cell with the all black column. Males that landed on the object (for B and D) have been plotted in the cell position in which the object was positioned (i.e., G7).

The distributions of where males passed over or landed on the downwind surface are also shown in Figure 2. When the vertical object was absent, all the males passed over or landed on the downwind surface within one column position of column 5, regardless of whether the downwind surface was 4 or 18 cm from the upwind surface. It should be noted, however, that a significantly greater proportion of males flew over or landed on the columns either side (i.e., columns 4 or 6) of column 5, when the downwind surface was at the greater distance (Figure 2A, C). When the vertical object was present, males passed over or landed (in one instance) on the downwind surface in columns highly skewed towards the side the object was on (Figure 2B, D). The distributions of these figures were significantly ($P < 0.001$) different from the distributions for the respective treatments without a vertical object. There was, however, no significant difference between the distributions of positions for the two treatments with vertical objects.

With the vertical object on the upwind surface, all but one of the males flew at a relatively high altitude (>2 cm) above the surface 18-cm downwind. For the corresponding treatment without a vertical object, all the males flew just above (<2 cm) the level of the downwind surface.

The distributions of landing positions on the upwind surface for both treatments without a vertical object were not different and were similar to that observed on the plain surface in experiment 1. That is, the majority of males landed on the downwind row, in line with the pheromone source; most of the remainder of the males landed on the pheromone source (Figure 2A, C). The distributions of landing positions, with regard to both rows and columns, on the upwind surface for the two treatments with the object were significantly different from the similar treatments without the object. In particular, males tended to land on the left-hand side (looking upwind) of the surface (i.e., the side of the surface that the object was on), and also tended to land farther upwind (Figure 2B, D). The latter effect is largely a reflection of the high percentages of males that landed on the vertical object (29.6% and 20.7%, respectively, for the 4-cm and 18-cm gaps between the two surfaces).

Experiment 3. No males landed on the downwind surface when the two surfaces were separated by 36 cm, regardless of whether there was a vertical object on the upwind surface or not (Figure 3). When there was no vertical object, most (24/33; 72.7%) males flew over the downwind surface at a relatively low altitude (within 2 cm of the level of the surface). Of these 24 moths, six flew under the surface. The remaining nine moths flew at an altitude between 2 and 5 cm above the level of the downwind surface. In contrast, when the vertical object was on the upwind surface, only one of 28 (3.6%) males flew within 2 cm of the level of downwind surface; this male flew under the surface. Three (10.7%) males flew between 2 and 5 cm above the level of the downwind surface, while 24 (85.7%) males flew at an altitude greater than 5 cm above the

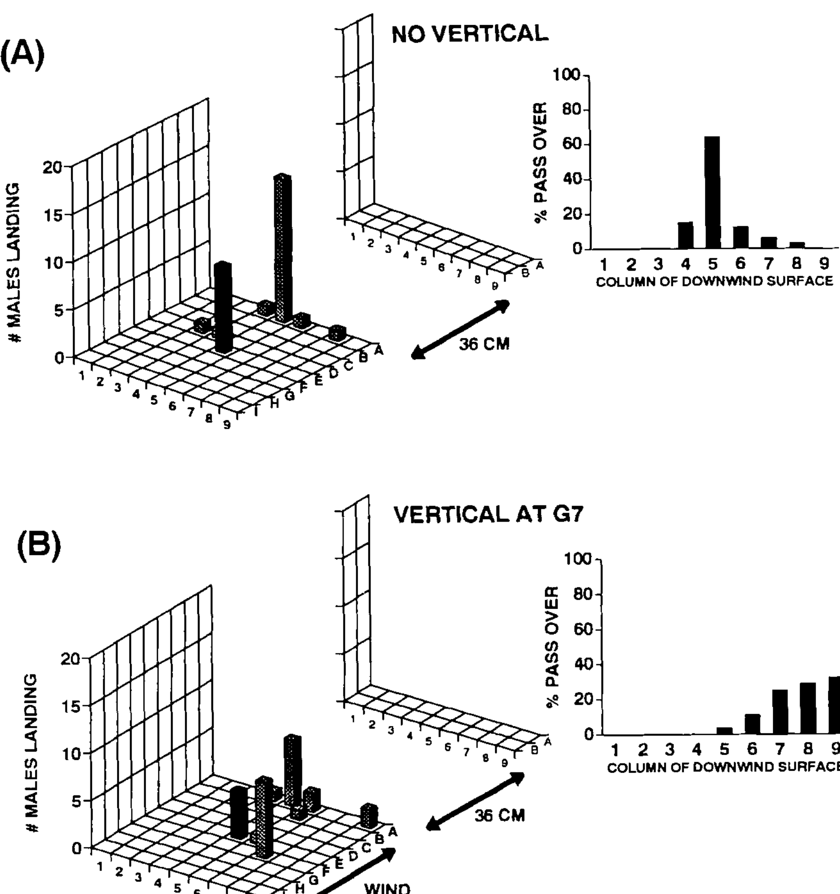

FIG. 3. Distribution of landing positions of male *Ostrinia nubilalis* on an 18 × 18-cm horizontal surface (upwind) and an 18 × 4-cm surface (downwind), and the distribution of column positions that males either landed on or passed over the downwind edge of the downwind surface when (A) the two surfaces were separated by 36 cm, and (B) the two surfaces were separated by 36 cm and a 2.5 × 10-cm vertical object was positioned on the upwind surface at cell G7. The position of the pheromone is indicated by the cell with the all black column. Males that landed on the object (for B) have been plotted in the cell position in which the object was positioned (i.e., G7).

level of the surface. The distributions of altitudes of males as they passed the downwind surface, when the vertical object was present or not, were significantly different ($P < 0.0001$).

The distributions of landing positions on the upwind surface (Figure 3) were similar to that observed in the previous experiments. That is, when there was no vertical object, most (20/31; 64.5%) males landed on the downwind row, and a high proportion (25/31; 83.9%) landed on the same column (5) as the pheromone source; nine of these males landed on the pheromone source. When the vertical object was present, a smaller proportion (12/27; 44.4%) of males landed on the downwind row, six (22.2%) landed on the source, and eight (29.6%) landed on the vertical object. The landing positions were skewed towards the side of the surface that the object was on. The distributions of landing positions were significantly different ($P < 0.001$) in both rows and columns.

The flight tracks (from a plan view) of males that flew over the downwind surface (36 cm downwind of the larger surface without a vertical object) were not different ($P > 0.05$), in either the total time that males flew through this region or in the distribution of time spent in 1-cm lateral columns (i.e., perpendicular to the direction of the wind), from those of males that flew through the same region without a downwind surface. In this region, typically, males exhibited two track reversals regardless of whether there was a downwind surface or not.

Experiment 4. The landing positions on the surfaces with the 10-cm and 20-cm vertical objects are shown in Figure 4. The distributions for both rows and columns were significantly ($P < 0.0001$) different between the two treatments. The most obvious difference was the larger proportion (22/32; 68.8%) of males that landed on the taller vertical object compared to the shorter object (12/32; 37.5%). Conversely, only one male (3.1%) landed on the source for the 20-cm treatment, compared to eight (25.0%) for the 10-cm treatment. For both treatments, most males that landed on the object did so on the bottom 5 cm of the object. Thus, of the 12 males that landed on the 10-cm object, nine landed on the lower 5 cm, while of the 22 males that landed on the 20-cm object, 18 landed on the lowest 5 cm, three landed on the 5 cm region above this, and the other one landed on the next highest 5-cm region.

Experiment 5. The mean altitudes (and SEM) in the five 7-cm zones downwind of the surface are shown for each of the three treatments in Figure 5. In all five zones there were significant differences in altitude between the four treatments. The highest mean altitudes were to the treatment with the 20-cm vertical object and lowest mean altitudes were to the treatment with just the horizontal surface. All treatments showed a decrease in mean altitude the closer males were to the surface (i.e., upwind).

Experiment 6. There was a significant difference in the distribution of land-

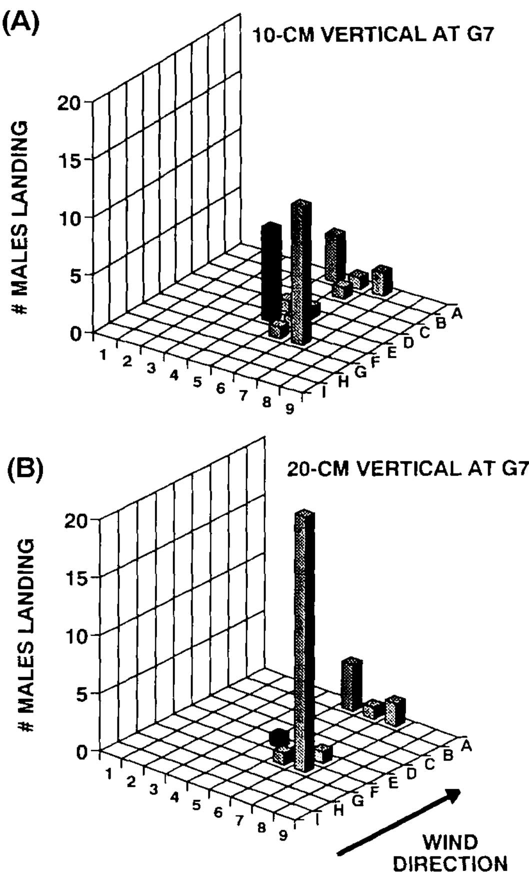

FIG. 4. Distribution of landing positions of male *Ostrinia nubilalis* on (A) an 18 × 18-cm horizontal surface with a 2.5 × 10-cm vertical object at position G7, and (B) an 18 × 18-cm horizontal surface with a 2.5 × 20-cm vertical object at position G7. The position of the pheromone is indicated by the cell with the all-black column. Males that landed on the object have been plotted in the cell position in which the object was positioned (i.e., G7).

ing positions in columns, but not in rows, between the surfaces with a black vertical object and the one with the white object. The most apparent difference between the two treatments was the low proportion of males that landed on the black object (3/36; 8.3%) compared to on the white object (13/36; 36.1%). Moreover, a greater proportion of males landed on the downwind row of the surface with the black object (17/36; 47.2%) compared to the surface with the

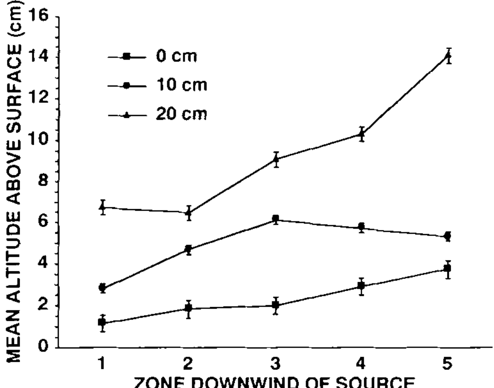

FIG. 5. Mean altitudes (±SEM) of male *Ostrinia nubilalis* in five 7-cm zones downwind of the downwind edge of an 18 × 18-cm horizontal surface (zone 1 is the closest to the surface) to three treatments; the horizontal surface (0 cm), the horizontal surface with a 2.5 × 10-cm vertical object (10 cm), and the horizontal surface with a 2.5 × 20-cm vertical object (20 cm). The pheromone source was in the middle of the surface.

white object (9/36; 25.0%). Similar proportions of males landed on the source for both treatments (Figure 6).

Experiment 7. The distribution of landing positions on the horizontal surface (Figure 7A) and the surface with the vertical object 10 cm upwind of the surface (Figure 7C) were not different. However, the distributions of landing positions on both these surfaces were significantly different from those on the surface with the same object immediately upwind of the surface (at position I7; Figure 7B), with respect to both columns ($P < 0.005$) and rows ($P < 0.001$). This was influenced by the five males that landed on the vertical object when it was immediately upwind of the surface (no males landed on the object when it was 10-cm upwind of the surface) (Figure 7).

Similar proportions of males landed on the source for all three treatments. However, greater proportions of males flew past (upwind of) the source when the vertical object was present (i.e., immediately upwind or 10 cm upwind of the surface; respectively, 79.3% and 53.1% of the males) compared to when it was absent (24.0% of the males). Of the males that flew past the source on the surface with the object immediately upwind, 11 of 18 that flew past the source (61.1%) touched the vertical object, but only five of these landed on the object. No males flew past the upwind edge of the surface for any of the treatments, and therefore no males touched the vertical object when it was 10 cm upwind of the surface.

Visualization of Smoke Plume. The smoke plume emanating from the hor-

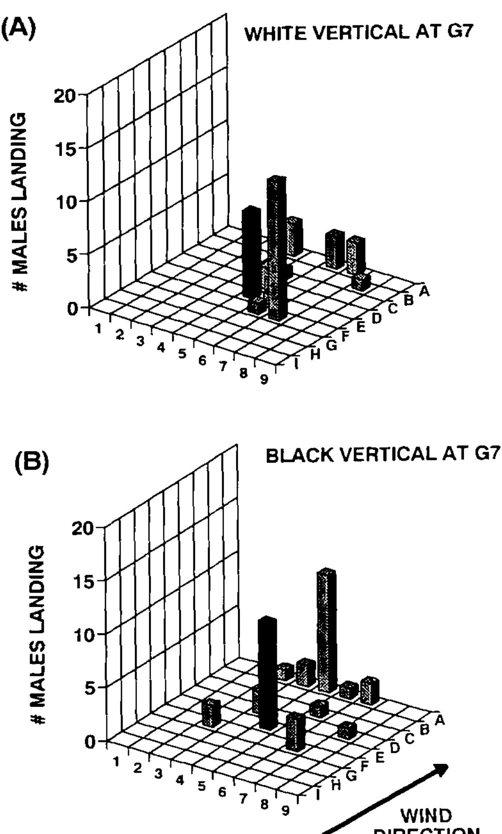

FIG. 6. Distribution of landing positions of male *Ostrinia nubilalis* on (A) an 18 × 18-cm horizontal surface with a white 2.5 × 10-cm vertical object at position G7, and (B) an 18 × 18-cm horizontal surface with a black 2.5 × 10-cm vertical object at position G7. The position of the pheromone is indicated by the cell with the all-black column. Males that landed on the object have been plotted in the cell position in which the object was positioned (i.e., G7).

izontal surface contained several defined wisps of smoke that became slightly more diffuse as they traveled downwind. The plume was centered in the middle of the tunnel but rose slightly as it came off the surface, such that, at about 1 m downwind, the center of the plume was approximately 5–10 cm higher than the level of the surface. When the 10-cm vertical object was placed at position G7, the smoke plume changed substantially. The structure was more diffuse, and the direction of the cloud of smoke, as it traveled downwind, was diverted

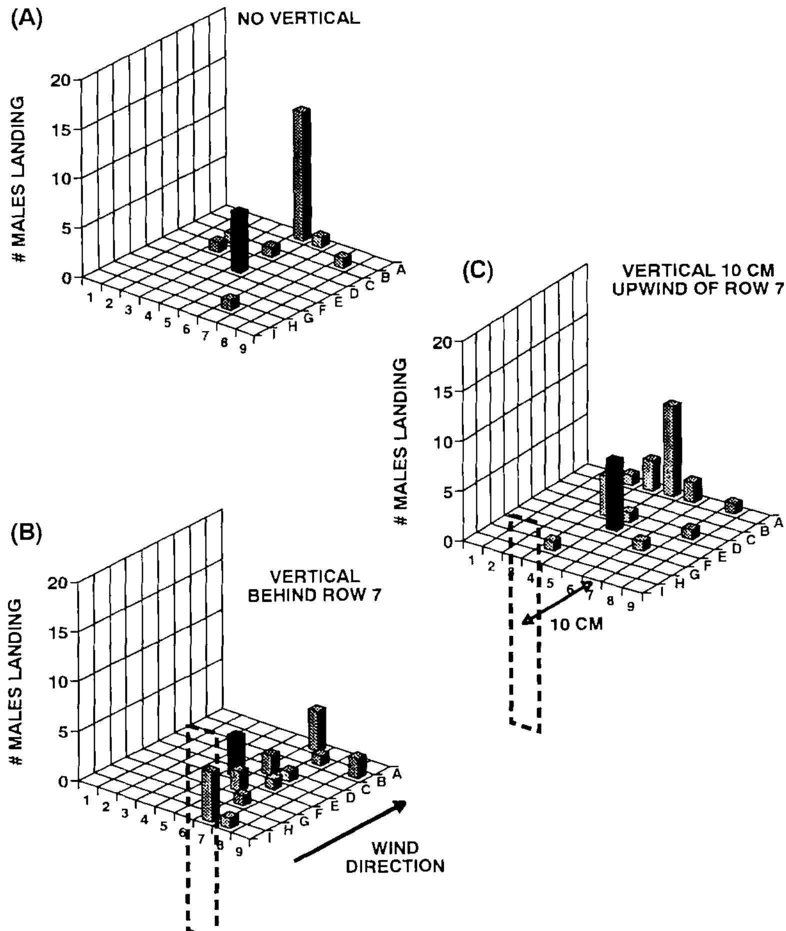

FIG. 7. Distribution of landing positions of male *Ostrinia nubilalis* on (A) an 18 × 18-cm horizontal surface, (B) an '° × 18-cm horizontal surface with a 2.5 × 20-cm vertical object located immediately upwind of column 7 of the surface, and (C) an 18 × 18-cm horizontal surface with a 2.5 × 20-cm vertical object located 10 cm upwind of column 7 of the surface. For all three treatments the surface was 10 cm above the floor of the tunnel (i.e., for B and C, the top of the vertical object was 10 cm higher than the surface). The position of the pheromone is indicated by the cell with the all-black column. Males that landed on the object (for B) have been plotted in the cell position in front of the object (i.e., I7).

slightly toward the left-hand side of the tunnel (i.e., the side of the surface on which the vertical object was positioned). Replacement of the 10-cm object by the 20-cm object did not appear to change further the structure or direction of the plume.

DISCUSSION

In response to a sex pheromone source on a horizontal surface, most male *O. nubilalis* fly upward in a zigzag track typical of pheromone-mediated fight observed in other species of moths (Baker, 1989) and land on the surface or source. To observe the relationship between the flight maneuvers and landing positions of *O. nubilalis*, we employed a simple experimental design in which a small surface was positioned downwind of the larger surface on which the pheromone source was placed. We had previously observed that a similar experimental setup apparently did not influence the flight tracks or landing positions of male *E. postvittana*, with virtually all males landing on the upwind surface (Foster and Harris, 1992). The video recordings of male *O. nubilalis* indicated that the flight tracks of males were not significantly affected by the presence of the downwind surface. Similarly, very few *O. nubilalis* males landed on the small, downwind surface, and the distribution of landing positions on the upwind surface was similar to that observed in the experiment (i.e., experiment 1) in which only the upwind surface was used, suggesting that the downwind surface did not affect where males landed on the upwind surface. The downwind surface was thus able to be used as a reference point for estimating flight position and maneuvers at certain distances from the upwind surface.

The experiments reported here demonstrate the close relationship between the pheromone-mediated flight and landing behaviors of male *O. nubilalis*. That is, the factors (such as olfactory and visual) that influence the flight behavior must also influence the landing behavior of males, either directly (by triggering landing) or indirectly by the male flying to a place where landing is triggered. In particular, they suggest that where a male lands is largely a consequence of his flight maneuvers that determine his track, altitude, and velocity. From the results, it is apparent that flight altitude has a great influence on where males land. Males flying to the horizontal surface were at an altitude close to that of the surface, and still descending, when near its downwind edge. Hence, most males landed on the surface near its downwind edge. However, males flying to the horizontal surface with a vertical object were substantially higher than the surface when they passed over the downwind edge, and this precluded them from landing. They continued flying until they either contacted the object or the source (which were, respectively, some 9 or 13 cm farther upwind).

With the 20-cm vertical object on the surface, the mean altitude of males

was even higher than for a 10-cm object or no object on the surface. In the region 7 cm downwind of the edge of the surface, males had a mean altitude almost 7 cm higher than the surface. Consequently, even though they were descending, few males landed at the downwind edge of the surface. Instead, most landed on the object. The large proportion of males that landed on the object rather than the source is explained by the fact that when males neared the source, their altitude, although declining (as evidenced by the large proportion of males that landed on the bottom 5 cm of the object), was probably still much higher than the source (the top of which was only 0.8 cm above the surface). They therefore did not land on the source but continued flying a short distance until they contacted the vertical object.

The flight tracks of males also appear to influence where males land in relation (laterally) to the pheromone source. Thus, when there was no vertical object on the surface, the flight tracks of males as they passed over the downwind surface tended to be centered in line with (downwind of) the pheromone source, and males tended to land on the upwind surface in line with (or on) the pheromone source. However, when the vertical object was placed on the surface, the flight track of males as they passed over the downwind surface (at least to distances 36 cm downwind of the upwind surface) tended to be skewed toward the source, and males tended to land toward (or on) the object. Males also passed over a broader distribution of column positions on the downwind surface when there was a vertical object on the upwind surface than when there was a not. Males landed in a broader distribution of columns on the upwind surface when there was a vertical object than when there was not.

The broadness of the distribution of columns that males passed over the downwind surface is probably an indication of the magnitude of the intertrack reversal distances of males at a given position downwind of the surface. At the closest distance (4 cm) between the two surfaces, nearly all the males passed over the downwind surface near its center (i.e., column 5), in line with the pheromone source, when there was no vertical object on the upwind surface. As the distance between the two surfaces increased, the distributions of males (with respect to column position) passing over the downwind surface was broader, but still centered around the column in line with the source. This is in accord with the expectation that the intertrack reversal distances would be greater farther downwind of the source (see Baker, 1989). Thus, the results also indicate that at a given distance, males flew in wider intertrack reversal distances when the object was present on the upwind surface than when it was not.

Thus, we see that the flight maneuvers that constitute the observed track and altitude also contribute to where the insects land. This is not to say that landing is an inevitable consequence of the downwind flight maneuvers. Certain features of the surface may only influence where the male lands at relatively close distances (see Foster and Harris, 1992), and hence a male may modify

his flight maneuvers when close to the source in response to say, certain visual features. For example, Zagatti and Renou (1984) found that male *Zygaena filipendulae* responded to certain visual cues associated with females when within 50 cm of the females. However, we stress that for a more through understanding of the landing behavior of male moths, it should be considered within the context of the flight behavior.

We used a small vertical object in these experiments because of its effect on the altitude and track of male *O. nubilalis* and other moths (Foster, unpublished results). That the placement of the vertical object on the surface had an affect on the flight track and altitude of male *O. nubilalis* is clear. Precisely which sensory modality is affected and thereby influenced these flight maneuvers is less clear. Male moths are known to use both olfactory and visual information during pheromone-mediated flight (Baker, 1989). The olfactory information is not derived purely from the presence or absence of the pheromone, but rather is thought to relate also to the fine structure (i.e., the instantaneous concentrations and their intermittency) of the pheromone plume (Murlis et al., 1992).

The turbulence created by placing the vertical object just behind and to the side of the pheromone source had a major effect on the plume structure (as evidenced through its effect on smoke from incense). In particular, the plume became more diffuse and apparently less structured than the plume off the surface without a vertical object. Thus, it is entirely plausible that the changes in flight track resulted from this change in plume structure. This is supported by the apparently wider intertrack reversal distances when the vertical object was present on the upwind surface than when it was not, which is consistent with lower-time-averaged pheromonal concentrations within the less-structured plume (Baker, 1989).

As well as changing the fine structure, the presence of the vertical object also affected the spatial position of the pheromone plume, with the plume appearing greater in volume and diverted slightly in the direction of the object and also slightly higher, than when the object was not present. This change in spatial position may explain the flight maneuvers of male *O. nubilalis*, which were similarly skewed toward the object and at a slightly higher altitude than when the object was not present.

While the vertical object affected the structure of the pheromone plume, it also potentially provided a visual stimulus that could have influenced the flight behavior of male *O. nubilalis*. Thus, the skewed flight toward the object and the higher mean altitude when the object was present (and to the 20-cm object) may, in addition to any effects resulting from the change in plume structure, be due to a visual stimulus from the object. Visual inputs during the pheromone-mediated flight of male moths are known to be important for the optomotor responses during control of flight track (Baker, 1989) and altitude (Preiss and Kramer, 1983, 1986; Preiss and Futschek, 1985. It is possible that as the male

moth approaches the surface, the image flow of the vertical object is used increasingly by the male for input into his optomotor responses and that his flight track and altitude are consequently skewed toward the object and higher above the surface. The results from experiment 5, in which the white or black vertical objects were tested, lend further support to the contention that the vertical object also (in addition to the changes in the plume structure) influences the flight maneuvers of males through the visual modality. In this experiment, with the black vertical object, the landing positions of males tended to be centered in line with the source rather than skewed toward the object (as it was with the white object), suggesting that the visual properties of the object influence the landing positions and the flight maneuvers of male *O. nubilalis*.

In the final experiment, the effect of position of the vertical object relative to the surface was tested. When the object was immediately upwind of the surface, it still affected where males landed on the surface; a large proportion of the males continued to fly past the source toward the object, and half of these males landed upwind of the source, either on the surface or on the object. When the object was 10 cm upwind of the surface, its effect on where males landed was less apparent. However, it still had a significant effect on their flight behavior, as nearly half of the males flew past the pheromone source. All but two of these males then flew downwind and landed either on the source or in front of the source. Interestingly, no males flew past the upwind edge of the surface. Presumably, the loss of contact with the pheromone combined with the approaching change in visual input (the apparent ground changing from just below the insect to over 10 cm below) resulted in males arresting their upwind progress. They then returned downwind where they probably established contact with the plume and landed.

Vertical objects within or near a pheromone plume have been reported to influence the orientation of several species of moths (Farkas and Shorey, 1974; Levinson and Hoppe, 1983; Charlton and Cardé, 1990). A recent report by Wyatt et al. (1993) found a similar effect for a predatory beetle. In a natural environment, wind turbulence and a rich and varied visual environment will likely result in more irregular flight tracks and less successful source location (see for example, Elkinton et al., 1987) than that observed in the more uniform and controlled environment of the wind tunnel. In future studies we intend to investigate how physical factors (e.g., the structure of trees) near a pheromone source in the field affect the flight and landing responses of male moths and, hence, how they influence the efficacy of male moths to locate a pheromone source.

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UP IN SMOKE: I. SMOKE-DERIVED GERMINATION CUES FOR POSTFIRE ANNUAL, *Nicotiana attenuata* TORR. EX. WATSON

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Abstract—Some postfire annuals with dormant seeds use heat or chemical cues from charred wood to synchronize their germination with the postfire environment. We report that wood smoke and polar extracts of wood smoke, but not the ash of burned wood, contain potent cue(s) that stimulate germination in the postfire annual plant, *Nicotiana attenuata*. We examined the responses of seeds from six populations of plants from southwest Utah to extracts of smoke and found the proportion of viable seeds that germinated in the presence of smoke cues to vary between populations but to be consistent between generations. With the most dormant genotypes, we examine three mechanisms by which smoke-derived chemical cues may stimulate germination (chemical scarification of the seed coat and nutritive- and signal-mediated stimulation of germination) and report that the response is consistent with the signal-mediated mechanism. The germination cue(s) found in smoke are produced by the burning of hay, hardwood branches, leaves, and, to a lesser degree, cellulose. Moreover, the cues are found in the common food condiment, "liquid smoke," and we find no significant differences between brands. With a bioassay-driven fractionation of liquid smoke, we identified 71 compounds in active fractions by GC-MS and AA spectrometry. However, when these compounds were tested in pure form or in combinations that mimicked the composition of active fractions over a range of concentrations, they failed to stimulate germination to the same degree that smoke fractions did. Moreover, enzymatic oxidation of some of these compounds also failed to stimulate germination. In addition, we tested 43 additional compounds also reported from smoke, 85 compounds that were structurally similar to those reported from smoke and 34 compounds reported to influence germination in other

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species. Of the 233 compounds tested, 16 proved to inhibit germination at the concentrations tested, and none reproduced the activity of wood smoke. By thermally desorbing smoke produced by cellulose combustions that was trapped on Chromosorb 101, we demonstrate that the cue is desorbed between 125 and 150°C. We estimate that the germination cues are active at concentrations of less than 1 pg/seed and, due to their chromatographic behavior, infer that a number of different chemical structures are active. In separate experiments, we demonstrate that cues remain active for at least 53 days in soil under greenhouse conditions and that the application of aqueous extracts of smoke to soil containing seeds results in dramatic increases in germination of artificial seed banks. Hence, although the chemical nature of the germination cue remains elusive, the stability of the germination cues, their water-solubility, and their activity in low concentrations suggest that these cues could serve as powerful tools for the examination of dormant seed banks and the selective factors thought to be important in the evolution of postfire plant communities.

Key Words—Fire, smoke, germination cues, *Nicotiana attenuata*, Solanaceae, seed dormancy.

INTRODUCTION

Fire has been an important evolutionary force sculpting the attributes of many plant communities and the individual species that make up these communities (Wright and Bailey, 1982). A suite of plant traits is recognized as being important in synchronizing regrowth, reproduction, and germination in the immediate postfire environment (Rundel, 1981). Perhaps most spectacular of these traits are those that cue seed germination with the occurrence of fire and are thought to be partially responsible for the dramatic response of the seed bank to fire (Stone and Juhren, 1951, 1953; Keeley, 1987, 1991; Thanos and Georghiou, 1988; Paige, 1992). Heat, inorganic nutrients, destruction of allelopathic compounds that inhibit germination, oxidation of seed coat, and unidentified compounds associated with blackened (charred) wood have been implicated as germination cues used by the seed bank for this synchronized germination response (Went et al., 1952; Christensen and Muller, 1975a, b; Keeley et al., 1985; Keeley and Pizzorno, 1986; Emery, 1992).

Fire is a natural component of the piñyon–juniper woodland and is a primary cause of secondary sucession (Madany, 1981). *Nicotiana attenuata* Torr. ex. Wats. (Solanaceae) is a native tobacco plant found as a summer annual in California, Nevada, Idaho, and Utah (Goodspeed, 1954; Brotherson et al., 1980). The plant can be found as a roadside weed after new road construction in previously undisturbed areas and is recognized as a component of the postfire annual community in both sagebrush and piñyon–juniper forest (Wells, 1959; Barney and Frischknect, 1974; Britton and Ralphs, 1978; Young and Evans, 1978; Wright et al., 1979; Wright and Bailey, 1982; Koniak and Everett, 1982;

Koniak, 1985). Native Americans are thought to have burned sagebrush to promote the growth of *N. attenuata* for medicinal and smoking purposes (Wells, 1959; Bye, 1972; Haberman, 1984).

We report here that volatile compounds found in wood smoke stimulate germination in some, but not all, populations of *N. attenuata*. We examine three mechanisms by which compounds in wood smoke might function to stimulate germination, report on the progress made in characterizing the germination cues of wood smoke with bioassay-driven fractionations of aqueous extracts of wood smoke, and examine the utility of the smoke cue as an ecological tool for the study of dormant seed banks.

METHODS AND MATERIALS

Combustion Experiments. Plant material was ignited (with a Bunsen burner or butane torch) in a stainless steel bowl attached to a portable vacuum pump that drew air (at approximately 50 ml/min) through the bottom of the bowl, a stainless steel screen (80 mesh), an atomizer (Pyrex ASTM 40–60, 12C) submerged in 50 ml of distilled water, and tubing attached to Pasteur pipets containing 20 *N. attenuata* seeds packed in glass wool located both upstream and downstream of 2.5–3 cm of an absorbant. In separate experiments, this combustion apparatus was used to compare the efficacy of trapping germination cues with the following three absorbants: Chromasorb 101 (Sigma Lot No. 89F1561, 80–100 mesh), activated charcoal (Sigma Lot No. 79F0765), and tenax (Hewlett Packard Lot No. 2252, 60–80 mesh). Homogenized and lyophilized *N. attenuata* leaf material (2 g) was burned, seeds before and after traps were assayed for germination, and the traps were loaded into a thermal desorption tube and thermally desorbed at 300°C with a helium flow of 20ml/min, using a short path thermal desorption unit (Scientific Instrument Services, Inc., Ringoes, New Jersey, model TD-2). Desorbed volatiles were trapped in a 10-ml syringe packed with ice, which was attached to the end of the thermal desorbtion unit. The syringes were rinsed with 1 ml of 9.8 mM aqueous KNO_3 and bioassayed for germination activity. Chromosorb 101 proved to be the absorbant of choice because it was an efficient trapper—seeds placed downstream of the absorbant did not germinate—and because the cue could easily be removed from the absorbant, both thermally and with rinses in methanol.

Although this apparatus did not quantitatively trap all the smoke produced during a combustion or quantitatively combust all material in the bowl, it nevertheless was useful for comparing the germination potential of smoke produced by burning different types of plant material and constituents of plant material (see Table 1 below). In these experiments, 2 g of material was ignited, and the evolved smoke was trapped on individual Chromosorb 101 traps, which were

eluted with methanol. The methanol was evaporated in air, taken up in 9.8 mM KNO_3, and bioassayed (see description of bioassay conditions below) over four decade dilutions (e.g., original, 10:1, 100:1, 1000:1, 10,000:1 v/v dilutions). Material was scored by the number of decades the extract could be diluted from the original extract and still produce a significant increase in germination compared to control trappings. Some of the materials—particularly sucrose, xylan, starch, and glucose—did not sustain their own combustions and had to be reignited several times. Control trappings consisted of trapping volatiles trapped when the butane torch was held over an empty combustion bowl.

An experiment was performed to compare the germination activity of trapped smoke and unburnt leaf and ash residue from a combustion of 5 g of *N. attenuata* leaf material with that found in commercially available "liquid smoke" (Potthast, 1993) (Wright's Lot No. 2318G5 Nabisco Foods, Inc., East Hanover, New Jersey 07936). Unburned leaf material and ash trapped on the screen was extracted in 50 ml of water. Smoke was trapped on Chromosorb 101, extracted in 5 ml methanol, dried, and combined with the 500 ml of the water trap. The combined smoke extracts were frozen, lyophilized, and taken up in 50 ml water. Ash, smoke extracts, and liquid smoke were bioassayed over four decade dilutions with four replicates at each dilution.

An experiment was performed to determine the temperature at which the smoke cue could be thermally desorbed from a Chromosorb 101 trap. Approximately 2.5 g of alpha-cellulose (Sigma Lot No. 69F0373) were burned, and the resulting smoke was captured on a trap that had been previously cleaned with methanol. After trapping, the chromasorb was removed, homogenized, and divided into eight equivalent fractions of approximately 50 mg each. Each fraction was then loaded into separate thermal desorption tubes that were thermally desorbed for 20 min at sequentially higher temperatures beginning at 75°C in increments of 25°C using the short path thermal desorption unit. Desorptions were conducted with helium as the carrier gas at a flow of 20 ml/min. After desorbing a tube at a particular temperature, each desorption tube and absorbant were repeatedly washed with 500 μl of methanol, and transferred to a 1-ml shell vial. The methanol was dried and taken up in 500 μl 9.8 mM KNO_3 for seed bioassays at the original concentration.

In all of the above experiments, seeds were exposed to smoke or smoke extracts at room temperature. In order to examine the role that heat from a fire might play in stimulating germination, four replicate batches of 20 seeds were separately heated in a GC oven at six temperatures (30, 50, 70, 90, 110, and 120°C) for 10 min and immediately hydrated with 9.8 mM KNO_3 and placed in a seed cup for a germination bioassay.

Seed Bioassays with Chemicals Found in Smoke. Seeds used in all the growth-chamber bioassays were from the second- to fifth-generation of glasshouse-grown inbred plants grown from seed collected from plants in 1988 on

the DI Ranch (T40S R19W section 9 of Utah). The original population of plants was found growing among charred stumps of blackbrush (*Coleogyne ramosissima* Torr.) in a gravel wash that had burned the previous year. Seeds were collected from dehisced pods on plants and kept at room temperature in a sealed scintillation vial. All seeds used in a given bioassay were from the same cohort of plants and were less than 2 years old. Batches of seeds would, on occasion, lose their dormancy unpredictably; when this happened, plants were grown to maturity and allowed to self pollinate in the glasshouse. The seeds from these plants would then be used for germination bioassays.

The seed germination bioassay was designed to maximize the germination rate of all seeds and produce at least 10% germination in untreated seeds in order to recognize when particular compounds and/or concentrations might inhibit seed germination. Since *N. tobaccum* seeds are well known to exhibit maximum germination under conditions of alternating light and dark, varying temperatures, and in the presence of KNO_3 at concentrations greater than 1 mM (Karssen and Hilhorst, 1992; Bewley and Black, 1982), we examined a range of these environmental conditions in order to optimize the germination rate of our dormant genotype. The conditions that produced the highest germination response were 14L: 10D photoperiod with 200 $\mu M/m^2/sec$ PAR, 30°C day–22°C night temperature cycle, and 9.8 mM KNO_3, and these were the conditions for our bioassays. Wells (1959) reported that *N. attenuata* seeds required a period of cold stratification in order to attain germination rates above 5%. We found no evidence for this requirement in our seeds. Each replicate consisted of 10 seeds in soufflé cups (Solo 1 oz., P100) containing approximately 5.5 g sterile sand. The sand in the seed cups was saturated with 9.8 mM KNO_3 so that seeds were just touching the water layer; previous experiments demonstrated that the amount of water in the seed cup influenced the rate of germination. Cups were sealed with transparent lids (Solo PL 1 lids) and placed in a growth chamber (Percival, Boone, Iowa, model E-54U). After 72 hr, in the growth chamber, seeds were examined daily for germination for up to seven days from the initial transfer to the cups. Germination was defined as the splitting of the seed coat and the emergence of the radicle as observed with a head-mounted 10 × magnifying lense. Previous experiments had demonstrated that seeds that did not germinate within 15 days of being exposed to smoke extracts would not germinate over the course of the next three months, and since these nongerminating seeds tended to decompose during this time, we considered these seeds not viable.

Pure chemicals and fractions of smoke extracts were tested for their ability to influence germination in the seed cup bioassay. Ten seeds were placed in a 1.0-ml shell vial with 0.1–0.5 ml of an aqueous solution containing the compounds or active fractions to be tested for the same amount of time (approx 1 hr) before being transferred with the test solution to the seed cup. Previous experiments had demonstrated that once dormant seeds had been soaked in

smoke-containing extracts for more than 15 min, germination could not be prevented by rinsing seeds with water. Most compounds and fractions (see Tables 2–4 below) were tested at an original concentration of 0.5 mg/ml and in some cases at one, two, or three decades of serial dilution (original, 10:1, 100:1, 1000:1). For water soluble compounds and fractions, serial dilutions were made with 9.8 mM KNO_3. For compounds and fractions not soluble in water, stock solutions and dilutions were made in methylene chloride and transferred to shell vials. The methylene chloride was removed by evaporation at room temperature and 0.5 ml of 9.8 mM KNO_3 was added to the shell vial. Seeds were added and allowed to soak for 1 hr before the seeds and their solutions were transferred to the seed cups. Each concentration was tested with at least three replicates, 10 seeds per replicate. Each experiment included three replicates of seeds treated with a 1:300 dilution of liquid smoke (House of Herbs, Inc., Passaic, New Jersey 07055; designated as "smoke" in Figures 1, 3–7 below) in 9.8 mM KNO_3 and three replicates of seeds soaked in water containing only 9.8 mM KNO_3 (designated as "water" in Figures 1, 4, 5 below). The percentage of all seeds germinating at each count for each seed cup were calculated and arcsine transformed for normality. Repeated-measures ANOVAs on the transformed percentages were used to analyze main effects. Analysis was performed with the MGLH ANOVA module from Systat Inc. (Evanston, Illinois).

In a separate experiment, the germination activity of three bottles of liquid smoke (Potthast, 1993) of two commercially available brands (House of Herbs, Passaic, New Jersey 07055; Wright's, Nabisco Foods, Inc. East Hanover, New Jersey 07936) were compared over four decades of dilution with three replicate batches of 10 seeds for each treatment at each dilution.

Bioassay-Driven Fractionation of Smoke Extracts. A variety of chromatographic techniques was employed to fractionate smoke extracts produced in the laboratory and the commercially available liquid smoke preparations, including packed-column GC on Chromosorb 101, wide-bore (0.53 μm) and narrow-bore (0.25 μm) capillary column GC (DB-1, DB-5 and Carbowax stationary phases), HPLC on C-18 columns and low-pressure liquid chromatrophy with LH-20, silica gel (of different chromatographic grades), Florisil, alumina (acidic, neutral, and basic grades), and columns packed with *N. attenuata* seeds.

Metals were determined with an atomic absorbtion spectrophotometer (Themo Jarrell Ash Corp., Franklin, Massachusetts), operated by Cornell University's Department of Pomology facility, in active fractions trapped from a packed column GC. Additionally, since we had established that passing an aqueous extract of smoke through a column packed with *N. attenuata* seeds removed all germination activity from the extract, we compared the concentrations of metals in smoke solutions before and after being passed through a seed column. Metals that significantly decreased in concentration after being passed

through a seed column or were present in the active fractions collected from the GC were tested in seed bioassays.

Since we have established that the germination cue can pass through a capillary GC column, most of our efforts in identification of the potential candidate compounds have been by GC-MS with or without derivatization with bis(trimethylsilyl)trifluoroacetamide (BSTFA), hexamethyldisilazane (HMDS), trimethylchlorosilane (TMCS), trimethylsilylimidazole (TMSI), and methyl bis(trifluoroacetamide) (MBTFA). Prior to separation, a number of clean-up steps were employed, including acid/base, solvent partitioning, and oxidation of phenolics by treatment with peroxidase enzymes and hydrogen peroxide. However, because the chemical basis of the germination response remains elusive, the details of these separation procedures will not be reported here. Compounds tentatively identified in active fractions by GC-MS were purchased or synthesized and tested for germination activity in the seed cup bioassays. These compounds are listed in Table 2 below. Other organic compounds found in wood smoke by other researchers but not explicitly identified in our active fractions were also tested for germination activity with the seed cup bioassay, and these are listed in Table 3 below. Other compounds that are structurally similar to those reported from wood smoke were also tested with the seed cup bioassay (Table 4 below). Last, other compounds, unrelated to compounds found in smoke but implicated in stimulating germination in other species were also tested in the seed bioassay (Table 5 below).

Heritability of Smoke-Induced Germination. Germination speed and uniformity of *N. rustica* seeds are known to have both additive and nonadditive components (Miura et al., 1988). We examined the overall heritability of smoke-induced germination in six native populations consisting of a least 30 *N. attenuata* plants during the summer of 1988. Seeds were collected from one plant from each population. Populations were at least 1 km apart in sections 1–21 of T40S R19W and sections 10–29 of T41S R17W of southwestern Utah. A subset of each seed collection was germinated with smoke extracts, and one plant from each population was grown in the glasshouse and allowed to self-fertilize. Seeds from these plants (offspring) were compared to those of their parents. Four replicates of 35 seeds of both smoke-and water-treated seeds from both parent and offspring generations from each of the six populations were tested with the seed bioassay in the same experiment to ensure that all seeds were tested under the same conditions. In order to standardize for differences in viability in the field-collected parent generation, the difference between water- and smoke-germinated seeds was expressed as proportions of total smoke-germinated seeds. Parent and offspring germination values were regressed against each other for an overall measure of heritability as determined by the slope of this regression.

Additional Seed Sources. In order to examine how many other species of *Nicotiana* might respond to smoke cues, bioassays with 1 : 300 dilutions of liquid

smoke were also performed with seeds from *Nicotiana* species that were known to exhibit dormancy or grow in fire-prone habitats. Specifically, we tested seeds of *N. attenuata* (accessions 7 and 7A), *N. debneyi*, *N. bigelovii*, *N. clevelandii*, *N. stocktonii*, *N. nesophila*, *N. corymbosa*, *N. noctiflora*, *N. spegazzinii*, *N. acuminata*, and *N. linearis* from the Tobacco Germplasm Collection at the USDA Crops Research Laboratory (P.O. Box 1168, Oxford, North Carolina 27565-1168).

Mechanisms of Germination Stimulation by Smoke. We propose that smoke-derived compounds could stimulate germination with one of the following three classes of mechanisms: (1) chemical scarification of the seed coat, (2) nutritive stimulation of germination (see for example Smith, 1973), and (3) signal-mediated stimulation of germination. To clarify how smoke extracts might stimulate germination, predictions of the first two hypotheses were examined. Chemicals present in smoke extracts may scarify the seed coat and remove dormancy imposed by the seed coat. Intact seed coats might function as a barrier to water or gas uptake by the embryo or mechanically constrain the growth of the embryo, as has been demonstrated in other species (Qi and Upadhyaya, 1993; Qi et al., 1993; Brits et al., 1993). Germane to the chemical scarification hypothesis is the observation that treatment of seeds with 5% hypochlorite for 15 min, followed by rinsing in water, stimulates the germination of dormant seeds in a manner that is statistically indistinguishable from that of smoke-treated seeds (repeated measures ANOVA of four replicates: contrast between hypochlorite- and smoke-treated seed germination rates $F_{3,4} = 1.805$; $P = 0.3$). On the other hand, both smoke- and water-treated seeds imbibe water and take up eosin dye (from a 1% solution); thus, it is not likely that the seed coat represents a significant barrier to water uptake. Nevertheless, the untreated seed coat may present a significant barrier to the diffusion of gases.

Three experiments were conducted to examine the chemical abrasion hypothesis. In the first experiment, we examine the integrity of the seed coat of 20 seeds that were soaking in a 1:300 dilution of liquid smoke in 9.8 mM KNO_3 and of 20 seeds that were soaked for 1 hr in only KNO_3 with scanning electron microscopy. Treatment with hypochlorite visibly (to the naked eye) deteriorates the seed coat, and smoke treatment might produce similar but less apparent abrasions in the seed coat. In the second experiment, we compared the germination rates of seeds that were abraded over the length of their seed coat with a file to the germination of seeds that were not abraded. Both abraded and unabraded seeds were treated either with smoke extracts in 9.8 mM KNO_3 or KNO_3. Four replicates of 10 seeds of each of the four treatments were tested in the seed cup bioassay. Abraded seeds had approximately 20% of their seed coat damaged, and if smoke extracts changed the permeability of the seed coat, we predict that abraded and smoke-treated unabraded seeds should have similar germination rates. The third experiment was designed to quench organic hydro-

peroxides and other oxidants that might be present in the smoke extract and might be functioning in a manner similar to that of hypochlorite. *tert*-Butyl hydroperoxide (Sigma Lot No. 40H0384) and hydrogen peroxide (Fisher Lot No. 902738) were used as model systems to design the quenching experiments. These two oxidants were found to produce a small germination response equivalent to approximately one third of that found in liquid smoke in a narrow concentration range (0.5–3.0% v/v). Seeds treated with higher concentrations would not germinate after smoke treatment and were considered dead. When these concentrations of hydroperoxide and *tert*-Butyl hydroperoxide were treated with an equal volume of 50 mM $NaSO_3$, germination was no different from controls treated with 50 mM $NaSO_3$, 50 mM $NaSO_4$, or 9.8 mM KNO_3. Moreover, the hydroperoxide solutions did not react with KI indicator paper after treatment with 50 mM $NaSO_3$; hence, we propose that organic hydroperoxides, perhaps transiently formed from organic ethers, would have been quenched by this treatment and that the treatment itself did not affect seed germination. Therefore, the third experiment involved four replicates of 10 seeds treated with one of the following four treatments: smoke extracts with 50 mM $NaSO_3$, smoke extract alone, 9.8 mM KNO_3 treatments, and 50 mM $NaSO_3$ treatments. All smoke extracts were made up in 9.8 mM KNO_3. We predicted that if the smoke extracts treated with 50 mM $NaSO_3$ failed to elicit germination above the control or water treatments, the smoke extract was likely to contain organic hydroperoxides that affect germination by means of an oxidative scarification of the seed coat.

In order to test the hypothesis that smoke extracts contain nutritive components that stimulate seed germination, we compared the growth of smoke-treated seeds with hypoclorite-treated seeds in sterile sand without any nutrient supplementation. We treated four replicates of 10 seeds with either 5% hypochlorite or smoke extracts. Treated seeds were grown in sterile sand in the growth chamber and individually weighed. Since smoke- and hypochlorite-treated seeds germinated at the same time, we used their biomass after seven days of growth as an indicator of nutritive components in the smoke extract.

Smoke Cues as Ecological Tools. Two criteria were deemed important to establish if these smoke-derived cues are to be used as an ecological tool to probe seed banks. Although smoke extracts clearly stimulate germination of dormant seeds in sterile quartz sand in our bioassay chamber, it was not clear that a similar response could be elicited in chemically and biologically more complex soils. Many soils contain large amounts of organic matter that might directly inactivate or harbor a microbial community that might inactivate the stimulatory activity of the smoke extracts. Second, the longevity of the cue in soil needed to be established. If smoke-derived cues are used by the dormant *N. attenuata* seed bank in order to synchronize germination with some aspect of the postfire environment (elevated nutrients, lower herbivory regimes, etc.),

the cue should be as long-lived as the changes in the important environmental characteristics. Since burned sites are typically revegetated within a month, we examined the cue's longevity in soil for 53 days.

To address the first question, an experiment was performed in the glass house with artificial seed banks created in fifteen 54-cm × 27-cm flats with a 3-cm-thick layer of nonsterile sand. Into the organic layer of each flat were mixed 116.6 mg of field-collected *N. attenuata* seeds. Seeds were from a bulk collection made in 1991 from a population of plants growing approximately 1 km from the Goldstrike gold mine (T39S R18W section 16) in southwestern Utah. Seeds were collected from a group of approximately 75 plants and stored at room temperature for 42 months. These plants were heavily infested with the seed-feeding negro bug (*Corimelaena* spp.) and had a viability of approximately 12%. Flats were randomly assigned to one of three treatment groups: (1) smoke A + B treatment, in which each flat was sprayed with 500 ml of a 300:1 aqueous dilution of liquid smoke on days 0 and 24; (2) smoke B treatment, which received the smoke treatment only on day 24 and received 500 ml of distilled water on day 0; and (3) a control treatment, which received 500 ml of distilled water on days 0 and 24. After the first treatment on day 0, flats were randomly placed on a glasshouse bench under 1000-W halogen lamps, which provided a minimum PAR of 250 $\mu M/m^2/sec$ for 16 hr/day. Flats were rotated weekly among positions on the greenhouse bench and watered when the subtending sand layer became noticeably dry. All emerging seedlings—defined by the presence of cotyledons emerging above the soil surface—were counted periodically for 24 days. On day 24, all seedlings were removed from all flats, and all flats were treated again with either water or smoke extracts as dictated by their treatment group. The number of growing seedlings was counted for an additional 25 days.

In order to examine the longevity of the cue in soil, 32 flats were prepared as described above except that seeds were not mixed in the organic layer. Sixteen of the flats were treated with 500 ml of the smoke extract, and all flats were arranged on a glasshouse bench and watered with 500 ml of water once a week. On days 0, 10, 20, and 53 after the initial smoke application, 100 seeds were mixed into the organic layer of four smoke-treated flats and four water-treated flats, and the maximum number of seedlings emerging after 15 days was expressed as a percentage of the number of seeds planted.

RESULTS

Combustion Experiments. For the comparison of smoke, ash, and liquid smoke, the highest germination activities for each component were found in bioassays performed on the 100:1 dilution; these values are plotted in Figure

1. The combustion of *N. attenuata* plant material produced the highest germination rates. In contrasts performed from a repeated measures ANOVA, we found that the water and ash treatments did not differ significantly (multivariate $F_{2,21} = 0.38$; $P = 0.82$) and exhibited very little germination over the 10-day experiment. Both liquid smoke and the true smoke extracts dramatically stimulated germination compared to the water control (multivariate $F_{2,21} = 27.97$; $P < 0.00001$), and the smoke extracts were not significantly (multivariate $F_{2,21} = 2.35$; $P = 0.087$) better than liquid smoke in stimulating germination. Hence, the germination activity is principally found in the smoke fraction and not in residual unburnt leaf material and ash.

When different plant materials were combusted in our smoke-trapping apparatus, the highest germination activity of smoke trapped on Chromosorb traps was found when plant material was burned (leaves, branches, or straw; Table 1). Significant germination activity compared with KNO_3-treated control seeds was found when pure cellulose was burned; marginal germination responses were found when cellobiose, glucose, and xylose were burned (Table 1). Because only one combustion was performed for each compound, statistical analysis of replicate bioassays performed on each burn was deemed inappropriate.

Bioassays performed on methanol extracts of Chromosorb traps desorbed at different temperatures after trapping the smoke from a cellulose combustion demonstrated that the germination cue could be desorbed from these traps between 125 and 150°C (Figure 2). Because only one desorption was performed at each temperature, statistical analysis of replicates in the germination bioassay

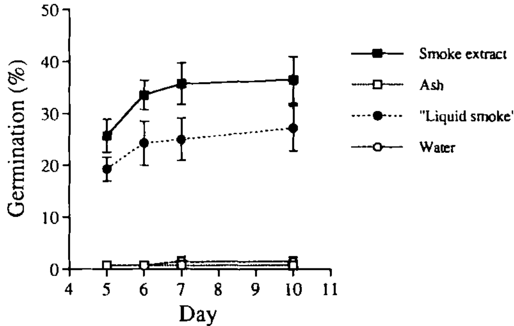


FIG. 1 Germination (mean ± SEM) percentages of four replicates of 10 *N. attenuata* seeds exposed to a 1 : 100 dilution with 9.8 mM aqueous KNO_3 of each of the following aqueous extracts (1) commercially available liquid smoke (solid circles); (2) smoke extracts produced by trapping volatiles from a combustion of 5 g of *N. attenuata* leaves on a Chromosorb 101 trap (solid squares); (3) unburned leaves and ash residue from the leaf combustion (open squares); and (4) 9.8 mM KNO_3 water control (open circles). SEMs are obscured by the symbols in the ash and water treatments.

TABLE 1. RELATIVE GERMINATION RESPONSE OF SMOKE TRAPPED FROM BURNED PLANT MATERIAL

Material combusted	Relative germination response
N. attenuata leaves	****
Acer negundo branches	****
Straw	***
α-Cellulose	**
d-(+)-Cellobiose	*
Glucose	*
d-(+)Xylose	*
d-(+)Trehalose	–
Arabinogalactan	–
Xylan	–
Starch	–
Sucrose	–
Palatinose	–

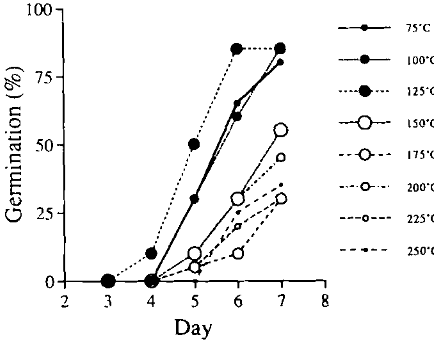


FIG. 2. Germination percentages of 10 *N. attenuata* seeds exposed to the extracts of eight different Chromosorb traps that had trapped the smoke from a combustion of 2.5 g of alpha-cellulose and were desorbed at eight different temperatures for 20 min in a short-path thermal desorbtion unit with helium as a carrier gas. The results demonstrate that the germination cue can be desorbed from Chromosorb 101 at temperatures between 125 and 150°C.

was deemed inappropriate. Because many of the compounds found in smoke slightly inhibit germination in the seed bioassay (Table 2), the increase in germination as the Chromosorb was desorbed at temperatures from 75 to 125°C (Figure 2) may reflect the thermal desorption of these germination inhibitors.

Germination activity of the commercially available brands of liquid smoke

TABLE 2. COMPOUNDS IDENTIFIED IN ACTIVE FRACTIONS OF SMOKE EXTRACT TESTED IN SEED BIOASSAYS

- Organic acids
  - Propionic acid[a,1]
  - Sorbic acid[4]
  - Benzoic acid[4,7]
  - Heptanoic acid[4]
  - Nonanoic acid[4]
  - Capric acid[4]
  - Myristic acid[1,7]
  - Palmitic acid[1,7]
  - Linolenic acid[1,7]
  - Oleic acid[3]
  - Phthalic acid[1,7]
  - Homovanillic acid (4-hydroxy-3-methoxyphenyl acetic acid)[2]
  - 3,4,5-Trimethoxybenzoic acid[2]
  - 2-Furoic acid[4,7,8]
- Phenols and Quinones
  - o-Cresol[4,8]
  - m-Cresol[4,8]
  - p-Cresol[4,8]
  - Catechol[4,8]
  - Resorcinol[4,8]
  - Guiacol[4,8]
  - 2,6-Dimethoxyphenol[3,5,6,7]

- Phenols and Quinones
  - 3,5-Dimethoxyphenol[3]
  - Eugenol (2-methoxy-4-(2-propenyl)phenol)[4,8]
  - Methyl syringol (2,6-dimethoxy-4-methylphenol)[4,5,6,7]
  - Ethylsyringol (2,6-dimethoxy-4-ethylphenol)[4,5,6,7]
  - 2,6-Dimethoxy-4-allylphenol[4,5,6,7]
  - 3,5-Dimethoxy-4-allylphenol[4]
  - Propylsyringol (2,6-Dimethoxy-4-propylphenol)[4,5,6,7]
  - 3,5-Dimethoxy-4-propylphenol[4]
  - Benzoquinone[4]
  - Hydroquinone[4]
  - Methyl-p-benzoquinone[4]
- Aldehydes
  - Vanillin (3-methoxy-4-hydroxybenzaldehyde)[2,7]
  - o-Vanillin (3-methoxy-2-hydroxybenzaldehyde)[2]
  - Syringaldehyde (3,5-dimethoxy-4-hydroxybenzaldehyde)[2,7]
  - 3,4,5-Trimethoxybenzaldehyde[2]
  - 2-Furaldehyde[1,7]
- Ketones
  - Acetophenone[2]
  - Methyl syringone (3,5-dimethoxy-4-methylacetophenone)[2,7]
  - Acetosyringone (3,5-dimethoxy-4-hydroxyacetophenone)[3,7]
  - Maltol (3-hydroxy-2-methyl-4-pyrone)[1,7]
  - Acetovanillone (4-hydroxy-3-methoxyacetophenone)[2,7]

TABLE 2. CONTINUED

Other organic compounds	Inorganic compounds
Styrene[3,7]	Calcium[4]
Naphthalene[3,8]	Cobalt[4]
Acenaphthene[3]	Copper[4]
Fluorene[3]	Iron (Fe^{2+} and Fe^{3+})[4]
Anthracene[3]	Magnesium[4]
9,10-Dihydroxyanthracene[3]	Manganese[4]
1,2,4-Trimethylbenzene[4]	Nickel[3]
Phthalic anhydride[1,6,7]	Sodium[4]
4-Chromanol[2]	Sulfur[4]
1,2,4-Trimethoxybenzene[2]	Zinc[4]
Homovanillyl alcohol[2]	Boric acid[2]
Vanillic acid methyl ester[4]	
3,4,5-Trimethoxytoluene[4]	
Syringic acid methyl ester[4]	
1,6-Anhydro-β-*d*-glucose[3,7]	
Furan[3,6]	
5-Methylfurfural[3,7]	
5-Hydroxymethyl-2-furaldehyde[3]	

[a] [1]Tested at concentrations of 1.5 mg/ml and one decade dilution. [2]Tested at concentration of 1 mg/ml. [3]Tested at concentration of 0.5 mg/ml. [4]Tested at concentration of 0.5 mg/ml and at one, two, or three decade dilutions. [5]Tested with and without treatment of laccase, peroxidase, or tyrosinase in separate trials. [6]Tested with and without treatment of 2,6-di-*tert*-butyl-4-methylphenol (BHT) at 0.2 mg/ml. [7]Tested in combination with other smoke constituents to mimic the composition of the active fraction. [8]Inhibitory at concentrations tested.

was compared in a repeated-measures two-way ANOVA with brand and dilution as main effects. A highly significant dilution effect was found ($F_{3,16} = 26.0$; $P < 0.0001$), with the highest germinations occurring at the 1 : 100 and 1 : 1000 dilution levels. No significant effect of brand or brand-dilution interactions was found ($Ps > 0.06$).

Seed Heating Experiment. Heating seeds to temperatures of 50°C for 10 min had no effect on germination, but heating seeds to 70°C or greater for 10 min killed seeds (e.g., seeds did not germinate after exposure to smoke extracts).

Bioassays Results. Over 70 compounds were tentatively identified by GC-MS and AA spectroscopy in active fractions of Chromosorb-trapped smoke and liquid smoke extracts and tested for germination activity in the seed bioassay (Table 2). An additional 43 compounds reported to be present in wood smoke (see references in Table 3) were tested for germination activity in the seed bioassay (Table 3). An additional 35 compounds, which are structurally similar to those found or reported to be present in wood smoke, were tested for germination activity in the seed bioassay (Table 4). Last, an additional 34 compounds reported to be important in stimulating or inhibiting germination in other species were tested (Table 5). Of these 233 compounds, none proved to stimulate germination over the range of dilutions found with extracts of wood smoke, and 16 proved to be inhibitory at the concentrations tested (see 8 in footnote a; Tables 2–5). The inhibition of germination could only be discerned if the seeds in the KNO_3-treated control groups for the particular bioassay had sufficient background germination rates to allow comparison. Hence the inhibition of germination should be viewed as a conservative estimate of this trait. Compounds that were repeatedly identified from active fractions were tested in combinations (see 7 in footnote a; Tables 2–5) reflecting their relative concentrations found in the active fractions. Since a family of methoxy phenols consistently chromatographed with the germination cue(s) both on HPLC and GC columns, we bioassayed these compounds separately with oxidizing enzymes (see 5 in footnote a; Tables 2–5) to test the germination potential of the corresponding quinones and with an antioxidant, BHT (see 6 in footnote a; Table 2–5), to ensure the stability of the phenol during the bioassay. Oxidation states can dramatically influence the germination potential of similar methoxy quinones for the seeds of parasitic plants (Chang et al., 1986; Chang and Lynn, 1986).

The chemicals responsible for the rapid germination of dormant seeds are clearly active in small quantities. A semipurified extract, from which we had identified more than 99.9% of all the organic material that could be detected by GC-MS, was found to be active in our bioassay at approximately 0.1 ng/seed. This suggests that the compounds are likely to be active at concentrations of less than 1 pg/seed. Moreover, germination activity in 1-min fractions collected from a 45-min capillary gas chromatographic separation found significant ger-

TABLE 3. COMPOUNDS REPORTED IN SMOKE TESTED IN SEED BIOASSAYS[a]

Organic acids
- Acetic acid[2]
- Pyruvic acid[4]
- *n*-Butyric acid[4]
- Isobutyric acid[4]
- Lactic acid[4]
- Tiglic acid[4]
- *s*-(+)-2-Methylbutyric acid[4,7]
- *n*-Valeric acid[4]
- Isovaleric acid[4]
- Levulinic acid[4]
- *n*-Caproic acid[4,7]
- Succinic acid[4,7]
- Malic acid[3,7]
- Caprylic acid[4]
- Phenylacetic acid[4]
- *p*-Hydroxybenzoic acid[2]
- Vanillic acid[2]

Phenols
- 4-Methylcatechol[3,7]

Aldehydes
- 3-Hydroxybenzaldehyde[3]

Ketones
- β-Hydroxybutyric acid lactone[2]
- γ-Hydroxybutyric acid lactone[3]
- Cyclopentanone[3]
- 2-Methyl-2-cyclopenten-1-one[4]
- 4,4-Dimethyl-2-cyclopenten-1-one[4]
- 1,3-Cyclopentadione[3]
- 2-Methyl-1,3-cyclopentanedione[3]
- 3-Methyl-1,2-cyclopentanedione[4]
- 1-Indanone[3,7]
- Pyran-4-one[4]
- 4-Hydroxy-2,5-dimethyl-3-(2H)-furanone[4]

Other organic compounds
- Acrolein[3,8]
- Crotonaldehyde[3]
- Propionic methyl ester[3]
- 2-Methylnaphthalene[3]
- Ethylbenzene[3]
- Veratrole (1,2-dimethoxybenzene)[3]
- 1-Phenyl-1-propyne[4,7]
- Biphenyl[3]
- Indene[3]
- Myoinositol[3]
- Glucose[3]
- 2-Methylbenzofuran[3]

[a] Data from Fiddler et al. (1970); Hruza et al. (1974); Ishiguro and Sugawara (1978); Maga (1992); Sakuma et al. (1981); Schlotzhauer and Chortyk (1987); Schlotzhauer et al. (1985, 1982); Schumacher et al. (1977). See Table 2 footnote.

mination activity in at least three distinct portions of the chromatogram, which suggests that there is more than one active structure.

Responses of Different Seed Sources. Of the different species of *Nicotiana* seed that were received from the Tobacco Germplasm Collection, only *N. attenuata* seeds from accession 7A exhibited a significant germination response to smoke-fractions in the seed bioassay. Accession 7 of *N. attenuata* and the other *Nicotiana* species germinated at the same rate regardless of their exposure to smoke extracts in the seed cup bioassays.

The seed collected from the six population of *N. attenuata* plants from southwestern Utah exhibited significant variability in the proportion of seeds that germinated after nine days of being exposed to smoke extracts in the seed cup bioassay. When these seeds were grown to maturity, seeds from these plants

TABLE 4. COMPOUNDS SIMILAR TO THOSE REPORTED IN SMOKE TESTED IN SEED BIOASSAYS[a]

Organic acids
- 3,3-Dimethylacrylic acid[4]
- β-Hydroxybutyric acid[3]
- *tert*-Butylacetic acid[4]
- 2,2-Dimethylbutyric acid[4]
- 3-Methylvaleric acid[4]
- 4-Methyl-*n*-valeric acid[4]
- α-Hydroxy-*n*-valeric acid[3]
- 2-Hydroxycaproic acid[4]
- Caprylic acid (octanoic acid)[4]
- Tartaric acid[4]
- 2-Oxohexanoic acid, sodium salt[4]
- Salicylic acid (2-hydroxybenzoic acid)[2,4]
- Gentisic acid (2,5-dihydroxybenzoic acid)[2]
- Protocatechuic acid (3,4-dihydroxybenzoic acid)[2]
- Isovanillic acid (4-methoxy-3-hydroxybenzoic acid)[2]
- Caffeic acid (3,4-dihydroxycinnamic acid)[2]
- 4-Propoxybenzoic acid[2,8]
- Ferulic acid (*trans*-3-methoxy-4-hydroxycinnamic acid)[2]
- 4-Butoxybenzoic acid[2]
- Syringic acid[2]
- *n*-Propylgallate[4]
- 3,5-Dimethoxy-4-hydroxycinnamic acid[4,7]
- 4-(3,4-Dimethoxyphenyl)-butyric acid[4]
- Kojic acid (5-hydroxy-2-(hydroxymethy)-4H-pyran-4-one[3,7]

Phenols
- 2-Methylresorcinol[4]
- 4-Ethylresorcinol[4]

Phenols
- Orcinol[3]
- 4-Propoxyphenol[2]
- 2,3-Dimethoxyphenol[2]
- 4-Hexylresorcinol[4,7]
- 4-Hexanoylresorcinol[4]
- Nonylphenol[4,7]

Aldehydes
- 2,3-Dimethoxybenzaldehyde[2]
- 2,4-Dimethoxybenzaldehyde[2]
- 2,5-Dimethoxybenzaldehyde[2]
- 2,6-Dimethoxybenzaldehyde[2]

Ketones
- Hydroxyacetone[3]
- α-Angelicalactone[4]
- 3-Methyl-2-cyclopenten-1-one[4]
- γ-Valerolactone[3]
- 6-Methyl-5-hepten-2-one[2,4,7]
- 2-Nonanone[2]
- d-Gluconic acid lactone[3]
- Benzylacetone[4]
- 2,5-Dihydroxyacetophenone[2,7]
- 2,4-Dihydroxyacetophenone[2,7]
- 2,6-Dihydroxyacetophenone[2,7]
- 3,5-Dihydroxyacetophenone[2,7]
- 2-Methoxyphenylacetone[4]
- 3-Methoxyphenylacetone[4]
- 4-Methoxyphenylacetone[3]

TABLE 4. CONTINUED

Ketones
- 4-(4-α-Hydroxyphenyl)-2-butanone[4]
- 2,4-Dihydroxy-3′-methylacetophenone[2,8]
- 4-(4-Methoxyphenyl)-2-butanone[4]
- 4-(4-Hydroxy-3-methoxyphenyl)-3-buten-2-one[4]
- 3,4-Dimethoxyphenylacetone[4]
- 2,2-Dihydroxy-5-methoxy-1,3-indandione[3]
- 2,6-Dimethyl-γ-pyrone[4]
- 4,6-Dimethyl-α-pyrone[4]
- 3-Hydroxy-2-methyl-4-pyrone[3]
- 4-Hydroxy-6-methyl-2-pyrone[4]
- 5,6-Dihydro-4-hydroxy-6-methyl-2H-pyran-2-one[4]

Other organic compounds
- *trans*-2-Methyl-2-butenal[4]
- Cyclohexanol[4]
- Propionic anhydride[3]
- 2-Nonanol[2,4,8]
- 1,4-Dimethylnaphthalene[3]
- Valeric anyhydride[3]

Other organic compounds
- 2-Oxocyclopentane carboxylic acid methyl ester[3]
- Phloroglucin (1,3,5-Trihydroxybenzene)[3]
- 4-Phenyl-1-butene[4]
- Methylbenzoate[4,8]
- 2,3-Dimethoxytoluene[2,4]
- Biphenylene[3]
- 4-Hydroxy-3-methoxybenzyl alcohol (vanillyl alcohol)[2]
- 4-Hydroxy-3-methoxyphenylethylalcohol (homovanillyl alcohol)[2]
- 1,2-Dimethoxy-4-propenylbenzene[4,7]
- 1,6-Anhydro-β-d-glucose-2,3,4-tribenzoate[3]
- 2-Hydroxy-1,4-napthoquinone[3]
- 2-(Hydroxymethyl)-anthraquinone[3]
- 2,5-Furandimethanol[3]
- Dibenzofuran[3]
- Thianaphthene[2,8]
- Tetramethylpyrazine[4]
- 7-Methylxanthine[3]

[a] See Table 2 footnote.

TABLE 5. ADDITIONAL COMPOUNDS TESTED IN SEED BIOASSAYS KNOWN TO AFFECT GERMINATION IN SPECIES OTHER THAN *Nicotiana attenuata*[a]

Organic compounds	Other organic compounds
Carbohydrates	Carbon dioxide
d(−)-Arabinose[3]	Urea[1,7]
d-Lyxose[3]	Thiourea[4]
d-(−)-Ribose[3]	Nicotinic acid[2]
d-(+)-Xylose[3]	Sodium acetate trihydrate[3]
d-Xyulose[3]	Methyl salicylate[4,8]
α-Cellulose[2]	Xylitol[3]
1,6-Anhydro-β-*d*-galactopyranose[3]	α-Naphthonitrile[4,7]
d-(+)-Cellobiose[2]	Umbelliferone[2]
Sucrose[3]	(−)-Menthol[4]
Palatinose[3]	2,4-Dinitrophenol[4]
d-(+)-Trehalose[3]	Methyl jasmonate[1,7]
Arabinogalactan[3]	Gibberellic acid (GA_3) (0.5%)
d-(+)-Cellotriose[3]	Lacase[2]
Xylan[2]	Peroxidase[2]
Starch[2]	Inorganic compounds
Maltotriose hydrate[4]	Hydrogen peroxide[3]
	Potassium cyanide[4,7]
	Hydroxylamine[4]

[a] See Table 2 footnote.

(offspring) were pooled and tested in the seed cup bioassays with seeds from the parents. Seeds from parent and offspring generations from the same populations were very similar in their germination responses to smoke extracts, as exhibited by the highly significant regression of offspring germination responses against parent germination responses (Figure 3; offspring, 0.990 ± 0.18; parent, 0.003 ± 0.13; $r^2 = 0.882$; $F_{1,4} = 30.04$; $P < 0.005$). Hence, the overall heritability of the responses to smoke extracts is very high; seeds from parent plants with a high degree of dormancy when tested with KNO_3 produce plants which in turn produce seed that also have a high degree of dormancy that can be removed by treatment with extracts of smoke. However, this analysis does not permit maternal effects to be separated from purely additive genetic effects.

Mechanism of Germination Stimulation. Seeds treated with smoke extracts or KNO_3 and examined with the scanning electron microscope did not look different (Figure 4). In some seeds, small fissures in the testa were apparent, but these were found in seeds from both treatments.

The results of the abrasion experiment (Figure 5) were analyzed with a two-way repeated measures ANOVA with abrasion and smoke treatments as main effects. Smoke-treated seeds had significantly higher germination rates

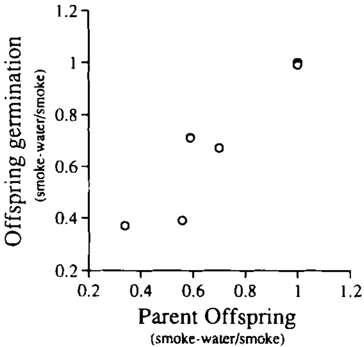


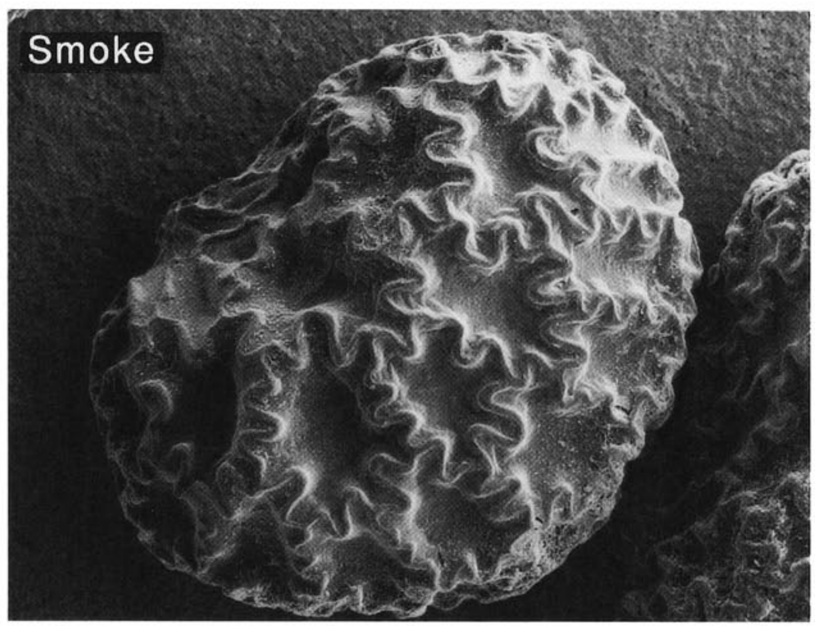
FIG. 3. Overall heritability of mean germination responses to smoke extracts of four replicates of 35 seeds from six populations of *N. attenuata* plants from southwest Utah. Germination responses to smoke are computed as the mean number of seeds germinating on day 9 in the smoke treatments minus the number germinating in the water (9.8 mM KNO_3) treatment expressed as a ratio of the number germinating in the smoke treatment in order to standardize for differences in the differences in seed viability among populations. Parents are seed collected from the field; offspring are the seeds produced after one generation of self-fertilizing plants grown from the parent seeds. Both parent and offspring seeds were tested at the same time. See text for regression statistics.

($F_{1,8}$ = 69.9; $P < 0.0001$) than those treated with KNO_3. The effect of abrasion was not significant ($F_{1,8}$ = 2.5; $P < 0.15$), and the abrasion–smoke interaction ($F_{1,8}$ = 0.08; P = 0.785) was also not significant.

Treatment of smoke extracts with 50 mM $NaSO_3$ did not significantly affect its ability to stimulate germination compared with seeds treated with either 50 mM $NaSO_3$ or 9.8 mM KNO_3 ($F_{2,10}$ = 78.2; $P < 0.0001$).

The results of these three experiments lead us to conclude that: (1) smoke extracts do not visibly damage the seed coat as does treatment with hypochlorite; (2) abrasion of the seed coat does not stimulate germination; and (3) smoke extracts treated to quench the organic oxidants stimulate germination in a manner similar to untreated extracts. We propose that these experiments demonstrate that the smoke extract does not stimulate germination via a mechanism involving scarification of the seed coat.

Seeds germinated with smoke extracts or hypochlorite and grown on sterile sand for seven days did not differ in biomass ($F_{1,38}$ = 0.05; P = 0.9), so we conclude that smoke extracts are not likely to function via a nutritive stimulation of germination. Hence, we are left with the hypothesis that smoke extracts are likely to stimulate germination with a smoke-specific signal molecule(s). Tetrazolium dye does not readily penetrate intact *N. attenuata* seed coats. When the seeds with abraded seed coats are treated with either smoke extracts or KNO_3



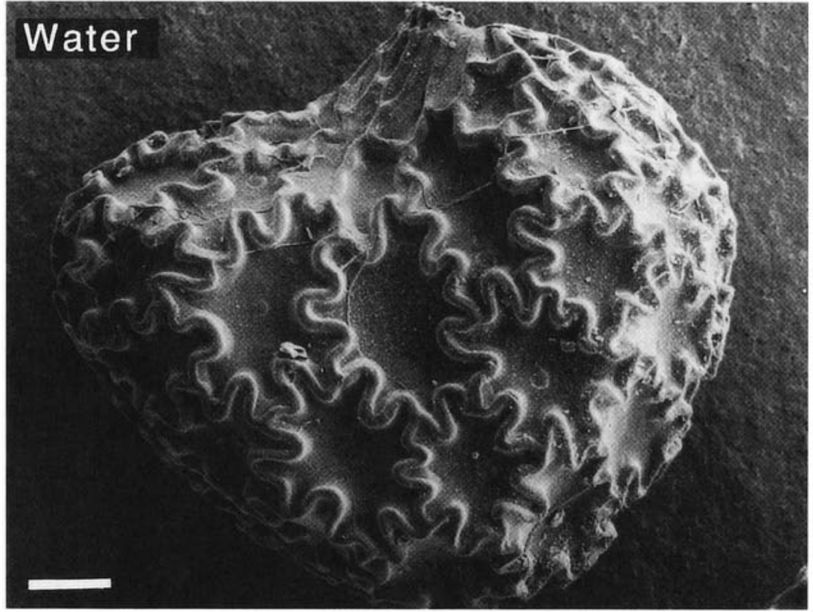


FIG. 4. Scanning electron micrographs of representative *N. attenuata* seeds after being soaked for 1 hr in a 1:300 dilution of liquid smoke in 9.8 mM KNO_3 (smoke) or only in 9.8 mM KNO_3 (water). Bar = 0.1 mm. Treatment with smoke extracts does not visably abrade the seed coat.

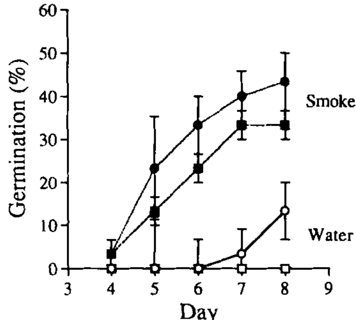


FIG. 5. Germination (mean ± SEM) percentages of four replicates of 10 *N. attenuata* seeds whose seed coats were abraded with a file over approximately 20% of the seed coat (squares) or left intact (circles) and either exposed to 1 : 300 dilution of liquid smoke in 9.8 mM KNO_3 (smoke; solid symbols) or 9.8 mM KNO_3 (water; open symbols). Mechanical scarification of the seed coat did not significantly affect germination. SEMs of the abraded seeds from water treatment are obscured by the plot symbol.

for 72 hr and soaked with 1% tetrazolium dye (Sigma Lot No. 111H0266), the embryo and endosperm of smoke-treated seeds turn bright red while KNO_3-soaked seeds remain pale. These results indicate that components of the smoke extract stimulate seeds to become metabolically active and are consistent with the smoke-specific signal molecule hypothesis.

Smoke Cues as Ecological Tools. The number of seedlings germinating in flats treated with smoke extracts or water were analyzed with two separate repeated-measures ANOVAs: the first analyzed the germination data through day 16, and the second analyzed the germination data from day 24 through the end of the experiment. Treatment of artificial seed banks with smoke extracts resulted in significant increases in seedlings germinating (Figure 6; $F_{2,12}$ = 41.98; $P < 0.0001$). Similarly, treatment of artificial seed banks with smoke extracts on day 24 that were previously treated with water also significantly ($F_{2,12}$ = 8.98; $P = 0.0041$) increased seedling emergence compared with artificial seed banks that were treated with water for the duration of the experiment. Treatment of artificial seed banks that had previously been treated with smoke extract with a second application of smoke extract did not significantly increase the rate of seedling emergence (Figure 6). Hence, dormant seeds that respond to cues in extracts of smoke were stimulated to germinate after the first application, but no further recruitment occurred with a second application.

The germination data from the cue-aging experiment were analyzed with a repeated-measures two-way ANOVA with time in soil and treatment as main effects (Figure 7). Both main effects were highly significant (treatment $F_{3,72}$ = 115; $P < 0.0001$; age of cue $F_{3,72}$ = 14.7; $P < 0.0001$), and the interaction

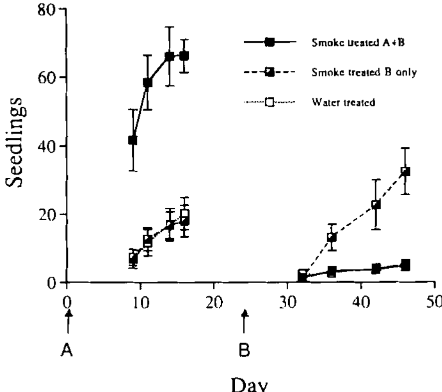


FIG. 6. Number (mean ± SEM) of *N. attenuata* seedlings emerging from five artificial seed banks each containing 117 mg of seed that were treated with either: (1) 500 ml of 300 : 1 aqueous dilution of liquid smoke on days 0 and 24; (2) 500 ml of 300 : 1 aqueous dilution of liquid smoke only on day 24 and 500 ml of distilled water on day 0; and (3) 500 ml of distilled water on days 0 and 24. On day 24, all seedlings were removed from all flats, and all flats were treated again with either water or smoke extracts as dictated by their treatment group. Plot symbols obscure SEMs on some dates.

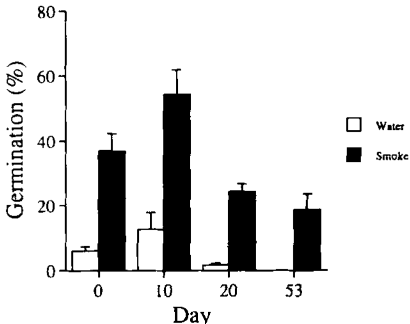


FIG. 7. The longevity of the germination potential of smoke-treated soil. Thirty-two flats of soil were treated either with 500 ml of 300 : 1 aqueous dilution of liquid smoke (black bars) or with 500 ml of distilled water (open bars) on day 0 in the greenhouse and watered weekly. On days 0, 10, 20, and 53, 100 *N. attenuata* seeds were planted in four smoke- and four water-treated flats and the germination (mean ± SEM) percentages 15 days after planting are depicted.

term was not significant ($F_{3,72} = 0.837$, $P = 0.477$). Hence, while the potential to stimulate germination of the smoke extract may change over time as the smoke-treated soil ages, the germination potential of smoke-treated soil retains its activity for at least 53 days.

DISCUSSION

Many fire-related cues are used by seeds of different species to synchronize the germination of dormant seeds with the postfire environment. These fire-related cues include mineral nutrients, heat, desiccation (Brits et al., 1993), charred wood, and components of wood smoke (Keeley, 1991). For the dormant seeds of *N. attenuata*, the components of wood smoke are clearly more important in stimulating germination than the other known fire-related cues. Although the chemical basis of the smoke-derived germination cues remains elusive, a number of important features of these germination cues have emerged from this study. Due to their chromatographic behavior, there are likely to be a number of active chemical structures that function at very low concentrations (less than 1 pg/seed). The structures are of intermediate volatility—those produced by the combustion of cellulose can be desorbed from Chromosorb 101 traps at temperatures above 125°C—are soluable in polar solvents, and tend to cochromatograph with methoxy phenols. Seeds will germinate after short exposures to aqueous extracts with the cue, and the germination cue remains active in soil for more than a month.

N. attenuata population are polymorphic in their degree of dormancy and the proportion of the seeds that respond to these cues. It is not known whether this polymorphism is related to the fire history of the population. The between-population variation in responsiveness to fire-related cues—particularly heat-related cues—has been found in other species (Stone and Juhren, 1953; Lotan, 1973). The variability among populations and the high degree of apparent heritability within populations of this trait suggest the tantalizing possibility that this trait could be transferred to agriculturally important crops in order to control germination and growth independently of water availability and temperature.

These smoke-derived germination cues may provide a useful experimental tool to ecologists for examining the factors thought to be important in the evolution of postfire plant communities. Various researchers (reviewed in Christensen and Muller, 1975a, b; Christensen, 1987; Keeley and Keeley, 1989) have argued that postfire plants, by synchronizing germination with the occurrence of fire, have been able to exploit: (1) a (potentially) elevated nutrient supply (particularly for nitrogen and phosphorus), (2) an allelochemical-free germination site, and reduced rates of attack from (3) herbivores and (4) pathogens. However, assessing the relative selective importance of these four factors is

difficult to do because fire simultaneously alters all four factors as well as many others. Yet, as we demonstrated with the artificial seed banks, we are able to stimulate germination in situ by applying aqueous extracts of smoke and therefore may be able to experimentally uncouple the above-mentioned selective factors from one another.

Clearly, we understand very little about the chemical ecology of the seed banks. Knowledge of the mechanisms that plants use to synchronize germination with particular environmental conditions will help us understand how plants disperse and aggregate in time.

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UP IN SMOKE: II. GERMINATION OF *Nicotiana attenuata* IN RESPONSE TO SMOKE-DERIVED CUES AND NUTRIENTS IN BURNED AND UNBURNED SOILS

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Abstract—*Nicotiana attenuata* is a native tobacco that is commonly found usually one growing season after fires in the blackbrush, sagebrush and piñyon-juniper forests of the Great Basin desert of North America. This plant also occurs in isolated dry washes and roadsides for many consecutive seasons. Postfire annuals are thought to synchronize their germination from the seed bank with the postfire environment in response to increases in (1) fire-related cues or (2) nutrient supply rates resulting from the mineralization of nutrients by fire, or (3) the removal of allelochemicals produced by the dominant vegetation occupying the sites before the burn or the microbial community associated with the dominant vegetation. We examine the effect of these three changes on the germination of *N. attenuata* seed from artificial seed banks made with burned and unburned soil taken in 1993 from under four dominant shrub species (*Coleogyne ramosissima*, *Yucca baccata*, *Lycium andersonnii*, *Purshia tridentata*) of an area that burned in 1992 and from two dry washes in which *N. attenuata* populations have persisted since at least 1988. We utilize our recent discovery that aqueous extracts of wood smoke contain potent germination cue(s) for this species and the established observation that nitrate stimulates germination in many *Nicotiana* species. In two experiments, we added smoke-derived germination cues and nutrients separately and in combinations to the artificial seed banks, measured germination rates, and inferred the effect of burning by the response of the seed banks to these additions. Germination rates of seed in burned soil were consistently higher than those in unburned soil collected from under all species tested; concentrations of nitrate, P, Mn, and Ca were also higher in burned than unburned soils. Because the addition of more cue and nitrate to burned soil increased germination rates, these soil components may not be at concentrations suffi-

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cient to saturate the germination response one year after a fire. The germination of seeds in soil collected from beneath unburned *Yucca bacatta* plants increased to the same level as that found under burned plants of the same species with the addition of cue and nitrate. Similarly, unburned bitterbrush soil attained the same germination potential with the addition of cue and a complete nutrient solution as burned bitterbrush soil. We conclude that the effect of fire on the germination potential of bitterbrush and yucca soil is due to increases in germination cue and nutrients. However, since the addition of cue and nutrients to the unburned soils under blackbrush and wolfberry shrubs did not elevate the germination potential of these soils to that found in burned soils, we conclude that these species alter the soil so as to inhibit germination and burning reverses this alteration. The presence of persistent *N. attenuata* populations in washes could not be attributed to particular chemical characteristics of these soils. Additions of cue dramatically increased germination potential of these soils, whereas the addition of nitrate did not. The concentrations of most mineral nutrients resembled those found in the unburned sites with the exception of Mn, Cu, and Zn, which were higher. However, these cations do not influence germination rates. Treatment of soil taken from these washes with cue resulted in significant increases in germination of *N. attenuata* seeds in the natural seed bank compared to water-treated controls, demonstrating that the plants growing in washes also produce dormant seeds that require the smoke cue for germination.

Key Words—Fire, seed dormancy, *Nicotiana attenuata*, smoke, germination cues, allelopathy, *Coleogyne ramosissima*, *Yucca baccata*, *Lycium andersonnii*, *Purshia tridentata*.

INTRODUCTION

Fire creates opportunities for colonization for many annual plants that may not otherwise be present in a community. A number of hypotheses have been proposed to explain the presence of annuals in the immediate postfire environment and their exclusion from later successional stages, including: (1) chemical inhibition by shrubs; (2) nutrient regimes or other abiotic environmental features altered by fire; (3) changes in herbivore and pathogen populations that prevent seedling establishment; and (4) production of fire-related cues that stimulate germination (Went et al., 1952; Keeley, 1991). This mixture of proximate and ultimate explanations for the dramatic response of the seed bank to fire represents hypotheses that address both how dormant seeds cue their germination to the postfire environment and which selective forces have led to the evolution of the postfire annual habit. Understanding the proximate cues that seed banks use to time germination would greatly facilitate the experimental examination of why the postfire environment is a habitat worth tracking.

Increases in inorganic nutrients, heat, and unidentified compounds associated with blackened (charred) wood and destruction of allelochemicals that inhibit germination and oxidation of the seed coat are direct consequences of fire and

have been identified as proximate germination cues used by the seeds of some species found to flourish after fires (Went et al., 1952; Christensen and Muller, 1975a,b; Wicklow, 1977; Keeley et al., 1985; Keeley and Pizzorno, 1986; Emery, 1992). Baldwin et al. (1994) have found that components of wood smoke stimulate germination in dormant *Nicotiana attenuata* (Solanaceae) seeds, a native tobacco found as a summer annual throughout southwestern North America (Goodspeed, 1954; Brotherson et al., 1980). Seeds will germinate after short-term (less than a minute) exposures to cooled wood smoke and polar extracts of wood smoke. The species of fuel used appears to have little influence on the response, and even the combustion of pure cellulose produces a significant germination response (Baldwin et al., 1994). However, the demonstration that a species responds to particular compounds with increased germination rates in the laboratory does not discount the importance of other proximate mechanisms for stimulating germination in the field.

The role of allelopathy in controlling germination has received considerable attention (see references in Keeley et al., 1985; Keeley and Keeley, 1989). In many of these studies, leachates from the dominant shrubs were applied to seeds in laboratory bioassays with little or no documentation of how accurately these extracts represent the chemical composition of the soil. Here we use the smoke-derived germination cues in order to examine the roles of nutrient increases and the removal of allelopathic compounds from soil by fire as proximate germination cues for *N. attenuata* seeds. In two experiments, we add smoke-derived germination cues and nutrients separately and in combinations to the artificial seed banks, measure germination rates, and infer the effect of burning by evaluating the response of the seed banks to these additions. We propose that allelochemicals inhibit germination if the addition of cue and a complete nutrient solution does not produce the largest observed germination response from a particular burned–unburned soil comparison. The prospects of using these smoke-derived germination cues as an experimental tool to compare the germination potential of different soils appear promising because we have demonstrated that: (1) the cues remain active for at least 53 days in soil under greenhouse conditions and (2) the application of aqueous extracts of smoke to soil containing seeds results in dramatic increases in germination of artificial seed banks (Baldwin et al., 1994).

Nicotiana attenuata plants can be found in populations in isolated washes that persist for many growing seasons (Wells, 1959; Baldwin unpublished data) or as an ephemeral component (usually for less than one growing season) of the postfire annual community in burned sagebrush, blackbrush, and piñyon–juniper forests of the Great Basin desert (Wells, 1959; Barney and Frischknect, 1974; Britton and Ralphs, 1978; Young and Evans, 1978; Wright et al., 1979; Wright and Bailey, 1982; Koniak and Everett, 1982; Koniak, 1985). We ask how a fire changes the germination potential of soils found under the dominant shrubs of

the blackbrush community and determine whether the fire-related edaphic factors can explain the persistence of populations in dry washes.

METHODS AND MATERIALS

Site Descriptions. A fire started with a lightening strike in a canyon bottom (elevation approx. 1160 m) on August 10, 1992 (personal communication H. Fletcher) in section 10 of T40S R19W located in southwest Utah and spread up a steep ridge to approx. 1400 m elevation, burning approx. 20 ha, and extinguishing itself within a week. The vegetation cover was predominately blackbrush (*Coleogyne ramosissima* Torr.), banana yucca (*Yucca bacatta* Torr.), wolfberry (*Lycium andersonnii* Gray), and bitterbrush [*Purshia tridentata* (Pursh) DC]. Only two junipers [*Juniperus osteosperma* (Torr.) Little] were burned in the fire. The fire spread into the "blackbrush site" of Bowns' (1973) research site, and the soil chemistry and taxonomy, precipitation data, and air temperature data for this area are thoroughly described (Bowns, 1973; Bowns and West, 1976). The vegetation composition for the area is described in Christian (1962). The soil is classified as Typic Paleorthid, loamy, mixed, thermic, and shallow and named the Cave Gravely Loam Series on slopes of 2–7% (Bowns, 1973). However, since most of the burn occurred on steeper slopes, this name may not apply.

From 1988 to 1992, no *N. attenuata* plants were found growing in the area that was burned in 1992, and neither Bowns nor Christian mention this species in their list of herbs growing in the blackbrush community of this area. However, during the growing season following the fire, over 70 plants were found growing among the charred stumps in the burned area. *N. attenuata* can also be found in persistent populations in dry washes and streambeds. We have observed (Baldwin unpublished data; Baldwin and Ohnmeiss, 1993) populations of plants growing every year since 1988 in nine washes located in the Motoqua, Goldstrike, and Shivwits area (T40S R17W–T41S R19W) of southwest Utah.

Soil Collection. The A_1 soil horizon layer barely exists between shrubs in this area and can be 5–10 cm thick directly underneath shrubs. Approximately 50 kg of A_1 layer was removed from beneath five randomly selected plants of each of the four dominant shrub species from within the burned area. Species were identified from the burned stumps or the leaves sprouting from the base of the blackened stumps. Soil samples were also collected from five plants of each of the four species from the adjacent unburned area within 60 m of the perimeter of the burned area but not closer than 5 m to the burned area. Voucher specimens were collected for each species. Soil samples from the upper 5 to 10-cm soil layer were removed on June 2–4, 1993, and stored in plastic bags for chemical analysis and use in artificial seed bank experiments (see below).

In addition, approx. 50 kg of soil were also collected from nine washes where large populations (>75) of plants were found growing and had been found every year since 1988. Soil was collected on June 4, 1993, after the spring germination had occurred and before any plants had started to produce flowers and seed. Hence any seeds present in this soil collection were from 1992 or earlier. Soil from washes with a small and large seed bank (washes 2 and 6, respectively, in Figure 1) was sampled for chemical analysis, sieved to remove *N. attenuata* seeds, and used in artificial seed bank experiments (see below).

Examination of the Natural N. attenuata *Seed Bank.* On June 5, 1993, 30 kg of all 49 soil collections (burned and unburned sites and washes) were divided into equal portions and spread into four 43-cm × 20-cm flats. Flats were arranged in rows in an open field on the property of the DI ranch (T40S R19W). Two trays from each soil collection were sprayed with 2 liters of water and two flats were sprayed with 1:50 aqueous dilution of liquid smoke (House of Herbs, Inc., Passaic, New Jersey 07055). Flats were watered twice daily, and the number of *N. attenuata* seedlings emerging were recorded daily for 20 days.

Soil Nutrient Analysis. A 1-kg aliquot of all soils was ground to a fine powder (850-μm mesh) in a Wiley Mill (Thomas Scientific) prior to chemical analysis and use in artificial seed banks. Chemical analyses were performed at the Cornell Nutrient Analysis Laboratories (Cornell University, 804 Bradfield Hall, Ithaca, New York 14853) using standard procedures. Briefly, samples were analyzed for pH (of 1 : 1 v/v soil–0.01 M $CaCl_2$ suspension), organic matter (by loss on ignition at 500°C for 2 hr), and "available" nutrients in oven-dried (110°C) samples by extraction with Morgan's solution (10% sodium acetate

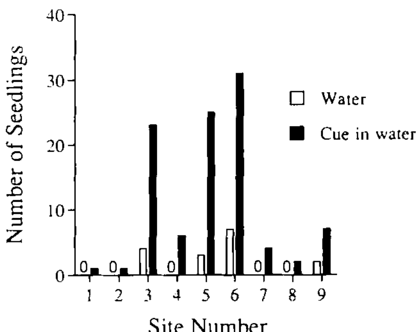

FIG. 1. Number of *N. attenuata* seedlings emerging from soil collected from nine dry washes where persistent populations exist. Soils were collected and treated either with water (open bars) or with a 1:50 aqueous dilution of liquid smoke (solid bars) and the number of seedlings emerging was monitored for 20 days. Soils that produced no seedlings are labeled with a 0.

buffered to pH 4.8 with 1:5 v/v soil–solution). K, Mg, Ca, Mn, Fe, Al, and Zn were determined by atomic absorption; P and N in NO_3 were determined colorimetrically by stannous chloride and hydrazine reduction, respectively. All nutrient values are expressed as milligrams per kilogram.

Artificial Seed Bank Experiments. Two experiments were performed in the laboratory with artificial seed banks created with soil collected in the field to which seeds from glasshouse-grown plants exhibiting a high degree of dormancy were added. Because *Nicotiana tobaccum* seeds are known to germinate in the presence of NO_3 at concentrations greater than 1 mM (Karssen and Hilhorst, 1992; Bewley and Black, 1982) and because burning increased soil nitrate concentrations (see Results), the first experiment examined the ability of increased nitrate and germination cue concentrations in soil after burning to explain the increased germination potential of burned soils. We propose that if additions of both nitrate and germination cue to burned soils increased the germination potential of these soils, then cue and nitrate are not at concentrations that saturate the germination response. We have established that a 1:300 dilution (v/v) of liquid smoke in 9.8 mM KNO_3 provides saturating concentrations of germination cue and nitrate for the germination of *N. attenuata* seeds (Baldwin et al., 1994). Second, we propose that if the addition of saturating amounts of nitrate and cue to unburned soils increases the germination potential of these soils to that found in similarly treated burned soils, then the effect of fire on a soil's germination potential can be adequately described by increases in these compounds. In the first experiment, three treatment groups were used: (1) 9.8 mM KNO_3; (2) 1:300 v/v liquid smoke in 9.8 mM KNO_3; and (3) 1:300 v/v liquid smoke in double-distilled water. Hence, contrasts of germination rates of seed banks in treatment groups 1 and 2 describe the effect of cue addition with saturation nitrate concentrations and contrasts with treatments 2 and 3 describe the effect of nitrate with saturating amounts of cue. Experiment 1 consisted of 42 soil collections (five collections of four species from burned and unburned sites plus two washes) $\times$ 3 treatments $\times$ 5 replicates = 630 artificial seed banks.

A second experiment was performed on those soils in which the effect of burning could not be described by changes in nitrate and germination cue, specifically, blackbrush, bitterbrush, and wolfberry soils. In this experiment, two treatment groups were used: (1) 1:300 v/v dilution of liquid smoke in a complete nutrient solution and (2) 1:300 v/v dilution of liquid smoke in double distilled water. The complete nutrient solution consisted of 9.8 mM KNO_3 in a nitrogen-free nutrient solution. The nitrogen-free nutrient solution consisted of 0.129 g/liter $CaSO_4 \cdot 2H_2O$, 0.123 g/liter $MgSO_4 \cdot 7H_2O$, 0.048 g/liter K_2HPO_4, 0.031 g/liter KH_2PO_4, 1.393 mg/liter $FeSO_4 \cdot 7H_2O$, 1.863 mg/liter $Na_2EDTA \cdot 2H_2O$, and 0.5 ml/liter of a micronutrient solution, consisting of 2.533 g/liter H_3BO_3, 1.634 g/liter $MnSO_4 \cdot 1H_2O$, 0.151 g/liter $Na_2MnO_4 \cdot 2H_2O$, 0.440 g/liter $ZnSO_4 \cdot 7H_2O$, 0.080 g/liter $CuSO_4 \cdot 5H_2O$, and 0.020 g/liter

$CoCl_2 \cdot 6H_2O$. This experiment examined the role of other nutrients in addition to nitrate that are potentially elevated by fire in explaining the increased germination potential of burned soils. We propose that if the addition of the complete nutrient solution and cue to unburned soils increases the germination potential of these soils to that found in similarly treated burned soils, then the effect of fire on a soil's germination potential can be adequately described by the increases in cue and plant nutrients. If, however, the germination potential of unburned soils was still lower than that of burned soils after the addition of cue and nutrients, we propose that these soils contain allelopathic factors that fire destroys. Experiment 2 consisted of 30 soil collections (five collections of three species from burned and unburned sites) $\times$ 2 treatments $\times$ 5 replicates = 300 artificial seed banks.

Nicotiana attenuata seeds used in the artificial seed bank experiments were from the second generation of glasshouse-grown plants grown from seed collected from plants in 1988 on the DI Ranch. Seed from glasshouse-grown plants were used in order to avoid infestation of the negro bug (*Corimelaena* spp.). The original population of plants was found growing among charred stumps of blackbrush in a gravel wash that had burned the previous year in a small garbage fire.

In both experiments, five replicate seed banks for each treatment of each soil type was prepared. Each replicate seed bank consisted of 10 seeds placed on the surface of 6 g of dry soil in a soufflé cup (Solo 1 oz., P100). Cups were treated with 3.0 ml of the appropriate solution. Treatments with nitrate therefore received 0.67 mg N as NO_3 in addition to that already present in the soil (which varied between 0 and 0.2 mg N as NO_3). Cups were sealed with transparent lids (Solo PL1 lids) and placed in a growth chamber (Sherer Controlled Environmental Lab, model cel.37-14, Marshall, Michigan) under conditions that maximized the germination of *N. attenuata* seeds (Baldwin et al., 1994), specifically, 14L:10D photoperiod with 200 $\mu M/m^2/sec$ PAR, 30°C day–22°C night temperature cycle. Artificial seed banks were rotated daily among positions within the growth chamber, after 48 hr in the growth chamber, seeds were examined daily for germination until day 7. Germination was defined as the splitting of the seed coat and the emergence of the radicle as observed with a head-mounted 10$\times$ magnifying lens. The percentage of all seeds germinating at each count for each seed bank was calculated and arcsine transformed for normality. Repeated-measures three-way ANOVAs on the transformed percentages were used to analyze main effects, and multivariate contrasts were performed from these ANOVAs to test specific hypotheses. The number of seedlings emerging from smoke-treated and control soils in the examination of natural *N. attenuata* seed banks was analyzed with the χ^2 test. Analysis was performed with the MGLH ANOVA module from Systat Inc. (Evanston, Illinois).

RESULTS

Natural N. attenuata *Seed Banks:* No *N. attenuata* seedlings emerged from any of the soil samples collected from either the burned or unburned, but otherwise undisturbed sites; hence the natural seed bank was clearly very small in this area. In contrast, a number of seedlings emerged from the soil collected from washes where persistent populations existed (Figure 1). Treatment of these soils with smoke cue resulted in a significant increase in the number of seedlings emerging compared with soils treated only with water ($\chi^2 = 60.83 P < 0.001$). Because the soil samples were taken in 1993 before any plants had flowered, the seeds in the seed bank were from the 1992 growing season and earlier. Moreover, because the soil samples were collected after the spring germination, we conclude that some proportion of the seed produced by plants growing in persistent populations in washes are dormant and require the smoke cue for germination.

Soil Chemistry. Burning significantly increased the concentrations of NO_3 N, P, Mn, Ca, and Mg in soil collected from under all species (Figure 2, Table 1) as determined by the two-way ANOVAs. Burning increased the K concentrations in yucca soils but decreased it in wolfberry soils as determined by the significant burning × species interaction (Table 1; Figure 2). Significant differences among the soils collected from under the different species were found for P, K, Ca, and Mg concentrations (mean ± SEM) and for soil pH (Table 1). No significant burn, species or interaction effects were found for Fe (0.345 ± 0.40 mg/kg), Cu (0.01 ± 0.007 mg/kg), Al (16.6 ± 0.6 mg/kg) and Zn (0.158 ± 0.021 mg/kg) concentrations or organic matter content (7.14 ± 0.65; all $Ps > 0.24$). The soil samples from the two washes were significantly ($Ps < 0.05$) higher in Mn (20.35 ± 0.950 mg/kg), Zn (0.69 ± 0.53 mg/kg), Cu (0.10 ± 0.10 mg/kg) concentrations and higher in pH (7.89 ± 0.07) than the soil samples found in the area adjacent to the burn.

Artificial Seed Bank Experiment 1. The germination rates of the artificial seed banks created from soil taken from the two washes were analyzed separately from the burned site data because the washes did not have a burn status treatment. From the three-way repeated-measures ANOVA it is clear that germination rates from artificial seed banks made with burned soil were consistently higher those made with unburned soil (Figure 3, Table 2). There were also significant differences among species and treatments and significant burn status × species and burn status × species × treatment interactions (Table 2), which were examined with the following contrasts. We were principally interested in the three following questions: (1) Does the addition of cue with saturating amounts of nitrate increase germination? (2) Does the addition of nitrate with saturating amounts of cue increase germination? and (3) For each soil type, can the addition of saturating amounts of both cue and nitrate to unburned soils

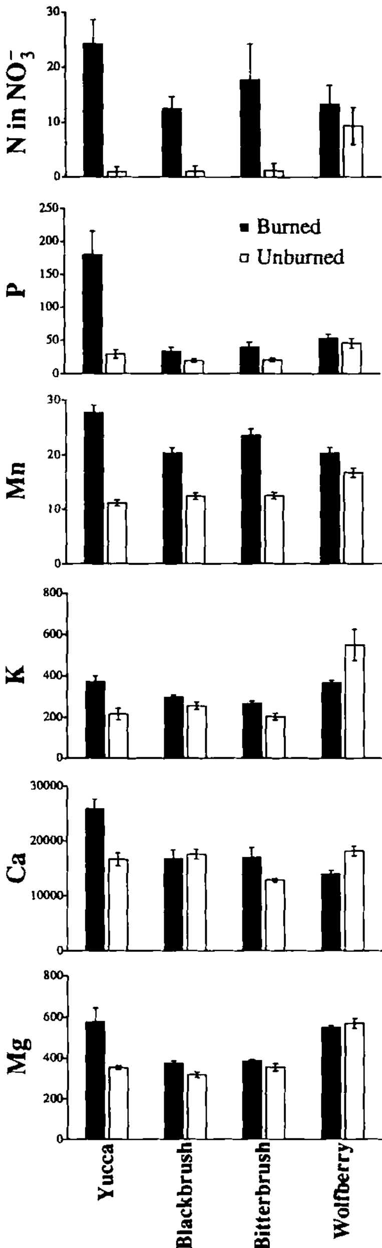

FIG. 2. Concentration (mean mg/kg ± SEM) of soil nutrients of the A_1 layer of soil taken from under five burned (solid bars) and unburned (open bars) yucca, blackbrush, bitterbrush, and wolfberry shrubs. Soil samples were collected in June 1993 and the burn occurred in August 1992.

TABLE 1. ONE-WAY ANOVA ON NO_3, P, MN, K, CA, MG, AL, AND pH OF A_1 LAYER OF SOIL TAKEN FROM UNDER BURNED AND UNBURNED YUCCA, BLACKBRUSH, BITTERBRUSH, AND WOLFBERRY SHRUBS.

Source of Variation	*df*	MS	*F*	*P*
(a) Nitrate (mg/kg^{-1})				
Burn status	1	1898.3	32.182	<0.001
Species	3	64.0	1.085	0.370
Burn status × species	3	165.6	2.807	0.055
Error	32	60.0		
(b) Phosphorus (mg/kg^{-1})				
Burn status	1	22662.4	23.528	<0.001
Species	3	13035.6	13.533	<0.001
Burn status × species	3	11736.6	12.185	<0.001
Error	32	963.2		
(c) Manganese (mg/kg^{-1})				
Burn status	1	963.3	30.471	<0.001
Species	3	16.6	0.524	0.669
Burn status × species	3	74.7	2.363	0.090
Error	32	31.6		
(d) Potassium (mg/kg^{-1})				
Burn status	1	4202.5	0.801	0.378
Species	3	96127.9	18.315	<0.001
Burn status × species	3	52566.2	10.015	<0.001
Error	32	5248.7		
(e) Calcium (mg/kg^{-1})				
Burn status	1	43699800	5.633	0.024
Species	3	75352800	9.712	0.001
Burn status × species	3	86271500	11.120	<0.001
Error	32	7758540		
(f) Magnesium (mg/kg^{-1})				
Burn status	1	52722.1	13.777	<0.001
Species	3	93656.6	24.473	<0.001
Burn status × species	3	28336.3	7.405	<0.001
Error	32	3826.9		
(g) Aluminum (mg/kg^{-1})				
Burn status	1	4.096	1.149	0.292
Species	3	22.410	6.285	<0.001
Burn status × species	3	2.818	0.790	0.508
Error	32	3.566		
(h) pH				
Burn status	1	0.025	1.714	0.200
Species	3	0.046	3.141	<0.001
Burn status × species	3	0.018	1.196	0.327
Error	32	0.015		

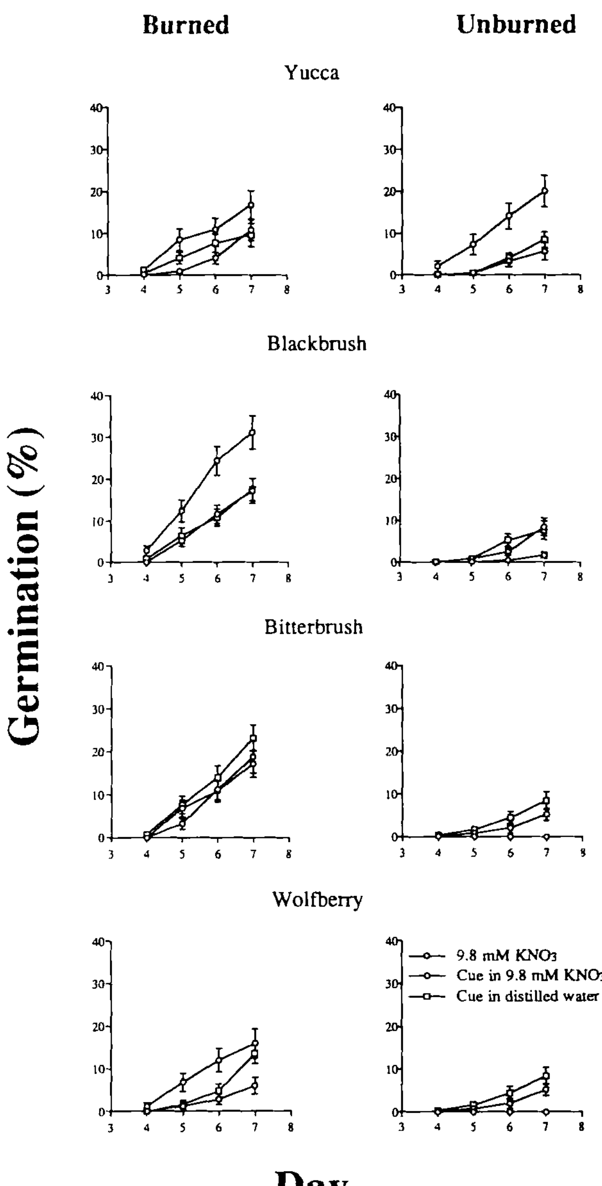

FIG. 3. Percentage germination (mean ± SEM) of 25 replicate artificial seed banks each containing 10 seeds on days 4–7 after treatment. Seed banks were made with soil collected from under five burned (left column) and five unburned (right column) yucca, blackbrush, bitterbrush, and wolfberry shrubs; five replicate seed banks were made from each soil collection. Seed banks were treated with either smoke cue in distilled water, cue with nitrate, or nitrate. The amounts of cue and nitrate are sufficient to saturate the germination response in sand. See Table 2 for statistical analysis of data.

TABLE 2. THREE-WAY REPEATED MEASURES ANOVA ON ARCSINE-TRANSFORMED PERCENT GERMINATION (DAYS 4–7), OVERALL MULTIVARIATE CONTRASTS TESTING EFFECTS OF CUE AND NITRATE ADDITION ACROSS BURN TREATMENTS AND SPECIES, AND MULTIVARIATE CONTRASTS BETWEEN BURNED AND UNBURNED SOILS BY SPECIES FOR TREATMENT CONTAINING SATURATING AMOUNTS OF CUE AND NITRATE.

Source of variation	*df*	MS	*F*	*P*
(a) Univariate between subjects effects				
Burn status	1	0.567	81.943	<0.001
Species	3	0.043	6.169	<0.001
Treatment	2	0.176	25.394	<0.001
Burn status × species	3	0.070	10.161	<0.001
Burn status × treatment	2	0.001	0.193	0.825
Species × treatment	6	0.010	1.390	0.216
Burn status × species × treatment	6	0.022	3.107	0.005
Error	576	0.007		
(b) Multivariate contrasts: Overall effect of cue and nitrate treatments				
Nitrate with cue versus nitrate in water	4, 573		13.930	<0.001
Cue with nitrate versus cue without nitrate	4, 573		4.932	<0.001
(c) Multivariate contrasts for the cue with nitrate treatment				
Burned vs. unburned—yucca	1, 576		1.377	0.241
Burned vs. unburned—blackbrush	1, 576		12.091	<0.001
Burned vs. unburned—bitterbrush	1, 576		7.583	<0.001
Burned vs. unburned—wolfberry	1, 576		4.064	0.003

produce germination rates that are equivalent to those found in similarly treated burned soil? In previous experiments (Baldwin et al., 1994) we had determined the concentrations of cue and nitrate that saturated the germination response of seeds placed in sterile sand. These three questions were examined with multivariate contrasts performed from the three-way repeated-measures (ANOVA) (Table 2). Significant effects of both cue with an excess of nitrate and nitrate with an excess of cue were found across species and burn treatments, demonstrating that these soils did not contain quantities of cue and nitrate that saturated the germination response. The addition of saturating amounts of cue and nitrate to unburned yucca soils was able to increase the germination rates to values that were statistically not distinguishable ($P = 0.241$; Table 2) from those of similarly treated burned yucca soils. This result demonstrates that the effect of burning on yucca soils can be explained in terms of increases of nitrate and cue in the soil. However, the addition of cue and nitrate did not have the same effect on soils collected from under blackbrush, bitterbrush, or wolfberry shrubs, which still had significantly lower germination rates (Ps < 0.003; Table 2) in unburned

soils than in burned soils when saturating amounts of cue and nitrate were added. We conclude that the effects of fire can not be explained solely in terms of cue and nitrate additions and explore these differences further in experiment 2.

The germination rates of seed in the artificial seed banks made with the soil taken from the two washes with persistent populations are depicted in Figure 4, and the germination data for days 5–7 were analyzed with a repeated measures two-way ANOVA. The day-4 germination data were not included in the analysis due to the large number of 0% germination values on this day. A significant treatment effect was found in the repeated measures two-way ANOVA (Table 3). This treatment effect was due to a significant increase in germination in response to cue in an excess of nitrate as determined by the significant multivariate contrast (Table 3). The multivariate contrast for the effect of nitrate with an excess of cue was not significant (Table 3). We conclude that germination in washes is limited primarily by the lack of smoke-derived germination cue in the soil.

Artificial Seed Bank Experiment 2. From the three-way repeated-measures

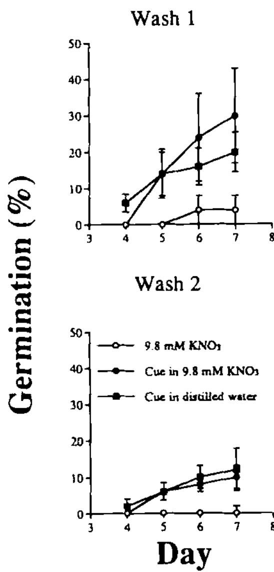

FIG. 4. Percentage germination (mean ± SEM) of five replicate artificial seed banks each containing 10 seeds on days 4–7 after treatment. Seed banks were made with soil collected from two dry washes with persistent populations of *N. attenuata*. Seed banks were treated with either smoke cue in distilled water, cue with nitrate, or nitrate at concentrations sufficient to saturate the germination response in sand. See Table 4 for statistical analysis of data.

TABLE 3. TWO-WAY REPEATED-MEASURES ANOVA ON ARCSINE-TRANSFORMED PERCENT GERMINATION (DAYS 5–7), AND MULTIVARIATE CONTRASTS TESTING OVERALL EFFECT OF CUE AND NITRATE TREATMENTS.

Source of variation	*df*	MS	*F*	*P*
(a) Univariate between subjects effects				
Site	1	0.309	2.931	0.100
Treatment	2	0.819	7.777	0.003
Site × treatment	2	0.048	0.457	0.639
Error	24	0.105		
(b) Multivariate contrasts: Overall effect of cue and nitrate treatments				
Nitrate with cue versus nitrate in water	3, 22		4.293	0.016
Cue with nitrate versus cue without nitrate	3, 22		0.314	0.815

ANOVA it is clear that germination rates from artificial seed banks made with burned soil were again consistently higher than those made with unburned soil (Figure 5, Table 4). Significant differences were found among species but the main treatment effect was not significant (Table 4). A significant burn status × treatment interaction was found (Table 4), and inspection of the data revealed that the only apparent treatment effect was in the unburned bitterbrush soils (Figure 5). We constructed a contrast from the ANOVA to ask the following question: For the bitterbrush soil type, does the addition of both cue and a complete nutrient mixture to unburned soils produce germination rates that are equivalent to those found in similarly treated burned soil? The multivariate contrast that compared the germination rates of burned and unburned bitterbrush soil treated with cue and complete nutrients were not significantly different ($P = 0.452$; Table 4). Hence, we conclude that the effect of fire on the germination potential of soil collected from under bitterbrush shrubs can be explained by increases in nutrients and cue in the soil. However, because the addition of cue and nutrients did not have the same effect on soils collected from under blackbrush and wolfberry shrubs, we conclude that these changes are not sufficient to account for the higher germination potential of burned soils from those two species. We propose that this result constitutes circumstantial evidence for the existence of allelopathic factors produced by these shrubs that can be removed by burning.

DISCUSSION

Because the addition of saturating amounts of germination cue and nitrate increased the germination potential of many of the burned soils, we conclude that burned soils did not contain quantities of cue and nitrate that saturated the

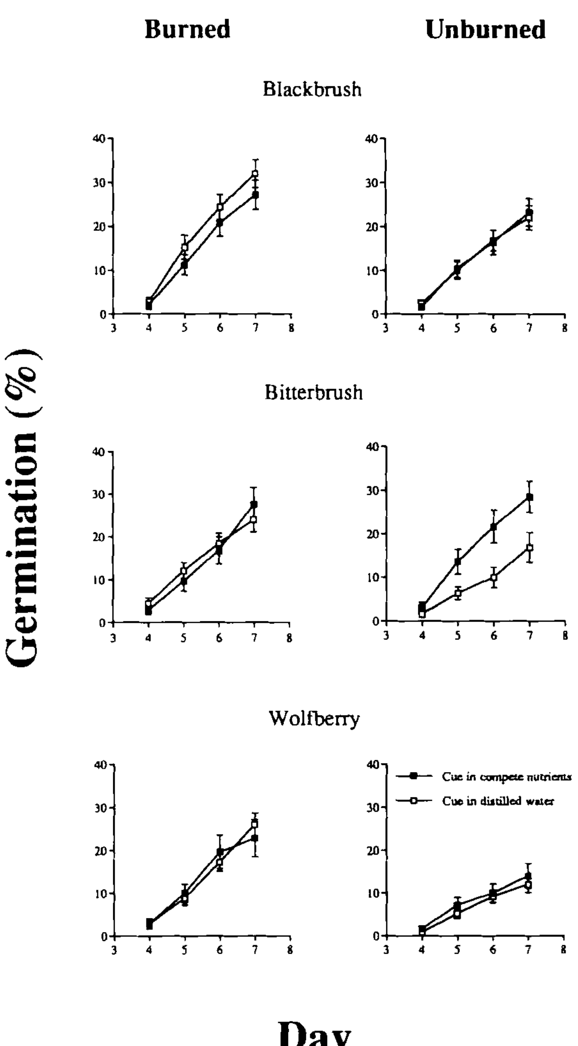

FIG. 5. Percentage germination (mean ± SEM) of 25 replicate artificial seed banks each containing 10 seeds on days 4–7 after treatment. Seed banks were made with soil collected from under five burned (left column) or unburned (right column) blackbrush, bitterbrush, and wolfberry shrubs. Seed banks were treated with either smoke cue in distilled water or cue with a complete nutrient solution. See Table 3 for statistical analysis of data.

TABLE 4. THREE-WAY REPEATED MEASURES ANOVA ON ARCSINE-TRANSFORMED PERCENT GERMINATION (DAYS 4–7), AND MULTIVARIATE CONTRAST FOR CUE WITH COMPLETE NUTRIENT TREATMENT FOR SOILS GATHERED UNDERNEATH BURNED AND UNBURNED BITTERBRUSH SHRUBS

Source of variation	*df*	MS	*F*	*P*
(a) Univariate between subjects effects				
Burn Status	1	1.374	11.434	<0.001
Species	2	0.516	4.298	0.015
Treatment	1	0.008	0.066	0.798
Burn Status × Species	2	0.096	0.802	0.450
Burn Status × Treatment	1	0.495	4.116	0.043
Species × Treatment	2	0.186	1.548	0.215
Burn Status × Species × Treatment	2	0.127	1.058	0.349
Error	288	0.120		
(b) Multivariate contrasts for cue with complete nutrient treatment				
Burned vs. unburned—bitterbrush	4, 285		0.921	0.452

germination response one year after the fire. Since (1) both nitrate and the germination cue are water soluable (Baldwin et al., 1994), (2) a majority of the yearly precipitation falls during the winter months for this area (Bowns and West, 1976) and the winter after the burn was one of the wettest in eight years (H.L. Fletcher, personal communication), and (3) the burned area is on the side of a steep canyon, it would not be surprising if a substantial proportion of the germination cue and nitrate that was deposited in the soil immediately after the burn had leached out of the soil by the time soil samples were collected the next season. Hence, we should view the germination potential of burned soils that result from the addition of cue and nutrients as representing a fraction of what might be found immediately after a fire. In laboratory experiments we have not been able to rinse the germination cue from seed once it was exposed to smoke and that the cue binds tightly to the seed (Baldwin et al., 1994), indicating that, despite its water solubility, the germination cue might not be easily leached from the seeds in the seed bank.

We conclude that the postfire germination of *N. attenuata* from the seed bank in soil under yucca and bitterbrush shrubs can be explained by increases in smoke-related germination cues and nutrients that occur for at least one growing season after a fire. However, for seed banks in soil under blackbrush and wolfberry shrubs, fire changes other aspects about these soils in addition to the concentration of cue and nutrients. These other factors may be allelochemicals produced by these shrubs or the associated microbial community, which burning

eliminates (Kaminsky, 1981). We have identified a number of chemicals from wood smoke that function as inhibitors of germination (Baldwin et al., 1994), but because burned soils have higher germination rates that unburned soils, the effect of these smoke-related inhibitors must be less than that of the putative inhibitors present in unburned soils. The approach we have used to identify the presence of germination inhibitors in unburned soils has the distinct advantage over the approach used in other studies (refs. in Keeley and Keeley, 1989) in that it does not rely on the use of leachates which may not reflect the chemical composition of the soil in the natural seed bank.

We also conclude that the presence of persistent populations of *N. attenuata* in washes and dry stream beds can not be explained by particular chemical characteristics of these soils or by the hypothesis that these plants do not produce dormant seeds. Plants found in persistent populations produce both dormant seeds that require exposure to chemicals present in wood smoke for germination and nondormant seeds that germinate whenever the conditions for growth are adequate. The persistence of *N. attenuata* populations in particular washes may therefore be due to lower herbivory rates and higher moisture regimes.

Although our experiments demonstrate that smoke-related germination cues, nutrients, and the destruction of putative allelopathic compounds are all potentially involved in the postfire germination response of the seed bank, the germination cue is likely the most important for the following reasons: addition of germination cue with either an excess of nitrate (experiment 1) or complete nutrients (experiment 2) accounted for a larger proportion of the variance in germination rates than did the addition of nutrients with an excess of germination cue as determined by the multivariate contrasts (Tables 2 and 3). Moreover, the addition of only the germination cue produced a large response from the natural seed banks taken from the washes (Figure 1) and the artificial seed banks made with soil from these washes (Figure 4) did not respond significantly to nitrate additions. If the smoke-derived germination cue is indeed the most important factor in stimulating dormant *N. attenuata* seeds to germinate from the seed bank, this cue may prove to be an important tool in determining the sizes and viabilities of seed banks and the rules governing germination from seed banks (Philippi, 1993). Moreover, by causing dormant seeds to germinate in situ without lighting a fire, this cue could be used to "uncouple" the selective factors (see Introduction) thought to be responsible for the evolution of the postfire annual habit. However, in situ experiments will have to await the identification and purification of the germination cue because many of the compounds identified from the crude smoke fractions (Baldwin et al., 1994), which are not responsible for the germination response, have antibiotic and antifungal (Ho et al., 1992; Wendorff et al., 1993) properties and would therefore confound the uncoupling experiments.

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DISTANCE OF RESPONSE TO HOST TREE MODELS BY FEMALE APPLE MAGGOT FLIES, *Rhagoletis pomonella* (WALSH) (DIPTERA: TEPHRITIDAE): INTERACTION OF VISUAL AND OLFACTORY STIMULI

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Abstract—Mature female apple maggot flies, *Rhagoletis pomonella* (Walsh), were released individually onto a single potted, fruitless hawthorne tree in the center of an open field. The tree was surrounded by four 1-m^2 plywood host tree models painted green or white, with or without synthetic host fruit odor (butyl hexanoate), and placed at one of several distances from the release tree. Each fly was permitted to forage freely on the release tree for up to 1 hr, or until it left the tree. Flies left the tree significantly sooner when green models with host fruit were present at 0.5, 1.5, or 2.5 m distance from the release tree than when these models were placed at a greater distance (4.5 m) from the release tree or when no models were present. Flies responded detectably to 1-m^2 models without odor up to a maximum distance of 1.5 m. These results suggest that female apple maggot flies did not detect green 1-m^2 models with odor 4.5 m away or models without odor 2.5 m or more away. Flies responded to white models with and without odor to a much lesser extent, both in terms of response distance and flight to and alightment upon models. Increasing model size to 2 m^2 increased the distance to 2.5 m at which flies responded to green models without odor. Decreasing model size to 0.5 m^2 reduced fly responsiveness to green or white models. The presence of host fruit odor alone, without the visual stimulus of a green model, did not influence residence time on the release tree.

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Key Words—*Rhagoletis pomonella*, Diptera, Tephritidae, fruit volatiles, visual response, olfaction, behavior, response distance.

INTRODUCTION

Over the past 20 years, intensive study of the foraging behavior of insects for food, water, shelter, mates, and oviposition sites has illuminated basic behavioral mechanisms and improved pest management strategies. The nature of information sources, tactics, orientation mechanisms, resource assessment and utilization, individual and environmental variability, and the role of experience have been the dominant topics in basic foraging research (reviewed in Hassell and Southwood, 1978; Finch, 1980; Papaj and Rausher, 1983; Visser, 1988; Papaj and Prokopy, 1989; Bell, 1990). Efforts to describe, model, and test foraging strategies maximizing proximal success and reproductive fitness of the forager have demanded a rigorous, comprehensive, and quantitative assessment of foraging behavior (Pyke, 1984; Stephens and Krebs, 1986; Houston et al., 1988).

Pest management techniques have benefited from foraging research through the proposal and application of efficient trap and attractant combinations (Coli et al., 1985; Chenier and Philogene, 1989; Leonhardt et al., 1990; Salom and McLean, 1990; Prokopy et al., 1990; Colvin and Gibson, 1992), disruptants and deterrents (Bartell, 1982; Van Steenwyk and Barnett, 1987; Miller and Cowles, 1990), aggregants and attractants (Dickens, 1989; Gray and Borden, 1989; Lewis and Martin, 1990), planting schemes designed to foil foraging strategies (Perrin and Phillips, 1978; Cromartie, 1981; Thiery and Visser, 1986; Nottingham, 1987b), and resource characteristics artificially altered to disguise quality (Boller et al., 1987).

Despite the attention these issues have received, the maximum distance at which resources are detected by foraging insects has remained largely undetermined, except for a very few species. Distance of response to a pheromone source has been demonstrated in convincing fashion for lepidopterans *Lymantria dispar* (L.) (Elkinton et al., 1987) and *Grapholita molesta* (Busck) (Baker and Roelofs, 1981; Linn et al., 1987, 1991), to host odor for dipterans *Lucilia cuprina* (Wiedemann) (Eisemann, 1988), *Delia antiqua* (Meigen) (Judd and Borden, 1989), and *Delia radicum* (L.) (Hawkes, 1974), and to male-produced pheromone for female tephritids *Anastrepha ludens* (Loew) (Robacker and Moreno, 1988).

Most theoretical predictions of the distribution of above-threshold concentrations of odor molecules have used Gaussian plume models, which average odor concentrations over some interval of time (Sower et al., 1973; Shapas and Burkholder, 1978; Stanley et al,. 1985). These models have failed validation

tests because above-threshold instantaneous odor concentrations persist at greater distances than those predicted for time-averaged, above-threshold concentrations (Elkinton and Cardé, 1984; Elkinton et al., 1984). Mathematical expression of instantaneous concentrations of odor molecules have not yet proven accurate or very useful (Murlis et al., 1992).

Speculation about maximum response distance has been generated from absolute density estimates based on trap catches (Howell, 1983; Dransfield, 1984), survey-trap optimum density analysis (Cunningham and Couey, 1986), trap-competition experiments (Wall and Perry, 1978, 1980, 1987; Tilden et al., 1983; Bradshaw et al., 1989; Byers et al., 1989), mark–recapture dispersal studies (Maxwell and Parsons, 1968; Coyne et al., 1987; Mason et al., 1990; Martinson et al., 1989; Harrison, 1989), and probability estimates for colonization of new crop plantings (Martinson et al., 1988). These studies have yielded measurements that may be positively correlated with distance of response, but do not definitively determine the distance at which response occurred.

The variety of potential mechanisms of detection and response available to insects in locating resources requires examination of multiple modalities to draw meaningful conclusions from foraging studies (Dethier, 1947; Kennedy, 1978; Bell, 1990). Specifically, the interaction of visual and olfactory cues has been reviewed (Prokopy, 1986) and reported in subsequent studies (Green, 1986; Prokopy et al., 1987; Nottingham, 1988; Tuttle et al., 1988; Torr, 1989; Warnes, 1989; Charlton and Cardé, 1990; Todd et al., 1990). Variable resource and environmental factors affecting stimulus apparency and external and endogenous influences on insect response demand careful consideration in experimental design, interpretation, and analysis (Mitchell, 1988).

Tephritid fruit fly foraging behavior (reviewed in Prokopy and Roitberg, 1989; Fletcher and Prokopy, 1991) and in particular the behavior of the apple maggot fly, *Rhagoletis pomonella* (Walsh) (=AMF) have been frequent subjects of research (Roitberg et al., 1982; Roitberg and Prokopy, 1982, 1984; Prokopy and Roitberg, 1984; Aluja, 1989). The economic importance of this major pest of commercial apple in North America and the relative ease of rearing and handling individual AMF in both field and laboratory settings have contributed to its popularity as a model organism.

Roitberg and Prokopy (1982) found that foraging AMF departed from host trees sooner when neighboring nonfruiting trees were nearby than when trees were further away or absent. This relationship was used to determine when neighboring trees were beyond the maximum distance of detection, i.e., when AMF foraged on a host tree as if no trees were nearby.

We undertook the following study to determine the maximum distance mature, host-seeking female AMF can respond to the visual and olfactory cues provided by host trees. We examined the influence of the size of visual cues,

presence or absence of olfactory cues, air temperature, relative humidity, and wind speed on the maximum distance of detection.

METHODS AND MATERIALS

All experiments were conducted during the summer months of 1986, 1987, and 1988 in an open 80 × 200-m field surrounded by nonhost trees in Amherst, Massachusetts, U.S.A. Artificial host tree mimics and synthetic host fruit odor were used as test stimuli. This eliminated natural variability in canopy size, structure, and spectral characteristics of reflected and transmitted light of real trees and in release rate and ratio of component compounds of real host fruit. These aspects vary both between individual trees and host fruit, and within trees and fruit over time, potentially contributing substantial error variation to assays using real trees and fruit (Averill et al., 1988).

A fruitless potted hawthorne tree (*Crataegus mollis* var. *toba*), pruned to ca. 1.5 m height, 0.75 m diam. canopy with ca. 125 leaves, was placed in the center of the open field. This tree was surrounded by green or white two-dimensional host tree models, with or without synthetic host fruit odor, at one of several distances from the tree (Figure 1). White models served as a control for any influence of a green model on air movement. Distance to the models was measured from the outer branch tips of the tree canopy. A no-model treatment served as an additional control and consisted of a vertical 1-cm-diam. stake with a wire cross-piece at 1.5 m height from which empty or odor-filled vials were suspended.

Tree models were 1-cm-thick plywood panels, 0.5, 1, or 2 m^2, painted with a mixture of oil pigments (83% cadmium yellow, 12% Winsor green, and

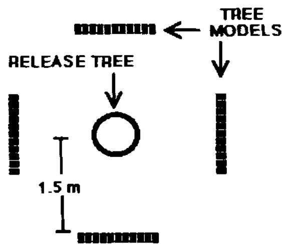

FIG. 1. Experimental set-up and overhead view of one treatment: 1-m^2 models at 1.5-m distance from the release tree. For each trial, a single fly was released onto the fruitless hawthorne tree and exposed to a treatment consisting of four tree models of one size (0.5, 1, or 2 m^2, or no models = control), one color (white or green), and one condition of synthetic host fruit odor (present or absent) surrounding the release tree at one distance (0.5, 1.5, 2.5, or 4.5 m). N = 800 flies tested.

5% Mars black, Winsor and Newton, London) to closely match the spectral reflectance pattern of apple foliage (Owens, 1982). The reverse side was painted with a non-UV reflecting white paint (675 White, Kyanize, Everett, Massachusetts). Each panel was perforated with 144, 4-cm-diam. holes/m^2 to approximate light and air penetration through real trees. Panels were nailed vertically to an upright white post mounted on a movable base such that the center of each panel was 1.5 m above ground.

Two clear polyethylene vials (Andler Israel & Son, Boston, Massachusetts) were partially filled with synthetic apple odor and hung at 1.5-m height at the sides of each model. Odor was released at a rate of ca. 500 μg/hr, equivalent to about 35,000 unripe or 330 ripe McIntosh apples (Carle et al., 1987). In 1986, this odor consisted of six components of the behaviorally active fraction of the volatile blend given off by apples after harvest (Fein et al., 1982; Reissig et al., 1985). A subsequent study showed that at least one of these components was not present in apples before harvest (Carle et al., 1987). Therefore, after 1986, butyl hexanoate, the major behaviorally active component of the volatile blend given off by fresh apple and hawthorne fruit, was used alone (Carle et al., 1987). Empty vials were used in the no-odor treatments.

Preliminary tests using artificial "smoke" ($TiCl_4$) confirmed that wind moved at least some air across the release tree from vial positions regardless of wind direction. The duration of this phenomenon appeared to decline with increasing distance between the models and the release tree.

Test flies were 14- to 21-day-old females, reared from fruit collected in nature and maintained in the laboratory according to methods reported in Roitberg et al. (1982). To ensure uniformity in the physiological state of flies as much as possible, and thus minimize error variability in test results, all pretest experience with fruit was standardized. Beginning 48 hr before transport to the field, flies were permitted free access to *C. mollis* fruit for a period of 24 hr. Immediately before testing, each fly was assayed for its propensity to oviposit (and presumably affirm a host-seeking mode) by being offered a single *C. mollis* fruit. Only those flies that oviposited in these assay fruit were used. Each fly was tested only once. Treatments were replicated an average of 20 times for a total of 800 trials. Tests were conducted between 0800 and 2000 hr EDST (Table 1), and the daily sequence of treatments was randomized throughout the course of the experiment.

During test days, flies were maintained with food and water in a shaded spot 40 m from the test arena. To begin a test, a single fly was carefully moved to the release tree while ovipositing in the assay fruit and transferred to a leaf at the lower center of the canopy as soon as oviposition was complete. For each test, four models of the same size, color, and odor condition were present around the release tree at one of the treatment distances (or no models = control).

Each fly was allowed to move freely within the release tree for up to 1 hr.

TABLE 1. ENVIRONMENTAL CONDITIONS AND DISTRIBUTION OF TIME OF DAY WHEN TESTS WERE CONDUCTED[a]

	Variable[a]		Mean (SD)		Range	
	Air temperature (°C)		28.0 (3.44)		19–37	
	Relative humidity (%)		35.8 (10.62)		15–80	
	Wind speed (m/sec)		4.5 (2.16)		0–11.2	
	Time of day (hr)[b]					
	0800–0959	1000–1159	1200–1359	1400–1559	1600–1759	1800–1959
Replicates (N)	40	181	197	220	131	31
% of total	4.9	22.6	24.6	27.5	16.3	3.8

[a]Temperature and relative humidity were recorded at the beginning and end of each test, and these values were averaged to give an individual test mean. Wind speed was measured continuously throughout each test. N = 800.
[b]Measured at 1-m height in the center of the open field, 10 m away from the release tree.
[c]Eastern daylight saving time.

During this time, two observers tracked its movement, recording number of leaves visited, time elapsed before departure, direction of departure over the first ca. 50 cm after leaving the tree, and whether or not the departing AMF alighted on a model. Wind direction at time of departure was noted by observing wind-caused displacement of a feather suspended from a thread within the tree canopy. Average wind speed was measured with a cup anemometer (Wind-Minder Indicator, Weathermeasure model W200-SI, Qualimetrics Inc., Sacramento, California), mounted at 1.5-m height. Temperature and humidity were recorded at the beginning and end of each trial. All data were collected using a Radio Shack 100 portable computer (Tandy Corp., Fort Worth, Texas), and later transferred to a Control Data Corp. Cyber 175/730 mainframe at the University of Massachusetts Computing Center for statistical analyses. Analyses were performed using BMDP (Dixon, 1985).

Because the data were incomplete or censored (i.e., 10.4% of flies remained on the tree for the full 60 min permitted), a survival-type regression analysis was performed using BMDP2L (Dixon, 1985). The effects of covariates model color, distance to model, model size, temperature, humidity, and wind speed on residence time on the release tree were fit using a Cox proportional hazards model. Proportions of AMF flying to and landing on the models were compared using logistic regression analysis (BMDPLR). Multiple regression (BMDP1R) analysis was applied to analysis of take-off flight direction when leaving the release tree in relation to wind direction at the time of departure.

RESULTS

The environmental parameters air temperature, relative humidity, and wind speed were highly variable (Table 1), and represented the wide range of conditions experienced by foraging AMF in nature. Seventy-three percent of all tests were conducted after noon.

When no tree models were present, flies remained on the release tree for ca. 31 min, regardless of the presence or absence of synthetic host fruit odor (Figure 2). Flies left the tree significantly sooner when (1) 1-m² green models with odor were present at 0.5, 1.5, or 2.5 m from the release tree, or 1-m² green models without odor were present at 0.5 or 1.5 m, (2) 0.5-m² green models with odor were present at 0.5 or 2.5 m, or 0.5-m² green models without

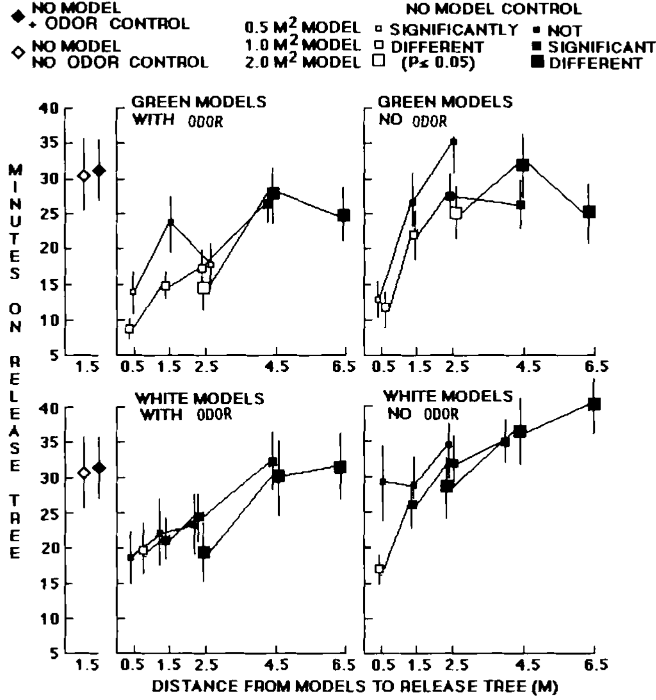

FIG. 2. Mean residence time of AMF on a fruitless hawthorne tree surrounded by four green or white tree models of various sizes, with or without synthetic host fruit odor, and placed at one of several distances from the tree (or no models = control). Single degree of freedom contrasts were used to compare treatment means to those of the no-model control.

odor were present at 0.5 m, (3) 2-m^2 green models with or without odor were present at 2.5 m, and (4) 1-m^2 white models with or without odor were present at 0.5 m.

Regression analysis indicated that model color and size, presence of odor, and distance between models and the release tree were very highly significant covariates influencing residence time on the tree ($P < 0.001$, Table 2). Air temperature was a highly significant covariate ($P < 0.01$), while relative humidity and wind speed were not significant covariates. Interactions between odor and color and between distance and size were also tested and found to be not significant. The regression model predicted that an AMF would leave the tree (1) 1.03 times sooner for each 1-degree increase in air temperature, (2) 1.39 times sooner when odor was present than when odor was absent, (3) 1.59 times sooner when green models were present than when white models of the same size were present at the same distance, (4) 1.33 times sooner for each 1-m^2 increase in model size, and (5) 0.80 times sooner (will remain longer) for each 1-m increase in distance between the release tree and the models.

The proportion of AMF landing on models approached 100% when green 1-m^2 models with or without odor were present at 0.5 m, and declined to less than 35% when these models were at 2.5 m distance (Figure 3). Lesser proportions of AMF landed on white models at these distances. Regression analysis indicated that model color, size, and distance were very highly significant factors influencing flight to and alightment on models (Table 3). The influence of odor was significant ($P < 0.05$).

Wind direction at the time AMF left the tree significantly influenced the direction AMF left the tree ($P < 0.001$, Table 4). Model color and distance

TABLE 2. PARAMETER ESTIMATES FOR COX PROPORTIONAL HAZARDS REGRESSION MODEL FOR RESIDENCE TIME OF AMF ON FRUITLESS HAWTHORNE TREE[a]

Variable	Coefficient	SE	P	Relative risk	95% CI for rel. risk
Temperature	0.033	0.010	<0.01	1.03	1.01-1.05
Odor	0.330	0.075	<0.001	1.39	1.20-1.61
Model color	0.462	0.076	<0.001	1.59	1.37-1.84
Model size	0.287	0.083	<0.001	1.3	1.13-1.57
Distance	−0.218	0.030	<0.001	0.80	0.76-0.85

[a]With each unit change in the value of a covariate, the risk of the AMF departing from the tree changes by a factor equivalent to the exponent of the coefficient, e.g., for each 1°C increase in temperature, the risk of an AMF departing increases 1.0333 times). Coded values for color were 0 (white models) or 1 (green models), and for odor were 0 (no odor) or 1 (odor). Because the effect of wind speed and relative humidity on residence time was not significant, these were dropped from the model. $N = 800$ total observations.

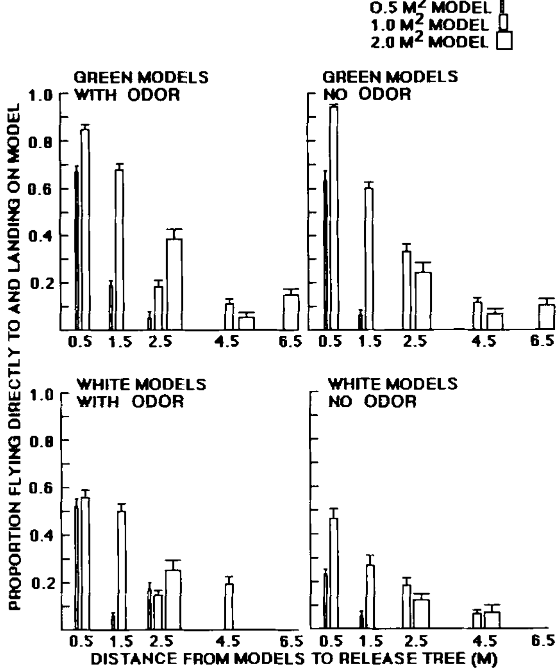

FIG. 3. Proportion of AMF (and SE) leaving the release tree and flying directly to and landing on the surface of a tree model. The 0.5-m^2 models were present at 0.5, 1.5, or 2.5 m, 1-m^2 models at 0.5, 1.5, 2.5, or 4.5 m, and 2-m^2 models at 2.5, 4.5 or 6.5 m. $N = 528$ flies.

from the release tree and time of day (before- or afternoon) were also significant covariates influencing the direction AMF left the tree.

The number of leaves visited was significantly positively correlated with residence time on the tree ($r = 0.38$ for 500 observations, $P < 0.001$). Total time on the tree, wind speed, relative humidity, and model size were significant or very highly significant covariates affecting number of leaves visited (Table 5).

DISCUSSION

AMF foraging on host trees were apparently unable to detect or respond to neighboring host tree models located beyond 2.5 m, regardless of size, color, or the presence or absence of synthetic host fruit odor. This is a relatively short distance in comparison to results of other studies of insect response to odor cues

TABLE 3. PARAMETER ESTIMATES AND STATISTICS FOR LOGISTIC REGRESSION MODEL PREDICTING PROPORTIONS OF AMF FLYING DIRECTLY TO AND LANDING ON TREE MODELS[a]

Variable	Coefficient	SE	*P*	Odds ratio	95% CI for odds ratio
Odor	0.411	0.210	<0.05	1.51	0.99–2.28
Model color	0.849	0.212	<0.001	2.34	1.54–3.55
Model size	0.861	0.238	<0.001	2.37	1.48–3.78
Distance	−0.828	0.099	<0.001	0.44	0.36–0.53
Constant	−0.508	0.273	<0.08	0.60	0.35–1.03

[a]With each unit change in the value of a covariate, the odds of an AMF flying to and landing on a model changes by a factor aproximately equivalent to the exponent of the coefficient, e.g., with each meter increase in distance between the release tree and the model, the odds of landing on the model decrease by a factor of approximately 0.44). Coded values for color were 0 (white models) or 1 (green models), and for odor were 0 (no odor) or 1 (odor). The effect of temperature, wind speed, and relative humidity were not significant and so were dropped from the model. $N = 800$ total observations.

TABLE 4. ESTIMATED PARAMETERS AND STATISTICS FOR MULTIPLE LINEAR REGRESSION MODEL PREDICTING DIRECTION IN WHICH AMF LEFT RELEASE TREE[a]

Variable	Coefficient	SE	*P*
Wind direction at time of departure	0.16	0.035	<0.001
Distance	−0.21	0.057	<0.001
Time of day (before or afternoon)	0.54	0.235	<0.02
Model color	0.46	0.216	<0.04
Constant	3.72		

[a]Overall $F = 11.65$ ($P < 0.001$), $df = 550$, $r^2 = 0.071$.

alone. Response distance maxima have been reported as at least 100 m for *D. antiqua* to dipropyldisulfide-baited traps (Judd and Borden, 1989), 15 m for *D. radicum* to brassica plants (Hawkes, 1974), 8 m for *A. ludens* to male-produced pheromone (Robacker and Moreno, 1988), and 20 m for *L. cuprina* to flystruck sheep (Eisemann, 1988). Response of Lepidoptera to pheromone has been demonstrated up to 80 m for *L. dispar* (Elkinton et al., 1987) and *G. molesta* (Baker and Roelofs, 1981; Linn et al., 1987, 1991).

Results here were remarkably similar to those of Roitberg and Prokopy (1982), who found that AMF foraged on a fruitless host tree for 32 min when no alternate host trees were nearby (vs. 31 min here). They reported a mean

TABLE 5. PARAMETER ESTIMATES AND STATISTICS FOR MULTIPLE LINEAR REGRESSION MODEL PREDICTING NUMBER OF LEAVES VISITED BY AMF FORAGING ON RELEASE TREE[a]

Variable	Coefficient	SE	*P*(F)
Total time on tree	0.01	0.001	$P < 0.001$
Wind speed	−2.11	0.535	$P < 0.001$
Relative humidity	−0.23	0.108	$P < 0.04$
Model size	−3.63	1.684	$P < 0.03$
Constant	37.44		

[a]Overall $F = 29.72$ ($P < 0.001$), $df = 550$, $r^2 = 0.18$.

within-tree foraging time of 16.4 min when fruitless hosts trees (ca. 2 m tall) were 1.6 m distant (vs. 14.9 min for 1-m^2 green models with odor at 1.5 m distance here), and 22.1 min when fruitless host trees were 3.2 m distant (vs. 16.6 and 26.7 min for green 1-m^2 models with odor at 2.5 and 4.5 m, respectively, here). Roitberg and Prokopy used real trees for neighboring hosts and counted time AMF spent actively foraging and not time spent motionless. Here all time on the release tree was counted. They did not consider the influence of host fruit odor on their results, although their experiments were conducted within a fruiting apple orchard with neighboring trees with fruit ca. 15 m distant.

Results here were also similar to those obtained by Aluja and Prokopy (1992), who observed AMF foraging in a patch of real fruitless host trees spaced 0.6 m apart from one canopy edge to the next. They found that on average, AMF left host trees within 5.3 min when odor was present, and 8.8 min when odor was absent (vs. 8.9 min with odor and 11.8 min with no odor associated with 1-m models at 0.5 m distance here, Figure 2).

Roitberg and Prokopy (1982) clearly demonstrated that AMF invested less search effort within a tree when alternate foraging sites were nearby. Their results fulfilled a prediction of optimal foraging theory that foragers should remain in a patch longer as travel costs between patches increase (Pyke, 1984). Results here indicated that, in addition to distance from nearby host trees, search effort in highly dependent on specific characteristics of nearby host trees, including size, color, and the presence of host fruit odor. Differences in these characteristics apparently affected the ability of AMF to detect the host tree models and may have also imparted information about the potential quality of the nearby host mimic. Differences in perceived quality may account for some of the differences in response observed here. Expected benefits from higher quality resources (e.g., larger size models, fruit odor present) may have counterbalanced costs and risks associated with travel from the release tree.

For nearly all models of the same size, color, and odor condition, response was graduated at distances between 0.5 and 2.5 m (Figure 2), not simply one residence time when models were apparent and a different residence time equal to the no-model control when models were beyond the maximum distance of detection. This graduation may have resulted from individual variation among flies in perceptual ability or responsiveness. Individual variation in various phases of foraging behavior due to genotype, environment, or experience has been demonstrated repeatedly (reviewed in Papaj and Rausher, 1983; Roitberg, 1990). By standardizing the adult AMF pretest environment and experience, these two sources of individual variation were, we hope minimized.

Alternatively or in addition, the relationship between the benefits of remaining within a patch and the cost of travel to nearby patches may have been continuous rather than discrete. Neighboring host tree models of the same type may have presented perceptible levels of difference in travel distance and associated risks and costs, creating or contributing to the graduated response evident here.

Interaction of Odor and Visual Stimuli. In assays here, host fruit odor and host tree visual stimuli acted synergistically. AMF did not respond to green 0.5- or 1-m^2 models without odor at 2.5 m distance, nor to odor alone at 1.5 m (no model treatment, Figure 2). However, the combination of odor and 0.5- or 1-m^2 models at 2.5-m distance elicited a significant response. Synergism between visual and olfactory cues during host-plant finding and acceptance has been reported for another dipteran, *D. antiqua* (Harris and Miller, 1982; Judd and Borden, 1991), and for a leafhopper, *Dalbulus maidis* (DeLong and Wolcott) (Todd et al., 1990).

The fact that AMF did not detectably respond to host odor in the absence of visual stimuli was an unexpected result. Subsequent work (Aluja and Prokopy, 1992; Green and Prokopy, unpublished) indicated that AMF were able to detect and respond to host fruit odor at a much greater distance than found here. Presumably AMF foraging in the presence of the no model + odor treatment were able to detect host fruit odor, but did not exhibit a discernible response. This lack of response clearly demonstrates the limitation of the current work as a definitive assay for distance of host tree stimulus detection. Rather, this set of experiments represented giving-up-time (GUT) assays for the maximum distance of response of foraging AMF to nearby host tree stimuli. The physiological state of AMF foraging among nonhost plants for host cues may be one of heightened sensitivity to host tree stimuli, and detection of and response to such stimuli may occur at a greater distance. Odor cues in particular may be more discernible from a distance than visual cues of host vs. nonhost trees.

Other studies have demonstrated that AMF foraging within a patch of fruitless host trees leave trees sooner, reach the edge of the patch sooner, and make more straight flights when host fruit odor is present at the edge of the

patch than when odor is absent (Aluja and Prokopy, 1992). The fact that host odor did not generate a detectable response in the absence of any visual stimuli here suggested that the costs and risks of travel over large open spaces remained high and outweighed any influence of odor cues indicating the presence of host fruit somewhere in the vicinity.

A factor not considered in the design of experiments here was the distance between odor vials, which varied according to model size and distance from the release tree. Additional experiments (Green and Prokopy, unpublished) have shown distance between odor sources surrounding AMF to be critical in determining response distance. In any case, two discrete odor sources per model was not representative of a natural situation where a tree may have several hundred point sources scattered over its entire silhouette, providing a vertically and horizontally diffuse odor plume. The height of odor sources (held constant at 1.5 m here) has been shown to influence response in other studies (Cuthbert and Peacock, 1975; Ono and Ito, 1989).

Influence of Color on Response. White models were intended as a control for the effect of green tree models on air movement between the models and the release tree. The significant effect of white 1-m^2 models at 0.5-m distance on fly residence time indicated that air movement or some other aspect of the white models did influence AMF foraging behavior, albeit to a much lesser degree than did green models. From most positions within the release tree canopy, the background behind all models was dark (woods or grass). White models perforated with holes (144 4-cm-diam. holes/m^2) may have represented sparsely foliated trees (i.e., the inverse image of green models). Several studies have confirmed the importance of contrast against background in resource location (Owens and Prokopy, 1984; Allen and Stoffolano, 1986).

Alternatively, perceptible white models may have simply offered a landing and perching site for AMF. The availability of such a perch may have reduced the costs and risks of travel between patches, especially given that AMF do not travel readily across open, grassy areas. In previous studies, numbers of AMF captured on sticky-coated white tree models were ca. 50% of captures on green models (Moericke et al., 1975), roughly comparable to proportions of AMF landing on white vs. green models here (Figure 3). Large white rectangles (1.2 $\times$ 2.4 m^2) with apples captured twice as many AMF as white rectangles without apples (Prokopy et al., 1973), but only 20% as many as yellow rectangles with apples.

Interaction of Stimulus Size and Distance. The 2-m^2 model at 2.5 m, the 1-m^2 model at 1.5 m, and the 0.5-m^2 model at 0.5 m were selected to occupy an approximately equivalent area in the visual field of AMF foraging within the release tree canopy (Figure 4). AMF responded as if these size–distance combinations were nearly equivalent when green models were used with odor. These sizes and distance combinations elicited different responses when odor was

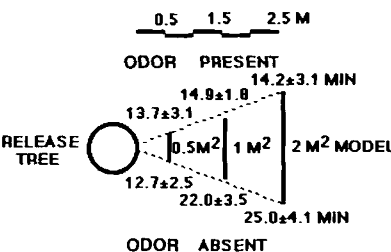

FIG. 4. Average residence time (±SE) on the release tree for AMF when 0.5-m^2 green models were 0.5 m from the release tree, 1-m^2 green models were 1.5 m from the release tree, or 2-m^2 green models were 2.5 m from the release tree hypothetically creating an image occupying the same area in the visual field of AMF foraging on the release tree.

absent. It is unclear why these disparate results were obtained. The experimental design permitted AMF to view the models from many vantage points within the release tree, including during occasional brief circling or looping flights just outside the canopy diameter. Perhaps as foraging time on the tree increased when odor was absent, AMF were able to obtain more information and judge the size–distance relationship more accurately.

In previous studies, increasing the size of sticky-coated red or yellow rectangles placed in open fields resulted in a proportional increase in the numbers of AMF captured per rectangle, but the numbers of AMF captured per square centimeter decreased with increasing size of yellow, although not red, rectangles (Moericke et al., 1975). Increases in ox-mimic size resulted in much greater than proportional increases in response by tsetse flies (Hargrove, 1980b). An increase, not always proportional, in AMF alighting on models with increasing model size was noted here for green models with odor at 0.5, 1.5, and 2.5 m distance, and without odor at 0.5 and 1.5 m (Figure 3).

Alightment on Tree Models. The proportion of flies landing on models after leaving the release tree did not give accurate information about the distance of detection of the model, because no information was available as to whether the fly detected the model while on the release tree or sometime after leaving it. The substantial proportion of AMF alighting on white models (although much less than for green models, Figure 3), and the significant influence of 1-m^2 white models at 0.5 m distance on residence time (Figure 2), support the possibility that white models may have at least provided intermediate perching sites for AMF if, in fact, they did not elicit a response due to a resemblance to sparsely foliated trees.

Take-Off Direction and Wind Direction at Time of Departure. In this study, the presence or absence of synthetic host fruit odor did not significantly influence direction of departure from the release tree. These results were in contrast to

those obtained in subsequent studies where take-off direction of AMF from a platform was random in the absence of odor stimuli, but directional in the presence of synthetic host fruit odor (Aluja and Prokopy, 1992; Green and Prokopy, unpublished). *Glossina* spp. (Bursell, 1987), *L. cuprina* (Eisemann, 1988), *D. antiqua* (Judd and Borden, 1988), *Psila rosae* F. (Nottingham, 1987b), and *D. radicum* (Hawkes, 1974; Nottingham and Coaker, 1987) also made more directional (upwind) flights in the presence than in the absence of host odors.

Results here were more influenced by other variables. The position of the sun in the morning vs. the afternoon (75% of tests were conducted after noon) may have created more or less apparent silhouettes for certain model positions. The fact that model color, distance to models, and time of day had a greater impact on departure direction than did wind direction (Table 4) suggested that this may have been the case. Significant deviation from odor-mediated upwind flight in the presence of visual targets at different distances and orientations has been demonstrated for tsetse flies (Torr, 1989; Gibson et al., 1991).

Effect of Wind Speed, Temperature, and Relative Humidity. It was surprising that wind speed was not a significant determinant of departure time from the tree, especially given the wide range and sometimes very high wind speeds experienced over the course of this study (Table 1). AMF were often observed crouching low on leaf surfaces during wind gusts, and increasing wind speeds resulted in significantly fewer leaf visits (Table 5). It was expected that "down time" would be reflected in higher residence times for trials during high winds. This finding may have represented: (1) continued assessment of the release tree and surroundings by AMF during wind-caused down time; (2) an internal, fixed-time GUT clock (Roitberg and Prokopy, 1984; Prokopy and Roitberg, 1989), set upon release on the tree and continuing to run during this time; or (3) GUT set by an energy expenditure clock rather than a fixed-time clock and continuing expenditure of energy during down time to maintain position during strong wind gusts, or possibly greater energy expenditure per unit time when foraging under higher wind speed conditions. High wind speeds significantly slowed rate of departure of alate aphids from host plants in the field or from artificial substrates or host plants in the laboratory (Walters and Dixon, 1984; Bottenberg and Irwin, 1991). Departure of aphids was delayed but not prevented at wind speeds as high as 10 m/sec.

The significant effect of increasing temperatures, reducing residence time (Table 2), was in agreement with nonsignificant trends reported for AMF by Roitberg and Prokopy (1984). However, temperature was not significantly correlated with foraging speed measured in number of leaves visited per second ($r = 0.0791$, NS), nor was temperature a significant influence on the total number of leaves visited (Table 5). Temperature may have: (1) increased foraging rates and/or energy expenditure in a way undetected by this assay and so speeded up the GUT clock, (2) acted in some other way to increase perception

of surrounding host tree stimuli, or (3) caused AMF to leave the release tree sooner to seek shelter and cooler temperatures. Studies of other systems have provided evidence that one or more of these possibilities may have occurred. Wing beat frequency of tsetse flies increased with increasing temperatures from 20 to 32°C (Hargrove, 1980a), and a bimodal behavioral response resulted. With increasing temperatures, a greater proportion of flies became active, but duration of flights decreased due to more rapid oxidation of metabolite reserves. Male Oriental fruit moths responded to an equivalent release rate of pheromone blend at twice the distance when temperatures were 25–28°C vs., 19–21°C, and male specificity of response increased at the lower temperature range (Linn et al., 1987). These authors hypothesized that higher temperatures may have increased release rates of pheromone; increased body temperatures, permitting greater levels of sustained flight; and/or altered interactions between chemical stimuli and receptors at the peripheral sensory level, optimizing response spectra or rates of disadaptation.

The small but significant influence of relative humidity on the number of leaves visited (coefficient = −0.23, $P(F) < 0.04$) is the first reported evidence of an effect of moderate humidity levels on AMF foraging. The regression model predicted a slight decrease in the number of leaves visited with an increase in humidity. Nottingham (1987a) demonstrated significantly greater than expected trap captures of *D. radicum* at 65–70% relative humidity, and trap catch increased linearly with increasing humidity.

Dispersal studies have indicated that marked AMF can travel at least 1572 m from a release site to apple orchards (Maxwell and Parsons, 1968), and marked AMF have been captured up to 45 m from a release site within an abandoned orchard (Neilson, 1971). Although results here in no way rule out directed movement over long distances, especially to odor cues, AMF foraging on host trees exhibit a very limited distance of response (maximum of between 2.5 and 4.5 m) to neighboring host trees of up to 2 m^2 in canopy size.

For AMF, color, size, and distance of nearby tree models and the presence or absence of host fruit odor may act alone and/or in combination to affect the apparency of stimuli and significantly impact GUT and decisions to alight in a continuous fashion. Environmental variables of temperature, relative humidity, and wind speed and direction also affect behavior of AMF in quantifiable ways. These findings may lead to improvement of conceptual models of AMF foraging to increase predictability of AMF movements under a broader range of resource and environmental conditions.

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IDENTIFICATION AND SYNTHESIS OF FEMALE SEX PHEROMONE OF ORIENTAL BEETLE, *Anomala orientalis* (COLEOPTERA: SCARABAEIDAE)

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Abstract—Females of the Oriental beetle, *Anomala orientalis* (Waterhouse), release a sex pheromone composed of a 9:1 blend of (*Z*)- and (*E*)-7-tetradecen-2-one. The double-bond position of the pheromone was determined by DMDS derivatization and interpretation of the fragmentation patterns produced by monounsaturated ketones. In a sustained-flight tunnel, males responded by flying toward female beetles and attempting to copulate with them. Both effluvium and whole-body extracts of OB females were analyzed, and the activity was found only in the airborne extracts. Flight-tunnel bioassays also showed that a synthetic 90:10 *Z/E* blend on a rubber septum was attractive and that the responses of males to this blend were equivalent to *Z* isomer alone, but much better than to the single *E* isomer.

Key Words—*Anomala orientalis*, Oriental beetle, Coleoptera, Scarabaeidae, sex pheromone, (*Z*)- and (*E*)-7-tetradecen-2-one, DMDS derivatization, flight tunnel.

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INTRODUCTION

In the United States, the Oriental beetle (OB), *Anomala orientalis* (Waterhouse), is also called the Asiatic beetle (Westcott, 1964). This species is a close relative of the Japanese beetle and Asiatic garden beetles, which form a group of very destructive pests in lawns, nursery plants, and forest trees. *A. orientalis* is presumably a native of the Philippine Islands (Tashiro, 1987) and widely distributed in the Asiatic and Malaysian countries (Essig, 1951). It was imported into the United States before 1908 and nearly destroyed all of the sugarcane in Hawaii. *Scolia manilae* (Ashm.), a digger wasp, was introduced into Hawaii from Philippine Islands in 1916 as the entomophagous parasite to prey particularly upon grubs of *A. orientalis* (Clausen, 1940; Essig, 1931; Westcott, 1964). After the OB was first discovered on the mainland United States in New Haven, Connecticut, by M.P. Zappe and B.H. Walden in July 1920 (Essig, 1931), its distribution gradually increased in the northeastern United States. Although the adult beetles do some damage by chewing the blossoms of flowers, the larvae kill grasses, especially of lawns, by eating the roots close to the soil surface (Arnett, 1985; Metcalf et al., 1951). It was reported that OB not only attacked various turf grasses, lawns, and nursery stock, but also was found on ageratum, cyclamen, iris, hollyhock, phlox, rose, bean, beet, onion, rhubarb, and strawberry (Westcott, 1964).

Although the OB has become a serious pest in the northeastern United States and other countries and treated with insecticides (Fleming, 1948), its sex pheromone communication system has not been reported. Thus, a project was initiated to determine if this species uses a sex pheromone for mate location. In our preliminary studies, we were surprised to find that at an elevated temperature (>25°C) during photophase, adult OB males responded strongly in the flight tunnel to the sex pheromone released from females by flying toward and touching the source. As our studies progressed, Dr. Leal provided us information and a synthetic sample of the sex pheromone of the Oriental beetle, *Blitopertha orientalis*, in Japan (Leal, 1993).

METHODS AND MATERIALS

Insects. Second- and third-instar larvae of OB collected from the Norwich Community Golf Course in Norwich, Connecticut, in October 1992 were stored individually in ~30-ml plastic cups containing soil and a small amount of grass seed for food. They were stored at 10°C for about six months. When adults were required, larvae were moved to a 25°C emergence room under a 16L:8D photoperiod, where they were checked daily until adults emerged (ca. 4–6 weeks). Upon emergence, imagos were sexed and moved to a rearing room under common environmental conditions (16L:8D, 25°C, and 50% relative

humidity). They remained there in their soil-containing larval rearing cups prior to testing in the flight tunnel.

Pheromone Collections and Aeration Apparatus. A total of 17 4-day-old female beetles were introduced into an aeration apparatus, which consisted of three three-neck glass bottles (500 ml) filled with a wetted Kimwipes tissue and connected to three Super Q (200 mg each, Alltech Associates, Inc. Deerfield, Illinois) traps (15 cm × 0.6 cm OD). The air was filtered with a charcoal (Activated Carbon, 6–14 mesh, Fisher Scientific) trap (7 cm × 1 cm OD) before being pulled through the apparatus with a water aspirator or a vacuum pump. Flow rate was controlled at ~1 liter/min. Female beetles were aerated continuously for several days (16 hr in the photophase at 27°C and 8 hr in the scotophase at 18.5°C). The Super Q traps were changed and tissues were wetted with water every 24 hr. The airborne volatiles were obtained by percolating each Super Q trap with four portions of glass-distilled hexane (0.5 ml/each), and the resultant hexane solutions were then combined and concentrated under a stream of nitrogen to a volume of about 100 μl. The concentrated airborne hexane extracts were fractionated on the GC. Each fraction was then rinsed with a total of 200 μl of glass-distilled hexane for the bioassays.

Female Whole-Body Extracts. Female OB whole-body extracts were obtained by rinsing 12 virgin females (approximately 6-days-old) with three portions of 100 μl of glass-distilled hexane. The extracts were then combined and concentrated to a small volume with a nitrogen stream.

Instrumentation. Isolation and purification of the active components were performed on a Shimudzu GC-8A packed column gas chromatograph equipped with a thermal conductivity detector and a modified chilled collector. Dry Ice was used to cool the collection tube. The collection tube was a 75-μl micropipet (VWR Scientific Inc., Philadelphia, Pennsylvania), which was cleaned by heating to 550°C in a muffle oven for 12 hr. Helium was used as a carrier gas at a flow rate of 30 ml/min. The TCD current was set up at 100 mA and the injector and detector temperatures at 220°C. The fractions were collected each minute from either a packed nonpolar column (3% OV-101 on 80/100 mesh, Supelcoport, 2 m × 0.5 cm OD, Supelco, Inc., Bellefonte, Pennsylvania) or a polar column (5% Carbowax 20 M on 100/200 mesh, Supelcoport, 2 m × 0.5 OD, Supelco). The oven temperature was programmed from 150°C to 210°C at 5°C/min and then held for 20 min. A Hewlett Packard 3390A integrator was used to record the GC traces.

Analytical work was carried out on a Hewlett Packard 5880A gas chromatograph equipped with an flame ionization detector (FID) detector, and a nonpolar SE-30 or a polar Carbowax Econo-Cap capillary column (30 m × 0.25 mm ID, 0.25 mm film thickness, Alltech Associates) in the splitless mode. The oven temperature was programmed at 100°C for 2 min, then 5°C/min to 220°C and held for 20 min. Nitrogen was the carrier gas and the flow rate was

2 ml/min. GC–mass spectrometry (GC-MS) was carried out with a Hewlett Packard 5890 gas chromatograph coupled to a HP 5970B Mass Selective Detector using the same SE-30 capillary column and conditions as above, but with helium as the carrier gas.

Chemicals. The two compounds identified and assayed as the sex pheromones were (*Z*)- and (*E*)-7-tetradecen-2-one. They were synthesized individually by a method described later in the structure confirmation section. The isomeric purity of both isomers was >99.5%, based on results with the capillary GC (FID). The starting materials, (*Z*)- and (*E*)-6-tridecen-1-ol, were purchased from Dr. S. Voerman at the Institute for Pesticide Research, The Netherlands.

Laboratory Bioassays. GC fractions and synthetic pheromones were assayed for activity in a flight tunnel (Miller and Roelofs, 1978) and by the electroantennogram (EAG) technique (Roelofs, 1984). Temperature and relative humidity in the flight-tunnel room were maintained at ~27°C and ~50% , respectively. The air flow rate was at ~0.4 m/sec. Males were tested during the photophase and at a light intensity of ~800 lux. All of the samples, including the whole-body extracts, crude airborne extracts, GC fractions, synthetic pheromones, and controls (solvent only) were placed at the upwind end of the tunnel on a piece of filter paper or a rubber septum, which were pinned vertically on a piece of cork placed on a metal platform at a height of 15 cm.

Male beetles were transferred from the rearing room to the flight-tunnel room, placed individually in a screen release case, and allowed to acclimate for at least 2 hr without any disturbance prior to a test. Male beetles were introduced into the tunnel by placing the release cage at the downwind end of the tunnel facing upwind on a 15-cm-high platform. One male was released for each assay and observed for a period of 5 min while responses were recorded. After each assay, the male was removed from the tunnel and a new male was introduced at the start of subsequent tests. All tests were replicated at least four times using a new male for each replication.

The live-female source consisted of three to six females (at least 5-days-old) held in a small screen cage at the upwind end of the tunnel and used as the standard pheromone source. The flight-tunnel results were analyzed by the adjusted significance levels for proportions ($P < 0.05$) (Ryan, 1960). In all cases, bars with the same letters are not significantly different.

The EAG responses of male antennae were displayed on a Tektronix 2212 digital storage and analog oscilloscope, and printed out on a Tektronix HC 200 9-pin matrix Tekprinter.

RESULTS AND DISCUSSION

Pheromone Isolation and Identification. Female whole-body extracts did not elicit a behavioral response in the flight tunnel, but the airborne extracts were very attractive to male OB. Effluvium collected with the aeration apparatus

was first fractionated on the GC with a nonpolar OV-101 column and fractions were assayed in the flight tunnel. Fractions were taken each minute up to 30 min, but only the 5- to 6-min fraction showed significant activity. This retention time corresponded to two hydrocarbon standards—pentadecane (4.9 min) and hexadecane (6.5 min)—under these conditions. On the polar Carbowax 20 M column, an active fraction was found at 9–10 min retention time. The active fraction from the polar Carbowax column was further fractionated on the nonpolar SE 30 capillary column with fractions taken each minute for 30 min. Flight-tunnel and EAG bioassays showed that only a single GC peak at 15.67 min elicited a significant response. The amount of pheromone produced appeared to be the highest in 5-day-old females (Figure 1).

The active component was analyzed by GC-MS. The spectrum exhibited a relatively small molecular ion at m/z 210, with a base peak at m/z 43. In another GC-MS study, the selected ion monitoring (SIM) mode was employed to increase sensitivity. Three ions, m/z 125, 152, and 210, were selected from the mass spectrum as the monitoring ions characteristic of the natural pheromone, and they revealed two components at 15.49 and 15.61 min in a ratio of 88:12. This ratio varied to 96:4 with different collections. The results suggested that the natural pheromone consists of two geometric isomers. By comparison of the mass spectrum with unpublished data (at that time) on the Oriental beetle of the Japanese species, *Blitopertha orientalis* (Leal, 1993), the sex pheromone produced by female *Anomala orientalis* in the United States was also postulated to be (*Z*)- and (*E*)-7-tetradecen-2-one.

Confirmation of the double-bond location of the pheromone was obtained by capillary GC-MS analysis of DMDS (dimethyl disulfide) derivatives (Francis and Veland, 1981; Buser et al., 1983; Dunkelblum et al., 1985). Two sulfide fragments of the adduct appeared at m/z 159 and 145 (Figure 2a), indicating

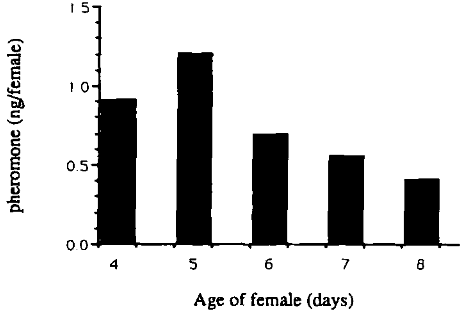

FIG. 1. Quantity of sex pheromone collected from 17 female Oriental beetles over a period of five consecutive days.

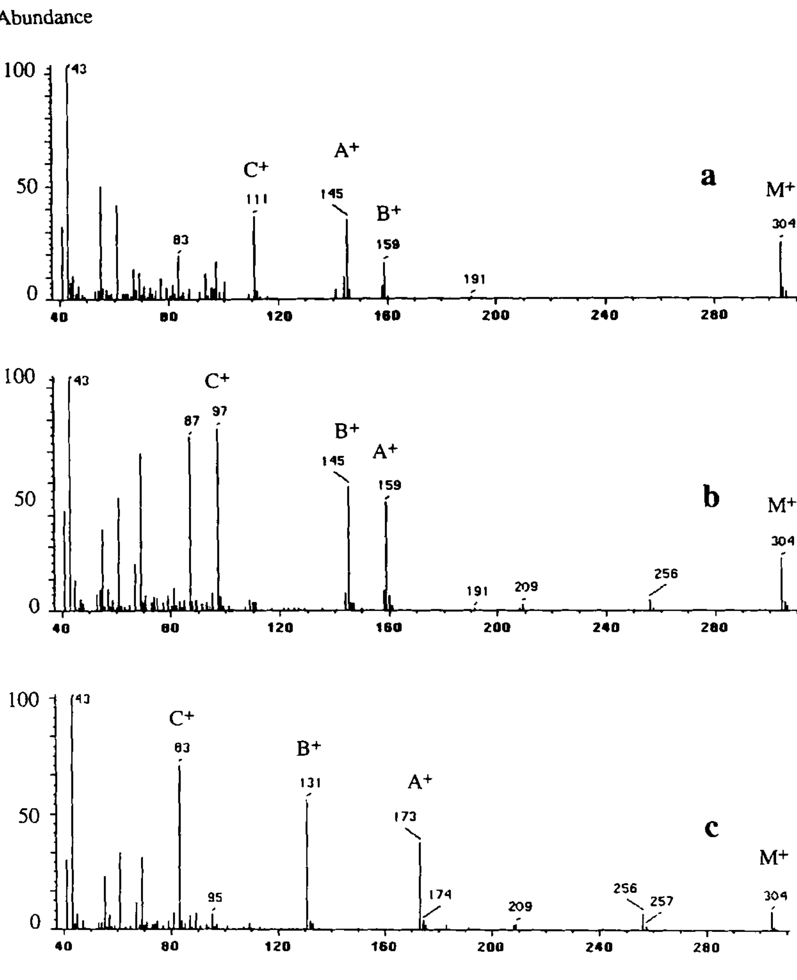

FIG. 2. Mass spectra of DMDS derivatives of three isomeric Δ-tetradecen-2-ones: (a) 7- (b) 6- and (c) 5-tetradecen-2-one. Molecular ion $M^+ = 304$. Fragment ions A^+, B^+, and C^+ are indicated.

that both 7- and 6-tetradecen-2-one were possible candidates for the natural pheromone.

Chemical Synthesis and Structure Confirmation. To confirm the above formulation, the (*E*)- and (*Z*)-7-tetradecen-2-one isomers were synthesized indi-

vidually. The starting material, (*E*)-6-tridecen-1-ol **(1)**, was oxidized to the corresponding aldehyde **(2)** with 1.5 equivalent of PCC (pyridinium chlorochromate) in methylene chloride for 2 hr. The aldehyde was converted to (*E*)-7-tetradecen-2-ol **(3)** by treatment with 1.2 equivalent of methylmagnesium bromide in anhydrous ether. The final product, (*E*)-7-tetradecen-2-one **(4)** was obtained from **(3)** by oxidation with PCC. These reactions are summarized in Scheme 1. The *Z* geometric isomer, (*Z*)-7-tetradecen-2-one, was prepared by a similar pathway starting with (*Z*)-6-tridecen-1-ol.

The mass spectra of the synthetic pheromones and their DMDS adducts were virtually identical to those of the isolated natural products (*Z* and *E* isomers gave the spectra). The GC retention times on both the nonpolar SE-30 and polar Carbowax capillary columns also were identical with those of the natural pheromone. The larger and faster eluting peak observed with the pheromone on the nonpolar capillary column was assigned to the *Z* isomer.

Derivatization. The DMDS derivatives of biologically active GC fractions and synthetic samples were prepared according to standard procedures (Dunkelblum et al., 1985), with the exception of only a 4-hr reaction time. The observed fragments at *m/z* 159 and 145 in the mass spectrum of DMDS adducts of (*Z*)-7-tetradecen-2-one (Figure 2a) could also be produced from its isomer, 6-tetradecen-2-one, so it was necessary to conduct further investigations to make an unambiguous assignment of the double-bond location. For this purpose, (*Z*)-6-tetradecen-2-one and (*Z*)-5-tetradecen-2-one (gifts from Dr. Leal) were studied.

The diagnostic sulfide fragment ions A^+ and B^+ is the mass spectra of DMDS adducts of linear monounsaturated alkenes, esters, and acetates usually are the prominent peaks, and sometimes are the base peaks in their spectra. The relative intensities of ions produced by secondary fragmentation, such as fragments $A^+ -48$ and $B^+ -48$ (decomposed via loss of CH_3SH), are always smaller than their parent ions A^+ and B^+. The transaction of $B^+ -48$, however, is found as the most predominant fragmentation in the DMDS adducts of these monounsaturated ketones compared to $A^+ -48$. Therefore, the ions, $B^+ -48$, can be used as the diagnostic fragments to locate the double-bond position in GC-MS

OH (1) —PCC / CH_2Cl_2→ H(C=O) (2) —CH_3MgBr / ether→

OH (3) —PCC / CH_2Cl_2→ O (4)

SCHEME 1. Synthetic pathway for Oriental beetle pheromone component, (*E*)-7-tetradecen-2-one.

analysis of DMDS derivatives of some monounsaturated ketones. The degradation of DMDS adduct of 7-tetradecen-2-one is illustrated in Scheme 2.

With this monounsaturated ketone, the relative intensity of the fragment ion, C^+ (*m/z* 111), is actually higher than its parent ion B^+ (*m/z* 159) (Figure 2a), whereas, fragmentation of A^+-48 (*m/z* 97) is not significant. Based on the fragment C^+ (*m/z* 111), the peak at *m/z* 159 can easily be assigned as the fragment B^+, and the double bond position in the natural pheromone can be unambiguously determined. The same degradation occurred in the mass spectra of DMDS derivatives of 6- and 5-tetradecen-2-one. The ion C^+ (*m/z* 97) is the second highest peak in the spectrum of the Δ6-isomer (Figure 2b). Even though the two sulfide fragments were similar to those obtained with natural pheromone, it is not difficult to determine that the ion at *m/z* 145 is the B^+ fragment (Scheme 3).

The B^+-48 fragment was also determined for the Δ5 isomer, which exhibits a capillary GC retention time that is identical with the natural pheromone. DMDS derivatization of (*Z*)-5-tetradecen-2-one produced a product with a molecular ion at *m/z* 304. Cleavage of the bond between the sulfur-substituted carbons led to two fragment ions, B^+ (*m/z* 131) and A^+ (*m/z* 173). The fragment C^+ (*m/z* 83) was obtained through further decomposition of B^+ with a loss of 48 units (CH_3SH) (Figure 2c). It was noted that in the case of the Δ5 and Δ6 isomers, the fragmentation of A^+-48 was not observed in the mass spectra of the corresponding DMDS derivatives.

The high intensity of the ion C^+, which is derived from the fragmentation

SCH₃ O +SCH₃
SCH₃ - CH₃SH
m/z 145 (A⁺)
m/z 304 (M⁺·) m/z 159 (B⁺) m/z 111 (C⁺)

SCHEME 2. Degradation pathway of DMDS adducts of 7-tetradecen-2-one.

SCH₃ O SCH₃ O
SCH₃ - CH₃SH
m/z 159 (A⁺)
m/z 304 (M⁺·) m/z 145 (B⁺) m/z 97 (C⁺)

SCHEME 3. Degradation pathway of DMDS adducts of 6-tetradecen-2-one.

of B^+-48, suggests that the neutral molecule, methanethiol (CH_3SH), is much easier to lose from its parent ion, B^+, than from ion A^+. It is obvious that this fragmentation is formed via a hydrogen rearrangement and that the carbonyl group is involved in the rearrangement (Scheme 4).

The neighboring-group participation can be proposed to explain this phenomenon. Initially, hydrogen (H_β) could be transferred to the oxygen of the carbonyl group, and then$_\gamma$ extracted by a pair of lone electrons on sulfur to complete the 1,3 hydrogen migration. The key fragment, allylic ion C^+, would finally be formed by elimination of methanethiol (CH_3SH) as a neutral species from the resultant. The change transfer could be the driving force in this fragmentation. The number of CH_2 can vary at least from 0 to 2. This differs from the McLafferty rearrangement, in which the transferred hydrogen is restricted to the γ position to the carbonyl oxygen as it forms a six-member ring transition state.

Laboratory Bioassays. A series of behavioral tests were conducted in the flight tunnel to monitor the activity of GC fractions from airborne collections, to verify the activity of synthetic pheromones, and to evaluate the response of male beetles to different *Z*/*E* ratios. When live 5-day-old females were placed in the emission screen cage and used as the pheromone source, males exhibited a directed upwind anemotaxis toward the source, but the males did not show any response to a blank control in all assays performed.

The sequence of behavior exhibited by males to synthetic pheromone was comparable to the responses to caged females. The initial activation response of males was manifested by restlessness, waving of antennae with open lamellae directed upwind, waving of forelegs, walking upwind, and preliminary wing extension. The oriented upwind flights were started with taking off from the platform, performing a wide zigzag flight pattern, and locking on to the plume and proceeding in upwind flight. Finally, the males approached and landed on the emission screen cage (filter paper or septum when active GC fractions or synthetic pheromones were used as the sources) and attempted to copulate with the source. This behavioral response sequence is very similar to that described for another scarab, the green June bug (Domek et al., 1990).

The entire upwind flight sequence was not completed by some males, especially those less than 4 days old. Bioassays with males of different ages, using three to six live females as the source, showed that males younger than

B^+ → → → C^+ + CH_3SH

n = 0, 1, 2...

SCHEME 4. Mechanism of elimination of methanethiol from fragment B^+.

4 days old did not fly, although some did activate. A batch of males was tested from their first day after emergence up to their ninth day using the different pheromone sources (three to six live females, 10 ng of *Z* isomer and 50 ng of *Z* isomer on filter paper). The results indicated that a significantly lower proportion of these males activated on their first to third day and that none flew to the source. With increasing age, greater numbers of males activated, took flight, and eventually touched the source (Figure 3). Therefore, only males that were more than four days old were used in the rest of flight tunnel tests.

Male OB responses were observed to various dosages and ratios of synthetic isomer. The isomers were located on the filter paper or rubber septa in different amounts and *Z*/*E* ratios and tested separately. With the filter paper, responses of males were not significantly different with either 50 ng *Z* isomer or 10 ng *Z* isomer compared with a live female (six) source (Figure 4). However, responses to the *E* isomer were significantly lower than to the *Z* isomer. Male responses to individual isomers and a blend (50 ng *Z*, 50 ng *E* and 50 ng 88 : 12 *Z*/*E* blend) relative to the live females (four to six) are compared in Figure 5, in which the proportions are averages of three trials. In these tests, there was not discrimination between the *Z* isomer alone and the blend, although there was a significant difference between responses to either the *Z* isomer or the blend and to the *E* isomer in all stages of male response. Interestingly, one male did fly all the way to 50 ng of the *E* isomer source in these studies.

A dosage study with rubber septa also was conducted to determine a usable range for field tests, as well as for future in-depth flight-tunnel tests. The rubber

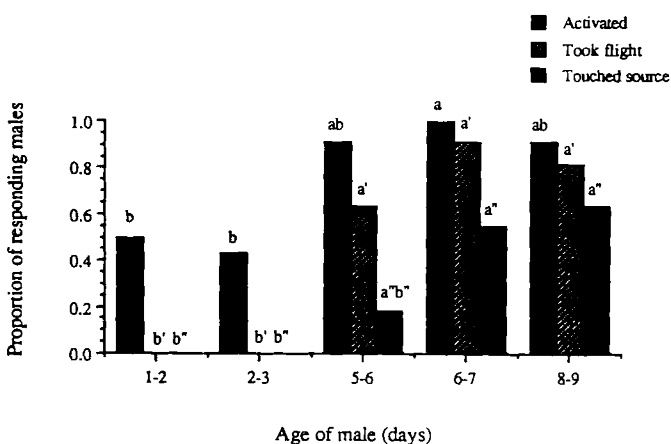

FIG. 3. Behavioral responses of OB males of different age to six live females. Bars (behaviors) superscripted by the same letter are not significantly different ($P < 0.05$. 1-2, $N = 10$; 2-3, $N = 14$; the rest, $N = 11$).

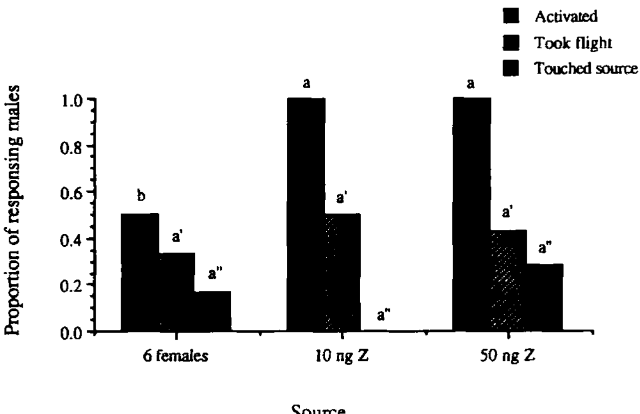

FIG. 4. Behavioral responses of OB males of different age to six live females, 10 ng *Z*, or 50 ng *Z* isomer. Separate comparisons were made for each behavior. Bars (behaviors) superscripted by the same letter are not significantly different ($P < 0.05$. Six females, $N = 6$; 10 ng *Z*, $N = 6$; 50 ng *Z*, $N = 7$).

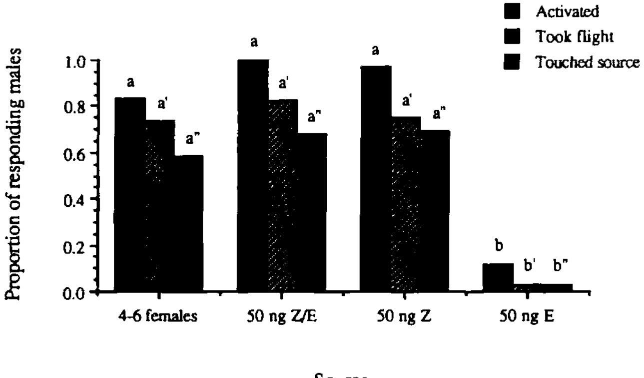

FIG. 5. Behavioral responses of OB males to a 50-ng source of the *Z*, *E* isomers and a 88:12 *Z*/*E* blend compared with four to six live females. Separate comparisons were made for each behavior. Bars (behaviors) superscripted by the same letter are not significantly different ($P < 0.05$. Trial 1, $N = 24$; trial 2, $N = 43$; trial 3, $N = 44$).

septa were baited with 1, 10, and 100 μg of a 90:10 *Z*/*E* blend. It was found that males were activated in significantly greater numbers to the 10-μg lure than to the 1-μg lure. Septa with the higher dosage (100 μg), however, elicited fewer completed flights. These data help to define the lower threshold level for com-

plete flight responses and the upper threshold level at which arrestment of upwind flight occurs in the flight tunnel.

The synthetic pheromone composed of a 90:10 *Z/E* blend (20 ng) elicited about a 1-mV EAG response with male antennae. The EAG value is comparable to the response of 17 female-day-equivalents of airborne collection.

Additional preliminary studies in the flight tunnel suggested that several other factors are important for OB behavior. First, virgin females in the emission cage were much more attractive than mated ones. Second, males older than four days flew to the pheromone source during photophase and scotophase. Third, when females were transferred from scotophase to the flight tunnel they did not elicit any male OB response during scotophase, although female beetles transferred from photophase did elicit complete responses from males during scotophase. These observations suggest that only virgin females OB release pheromone and they release it only during the photophase, or at least the calling period is initiated in photophase. These results are in agreement with the field-trapping studies with the OB (Facundo et al., 1994).

CONCLUSIONS

The sex pheromone isolated from female Oriental beetle, *Anomala orientalis*, was identified, using GC, GC-MS, along with flight-tunnel bioassays, to be a 96:4 to 88:12 mixture of (*Z*)- and (*E*)-7-tetradecen-2-one. The double-bond position of the pheromone (monounsaturated ketone) was determined by DMDS derivatization and interpretation of the diagnostic ions in their mass spectra. Behavior studies with male OB in the flight tunnel showed that 10 μg of a 90:10 *Z/E* blend on a rubber septum was an attractive source. The same pheromone with a similar *Z/E* ratio also was found in the Japanese Oriental beetle, *Blitopertha orientalis* (Leal, 1993). It is not known if these populations are the same species.

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LIPIDS OF CYANOBACTERIUM *Aphanizomenon flos-aquae* AND INHIBITION OF *Chlorella* GROWTH

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Abstract—The inhibition of the growth of the green alga *Chlorella pyrenoidosa* by the lipids of the cyanobacterium *Aphanizomenon flos-aquae* is associated with the fractions containing long-chain unsaturated fatty acids. A sterol present in *Aphanizomenon* has been identified as poriferasterol.

Key Words—*Aphanizomenon flos-aquae, Chlorella pyrenoidosa*, growth inhibition, lipids, fatty acids, poriferasterol.

INTRODUCTION

The major nonpigment lipids of cyanobacteria consist of monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglyerol (DGDG), phosphatidylglycerol, and sulfoquinovosyldiacylglycerol (Murata and Nishida, 1987). Other lipids occurring in lesser amounts include fatty acids, sterols, hydrocarbons, and heterocyst glycolipids (glycosides of long chain diols, triols, and hydroxy acids). The cyanobacteria do not appear to contain phosphatidylcholine, -ethanolamine, -serine, -inositol, and diphosphatidylglycerol (cardiolipin).

Studies of the lipid components of *Aphanizomenon flos-aquae* itself have been limited. The carotenoid pigments have been investigated by Hertzberg and Liaaen-Jensen (1966), and the presence of C_{28-29} 1,13-diols and C_{30-32} 1,15-diols has been reported by Morris and Brassell (1988).

This report describes the general lipid profile of *A. flos-aquae* and the activity of the lipid fractions in inhibiting the growth of the green alga *Chlorella*

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pyrenoidosa. This is part of an ongoing program on the ecology and toxicity of freshwater cyanobacteria.

METHODS AND MATERIALS

Organism. *Aphanizomenon flos-aquae* strain NH-1, which was originally isolated by Dr. W.W. Carmichael, Wright State University, Dayton, Ohio, from a toxic bloom that occurred in a farm pond in Durham, New Hampshire, was cultured in the laboratory using ASM-1 medium (Carmichael and Gorham, 1974). The cells were harvested by centrifugation, lyophilized, and stored in a freezer.

Extraction of Lipids. Lyophilized *A. flos-aquae* cells (10 g) were added to 250 ml of methanol and the mixture brought to a boil. After cooling, 500 ml of chloroform was added. The mixture was stirred for 1 hr, then filtered through Whatman No. 1 paper. The extract was concentrated to dryness in vacuo and the residue extracted with 300 ml of chloroform—methanol (2:1). The mixture was filtered, the filtrate taken to dryness, and the residue extracted with 150 ml of chloroform—methanol (2:1). The final extract was filtered and taken to dryness to yield 2.28 g of lipids as a black syrup.

Silicic Acid Chromatography. The lipid residue was dissolved in a small volume of chloroform and applied to a silicic acid column (2.6 × 18 cm) formed from a slurry of 50 g of precipitated silicic acid (Fisher Scientific Co.; sample previously heated for 1.5 hr at 110°C) in chloroform. The column was developed using a solvent sequence similar to that described by Vorbeck and Marinetti (1965). The details and results are shown in Table 1.

Fraction A. Fraction A (0.116 g) was dissolved in 1 ml of chloroform and streaked along the origin of an E. Merck Silica Gel 60 preparative TLC plate (20 cm × 20 cm × 2 mm) and developed in chloroform—methanol—acetic acid—water (85:15:10:3.7). A dark orange band of carotenoid pigments (weight: 69 mg) was visible at the solvent front. Immediately below the solvent front was a major band, made visible by spraying a narrow vertical end section of the plate with 50% sulfuric acid and heating. This band was scraped out and eluted with 50 ml of chloroform—methanol (2:1). The eluate was concentrated to dryness in vacuo to yield 40 mg of a yellow syrup containing crystals. This was recrystallized from methanol to yield 2.3 mg of feathery crystals melting at 125–133°C (fraction A1). A second crop of crystals (fraction A2) (4 mg) was obtained. The filtrate was concentrated to dryness to yield fraction A3.

Fraction B. Very darkly pigmented fraction B (206 mg) was dissolved in chloroform—methanol (2:1) and streaked along the origins of two E. Merck Silica Gel 60 preparative TLC plates and developed in chloroform—methanol—acetic acid—water (85:15:10:3.7). The main fraction was located as described for fraction A. This zone was scraped out, eluted with

TABLE 1. SILICIC ACID CHROMATOGRAPHY OF *Aphanizomenon flos-aquae* LIPIDS

Fraction	Eluting agent	Volume Fraction (ml)	Appearance	Dry wt. (g)	Prominant components (by TLC)
A	$CHCl_3$	130	Orange	0.127	Orange pigments, fast moving compounds
B	$CHCl_3$	220	Inky black	0.225	Dark green pigments, fast moving compounds
C	$CHCl_3$: acetone (1 : 1)	55	Inky black	0.312	Dark green pigments, MGDG
D	$CHCl_3$: acetone (1 : 1)	325	Brown	0.138	MGDG
E	Acetone	150	Light orange brown	0.162	DGDG, phosphatidylglycerol
F	$CHCl_3$: MeOH (9 : 1)	90	Light brown	0.011	DGDG, phosphatidylglycerol
G	$CHCl_3$: MeOH (9 : 1)	87	Brown	0.015	DGDG, phosphatidylglycerol
H	MeOH	220	Dark brown	0.921	Sulfolipid, other polar lipid material
Total recovery				1.911	(84%)

chloroform—methanol (2 : 1), concentrated, and the preparative TLC step repeated. Most of the dark pigments had been removed. The material from the second preparative TLC (89 mg) was dissolved in 1 ml of chloroform, streaked onto two preparative TLC plates, and the plates developed in chloroform to yield two fractions. The faster moving fraction was streaked onto an E. Merck Silica Gel 60 analytical TLC plate (20 × 20 cm) and developed in chloroform to yield crystalline fraction B1 (4 mg). The slower moving fraction was streaked onto a preparative Silica Gel 60 TLC plate and developed in chloroform—methanol (96:4) to yield fraction B2 (43 mg) as feathery crystals.

Fractions C and D. Fractions C and D were combined (424 mg), dissolved in a small amount of chloroform—methanol (2 : 1), streaked onto two E. Merck preparative Silica Gel 60 TLC plates, and developed in chloroform—methanol—acetic acid—water (85 : 15 : 10 : 3.7). The major band, located as in fraction A, was scraped out, eluted with chloroform—methanol (2 : 1), and the extract concentrated in vacuo. The preparative TLC was repeated twice more to give 104 mg of the major band. Recrystallization from methanol yield fraction CD1 (white crystals, 7 mg, mp 137–141°C). Fraction CD2 was recovered from the mother liquors.

Chlorella *Paper Disk Assay Procedure.* The paper disk assay procedure was essentially as previously described (Ikawa et al., 1985). The procedure for the preparation of the *Chlorella*-seeded agar plates was simplified as follows.

Medium (200 ml) containing 2 g of agar was brought to boiling, and, when cooled to 50°C, was inoculated with cells of *Chlorella pyrenoidosa* (UTEX strain 395) washed from three agar slants of *C. pyrenoidosa* (UTEX strain 395) with 3 ml each of sterile water, and immediately poured into Petri dishes before hardening. Fractions were dissolved in 95% ethanol and applied to 6-mm paper disks (Difco) for testing.

Physical Methods. Proton NMRs were run on a Bruker AM 360 NMR spectrometer at 360 MHz. Carbon spectra were decoupled at 90 MHz. Mass spectra were obtained with a Hewlett-Packard model 5988 quadrupole mass spectrometer at an ion source temperature of 200°C and an ionization energy of 70 eV. For GC-MS, the mass spectrometer was coupled to a Hewlett-Packard model 5890 GC equipped with a capillary column (25 m × 0.2 mm ID) coated with a 0.5-μm film thickness of HP-1 cross-linked methyl silicone gum. Methyl esters of fractions A3 and B2 were prepared using dimethylformamide dimethyl acetal (Methyl-8 Concentrate, Pierce, Rockford, Illinois).

Thin-Layer Chromatography (TLC). The major lipid classes in each fraction were identified by TLC on Silica Gel 60 plates (E. Merck) in chloroform—methanol—acetic acid—water (85:15:10:3.7, v/v) (Nichols, 1970). Compounds were detected by spraying with 50% sulfuric acid, followed by heating with a heat gun. Carbohydrate-containing components were detected by spraying with periodate followed by benzidine (Grado and Ballou, 1961). TLC for fatty acid components were carried out using Silica Gel 60 plates (E. Merck) in hexane—diethyl ether—acetic acid (45:5:0.5) and Si—C_{18} reverse-phase plates (J.T. Baker Chemical Co.) in acetic acid—water (9:1). After chromatography for fatty acids, the plates were thoroughly dried overnight in a hood to remove the acetic acid. The plates were then sprayed with 0.05% rhodamine 6G in ethanol and the fluorescence observed under long-wavelength UV or by periodate—benzidine. Similarly to carbohydrates, unsaturated fatty acids could be detected with the periodate—benzidine spray. Saturated fatty acids showed no reaction with this spray. The upper one third of the reverse-phase plates in acetic acid—water (9:1) reacted strongly with the periodate—benzidine spray and was not useful in this region.

RESULTS AND DISCUSSION

Lipid Profile. Table 1 shows the approximate lipid composition of *A. flos-aquae*. Besides the orange and dark green pigments, major components could be identified by TLC as monoglycosyldiglyceride (MGDG), diglycosyldiglyceride (DGDG), phosphatidylglycerol, and sulfolipid.

Fractions A1 and B1. Both fractions A1 and B1 on mass spectrometry gave a strong molecular ion at 412, and their mass spectra indicated that they were

either stigmasterol or its C-24 epimer poriferasterol. A comparison of the chemical shifts of the methyl group protons of fraction A1 and stigmasterol, especially of C-27 and C-29 (Table 2), and the reported differences between poriferasterol and stigmasterol (Khalil et al., 1980; Chiu and Patterson, 1981), indicated that the compound was poriferasterol. Comparison of the differences in the ^{13}C chemical shifts of ring D and the side chain carbons between fractions A1 and stigmasterol and the reported differences between poriferasterol and stigmasterol, especially at C-16, C-20, C-21, and C-29 (Wright et al., 1978) (Table 2), also indicated that fraction A1 was poriferasterol with the β configuration at C-24.

Kohlhase and Pohl (1988) reported the presence of significant percentages of 24-ethylcholest-5,22-dien-3β-ol (stigmasterol or poriferasterol), 24-ethylcholest-5-en-3β-ol (sitosterol or clionasterol), and 24-ethylcholestan-3β-ol (stigmastanol or 5,6-dihydroclionasterol) in the sterols of the cyanobacteria of the genera *Anabaena*, *Nodularia*, and *Nostoc*, but the configuration at C-24 was not determined. Other studies have shown that C-24 ethyl and methyl groups are predominantly α (*S* if the side chain is unsaturated at C-22, and *R*, if the side chain is saturated) in vascular plants and β(*R* if the side chain is unsaturated,

TABLE 2. ^{13}C CHEMICAL SHIFTS OF RING D AND SIDE CHAIN CARBONS AND METHYL GROUP ^{1}H SHIFTS OF 24-ETHYL-CHOLEST-5,22-DIEN-3β-OLS

C	^{13}C (δ, ppm)		^{1}H (δ, ppm)	
assignments[a]	Fraction A1	Stigmasterol	Fraction A1	Stigmasterol
13	42.21	42.21		
14	56.85	56.86		
15	24.34	24.37		
16	28.82	28.94		
17	55.92	55.93		
18	12.05	12.05	0.697(s)	0.698(s)
19	19.40	19.41	1.011(s)	1.011(s)
20	40.44	40.53		
21	20.94	21.12	1.023(d, J = 7.9)	1.021(d, J = 7.3)
22	138.29	138.34		
23	129.31	129.25		
24	51.21	51.24		
25	31.84	31.89		
26/27	18.94	18.99	0.844(d, J = 6.5)	0.847(d, J = 6.4)
27/26	21.17	21.23	0.791(d, J = 6.4)	0.796(d, J = 6.6)
28	25.39	25.42		
29	12.44	12.27	0.811(t, J = 8.0)	0.805(t, J = 7.0)

[a]Wright et al. (1978).

and *S* if the side chain is saturated) in fungi and algae (Nes et al., 1976). Our results show the presence of the β configuration at C-24 in the cyanobacterium *A. flos-aquae*.

Fractions A2, A3, and B2. Fraction A2 was identified as palmitic acid from its mass spectrum ($M^+ = 256$). GC-MS of the methyl esters of fraction A3 showed principally palmitic acid ($C_{16:0}$) (~60%) with lesser amounts (~20% each) of $C_{16:1}$, and $C_{18:1}$, and trace amounts of stearic acid ($C_{18:0}$). GC-MS of the methyl esters of fraction B2 showed principally palmitic acid (~50%); lesser amounts (~10–20% each) of $C_{16:1}$, $C_{18:1}$, and $C_{18:2}$; and trace amounts of $C_{16:2}$, stearic acid, and $C_{18:3}$. These results were corroborated by TLC. The reverse phase Si—C_{18} system showed the presence of $C_{18:1}$ and also $C_{18:2}$ and/or $C_{16:1}$ acids. It can thus be concluded that both fractions A3 and B2 contain principally palmitic acid and smaller but significant amounts of $C_{16:1}$ (palmitoleic or palmitelaidic acids), $C_{18:1}$ (oleic acid), and $C_{18:2}$ (linoleic acid).

Fraction CD1. Fraction CD1 chromatographed as monoglycosyldiacylglycerol. On methanolysis it yielded a mixture of fatty acid methyl esters, which on GC-MS, indicated mostly palmitic acid, but also the presence of stearic acid, $C_{18:1}$ (oleic acid), and $C_{18:2}$ (linoleic acid). The other products of the methanolysis were identified by TLC as galactose and glycerol, indicating that the compound is a monogalactosyldiacylglycerol (MGDG).

Activity of Lipid Fractions of A. flos-aquae *on Inhibition of* Chlorella *Growth.* The activity of the various lipid fractions in inhibiting the growth of *Chlorella* is shown in Table 3. Measurable activity was observed in fractions A3 and B2. These are fractions that contain largely palmitic acid but also significant amounts of $C_{16:1}$, $C_{18:1}$, and $C_{18:2}$. Although palmitic and oleic ($C_{18:1}$) acids show no activity at 10 mg/ml or even at higher concentrations (McGrattan et al., 1976; Ikawa et al., 1984) , unsaturated acids such as palmitoleic (*cis*-$C_{16:1}$) (unpublished results) and linoleic (*cis*, *cis*-$C_{18:2}$) acids show inhibiting activity in the 1–2 mg/ml range and can account for the growth inhibiting activity of these fractions. Linoleic and linolenic ($C_{18:3}$) acids have been shown to be the autolytic substance in the freshwater cyanobacterium *Phormidium tenue* (Murakami et al., 1990) and the substances produced by the green alga *Chlamydomonas*, which inhibit the growth of *Hematococcus* and other green algae including itself (McCracken et al., 1980). Monogalactosyldiacylglycerols (MGDGs), which are released into the growth medium by the cyanobacterium *Phormidium tenue*, also autoinhibit the growth of this organism, especially if they contain unsaturated fatty acids such as linoleic and linolenic acids (Murakami et al., 1991). These authors suggest that the liberation of the unsaturated fatty acids from the MGDGs by esterases in the cyanobacterium is responsible for the autolytic effects of the MGDGs. We could demonstrate only minimal activity at best with fraction CD1, the MGDG from *A. flos-aquae*, at 1 mg/ml. Fraction CD2, which contained the bulk of the MGDG, failed to show any

TABLE 3. INHIBITION OF *Chlorella* GROWTH BY *A. flos-aquae* LIPID FRACTIONS

Fraction[a]	Net diameter of inhibition zone (mm)[b] at EtOH concn. (mg/ml) of			
	10	5	2	1
Palmitic acid (Fraction A2)	0			
Fraction A3 (fatty acids)	4	0		
Fraction B1 (poriferasterol)				0
Fraction B2 (fatty acids)	5	0		
Fraction CD1 (MGDG)				±
Fraction CD2 (MGDG)	0			
MGDG (whole wheat flour)			0	
Fraction E (DGDG, phosphatidylglycerol)	4	0		
DGDG (whole wheat flour)			0	
Fraction H (sulfolipid, other polar lipids)	±			

[a]MGDG and DGDG from whole wheat flour were purchased from Sigma Chemical Co., St. Louis, Missouri.
[b]Total diameter of inhibition zone less paper disk diameter (6 mm) after two to three days (two-day boundaries were sometimes diffuse and difficult to measure. ± indicates slight or questionable inhibition (ca. 2 mm).

activity at 10 mg/ml. Fraction CD1 contained mostly palmitic acid and some stearic, $C_{18:1}$, and $C_{18:2}$ acids. No activity was observed with wheat flour MGDG at 2 mg/ml.

Fraction E, which consists of DGDG and phosphatidylglycerol, showed activity at 10 mg/ml. Murakami et al (1991) reported that the DGDGs of *Phormidium tenue* were less potent than the MGDGs in autoinhibiting the growth of the cyanobacterium. We found that wheat flour DGDG was inactive at 2 mg/ml. Fraction H, consisting of sulfolipid and other polar lipids, showed very weak activity.

We can conclude that at least part of the inhibition of *Chlorella* growth by *A. flos-aquae* extracts is associated with the lipid fractions containing free fatty acids and that the unsaturated fatty acids are probably the active species. Thus, there is a growing body of evidence indicating that unsaturated fatty acids may play an important role in limiting the growth of green algal and cyanobacterial populations in freshwater ecosystems through inter- and intraspecific chemical mechanisms.

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CUTICLE ALKANES OF HONEYBEE LARVAE MEDIATE ARRESTMENT OF BEE PARASITE *Varroa jacobsoni*

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Abstract—The ectoparasitic mite *Varroa jacobsoni* invades worker brood cells of the honeybee *Apis mellifera* during the last 20 hr before the cells are sealed with a wax cap. Cuticle extracts of 8-day-old worker honeybee larvae occupying such brood cells have an arrestment effect on the mite. The mites run for prolonged periods on the extract, systematically returning onto the stimulus after touching the borders of the treated area. Mites increase walking speed and path straightness in response to increasing doses of a nonpolar fraction of the cuticle extract. Saturated straight-chain odd-numbered $C_{19}-C_{29}$ hydrocarbons were identified by thin-layer argentation chromatography and gas chromatography-mass spectrometry as the most active constituents, with branched alkanes also contributing to the arrestment effect of this active fraction. Analysis of the behavior responses to synthetic *n*-alkanes indicate that the response is probably based on a synergism between the different alkane components of the fraction rather than to an individual compound.

Key Words—*Varroa jacobsoni*, Acari, Varroidae, mite, *Apis mellifera*, Hymenoptera, Apidae, honeybee, chemoreception, host selection, cuticle, hydrocarbons, alkanes.

INTRODUCTION

The ectoparasitic mite *Varroa jacobsoni* Oud. (Acari, Varroidae) threatens colonies of honeybees *Apis mellifera* L. worldwide. It enters brood cells of male

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honeybee larvae 20–40 hr and cells of female worker larvae 0–20 hr before operculation (Boot et al., 1992). Worker bees seal the cells of 9-day-old worker and 10-day-old drone larvae with a wax cap (Winston, 1987). The mite reproduces during host development within the brood cell. On emergence of the young bee, mother and daughter mites leave the brood cell and the males die. Being ectoparasitic, the mites feed on host hemolymph.

The short time span of 20 hr during which the mites invade brood cells suggests recognition of hosts of the appropriate age by *Varroa*. Indeed. three fatty acid esters (methyl palmitate, ethyl palmitate, and methyl linolenate) identified from 9-day-old drone larvae proved attractive to *Varroa* in an olfactometer (LeConte et al., 1989). In addition, palmitic acid, the probable precursor of methyl palmitate, is present in the headspace over 8-day-old worker larvae and attracts the mites, eliciting an upwind walking response when presented in an airstream (Rickli et al., 1992). These products may therefore serve as host-finding cues. After operculation, the bee larva spins a cocoon. During this period, the mite is highly mobile but shows a strong attachment for the free surface of the larva (Donzé and Guerin, 1994). Two functions of this behavior appear plausible: (1) to avoid being crushed between the larval body and cell wall during cocoon spinning and (2) to avoid being excluded from the larva by the newly spun cocoon on the cell wall.

We have observed that mites show an arrestment response on a substrate treated with cuticle extract of 8-day-old worker larvae in vitro. Mites walking on the area treated with this extract systematically change their walking direction upon touching the border of the extract to return to the treated area (see Figure 1 below). In this study, we identify chemostimuli mediating this arrestment response of *Varroa*. Furthermore, an analysis of tracks made by *Varroa* on the treated area describes major features of the mite's response to the cuticle extract and one of its components.

METHODS AND MATERIALS

Use of Abbreviations: straight-chain alkanes are abbreviated as n-C_x where x indicates the number of carbon (C) atoms followed by the number of double bonds. *br*-C*x* stands for internally branched alkanes, most of them monomethyl alkanes, but also including some dimethyl alkanes. Thus *br*-$C_{25:0}$ signifies a chain of 25 C atoms with either one or two methyl groups at an unspecified location within the chain, the molecule having no double bonds.

Mites. Lots of 60–100 *Varroa* visible on the surface of adult bees in heavily infested bee colonies were collected and held for two to seven days on their hosts in the laboratory before bioassay. Some 15–30 min before a test, a group of 10–15 mites was removed from the bees and held in a humidified glass tube

at room temperature (18–21°C) until a test run. Each test solution was assayed with mites of at least two different lots on different days. Tests were conducted with batches of test solutions all assayed on the same day and accompanied by at least one solvent control. The daily sequence of solutions was randomized. Prior to tests of fractions of bee cuticle extract and dilution series, some mites were subjected to negative (solvent) and positive (active fraction of extract) controls to ascertain the responsiveness of the lot of mites being employed in the bioassays.

Cuticle Extract and Fractionation. Eight-day-old *A. mellifera* worker larvae were extracted using their size and weight (over 120 mg) (Thrasyvoulou and Benton, 1982) as an index of age. These larvae were chosen because of the relative ease of access to them before operculation. Fifty to 200 larvae were submerged for 15 min in 10 ml *n*-hexane (Merck, analytical grade) and the resulting extract concentrated to 1 ml under a gentle flow of nitrogen. All extracts were stored at −20°C. Thin-layer chromatography (TLC) plates (Merck, ready-made Silica Gel 60 analytical plates) were conditioned by running them twice in a mixture of methanol—chloroform 1:2 and drying. After loading 0.2–1.0 ml of extract (20–100 larva equivalents, leq), the plates were fully developed in hexane followed by toluene, and to two thirds in hexane—diethylether—acetic acid 70:30:1. A strip was cut off the plate and developed by charring with 50% H_2SO_4 and heating to 140°C until bands were visible. Bands corresponding to the fractions thus visualized were then scraped from the rest of the plates and extracted with dichloromethane (CH_2Cl_2). Fractions were concentrated under N_2 to a volume equivalent to that of the extract and then tested at a level corresponding to 12 leq on the test arena. The most apolar fraction, F1, was further separated into complex wax esters and front-running hydrocarbons (HC) by separation of F1 alone in hexane on the same TLC plates. This HC fraction (F1A) was found active and tested in amounts ranging from 0.6 to 12 leq.

To distinguish between saturated and unsaturated alkanes present in fractions F1 and F1A (above), it was necessary to separate saturated HCs from the rest. For this, cuticle extract was separated in hexane on TLC plates impregnated with silver nitrate (Aitzetmüller and Guaraldo Goncalves, 1990) where only *n*- and *br*-alkanes migrated. The nonmigrating fraction of more polar products was bioassayed as such. In addition, alkenes and alkadienes were purified from this more polar material by elution in hexane on ready-made TLC plates and tested as "alkenes." The silver nitrate-developed fraction containing the saturated alkanes was further separated into straight-chain and branched alkanes by adsorption of the straight-chain products onto a molecular sieve of zeolith $CaAlSi_3O_8$ (O'Connor et al., 1962). After conditioning the sieve (Merck 5705, pore size 0.5 nm) at 260°C under vacuum (0.6 mm Hg) for 4 hr, 1 g was added to the sample of saturated compounds dissolved in 2–3 ml isooctane and refluxed at 110–115°C for 8 hr. The nonadsorbed *br*-alkanes were recovered from the

isooctane by concentration under N_2. Recovery is never complete, and in this case some *br*-alkanes were also adsorbed. The initial amount of HCs exposed to the molecular sieve was 50–70 leq, the amount bioassayed was 16 leq *br*-alkanes (12 $\mu g/cm^2$).

Identification and Quantification. Gas chromatography–mass spectrometry (GC-MS) was employed to identify constituents of active TLC fractions. Samples were injected on-column onto a 15-m BGB (Zürich) high-temperature/high-resolution fused silica capillary column in a Hewlett Packard 5890 Series II gas chromatograph coupled to a HP 5971A mass-selective detector. The nonpolar column (5% phenyl, 95% methylpolysiloxane, 0.25 mm ID, and 0.12-μm film thickness) was temperature programmed either from 60°C after 2 min (He with 16 kPa head pressure, constant flow) or from 100°C after 2 min (He at 28 kPa, constant flow) at 10°/min to 370°C. The mass selective detector (190°C) was set to a scan range of m/z 50–650. Retention times and mass spectra of unknowns were compared with those of authentic samples.

Gas chromatography with flame ionization detection (GC-FID) allowed us to estimate accurately the quantities of the compounds present in the different fractions. For this a Carlo-Erba HRGC 5300 (Mega Series) equipped with a 30-m nonpolar high-resolution fused silica capacity DB-5 column (J&W, California: 0.32 mm ID and, 1-μm film thickness) was employed with splitless injection at 240°C and the FID detector at 300°C. The column was temperature programmed for a fast analysis from 200°C after 2 min at 5°/min to 340°C and held for 16 min or, for more detailed analysis, from 60°C after 2 min at 5°/min to 300°C and held for 80 min with He at a flow rate of 1.35 ml/min. Quantification of compounds identified by GC-MS was made by analysis of five separate extracts of 100 larvae held for 15 min in hexane and concentrated to 1 ml. Of each extract, 0.1 leq with 50 ppm of n-$C_{24:0}$ as internal standard were injected. In this study, only components making up more than 1% of the cuticular extract were quantified.

Bioassay and Data Analysis. Three concentric circles of 12, 24, and 36 mm diameter were drawn with a fine 0.1-mm ink pen of the underside of a semipermeable biological membrane (Baudruche, John Long Inc.) (Figure 1). This membrane was then washed twice in hexane and once in acetone and stretched over a small water bath providing a humidity of ≥90% and a temperature of 32°C on the surface of the membrane. Some 50–200 μl of the test solution were spread with a 10-μl micropipet on the band between the inner and middle circles called the treated area. The surface of the treated area (3.4 cm^2) made up roughly 1.7 times the surface of an 8-day-old larva (1.8–2.4 cm^2). A mite was deposited with a fine brush in the center of the area. After 300 sec or upon leaving the outer circle, which ever came first, the test was terminated and the mite removed.

Synthetic saturated and monounsaturated odd-numbered n-C_{19} to n-C_{29}

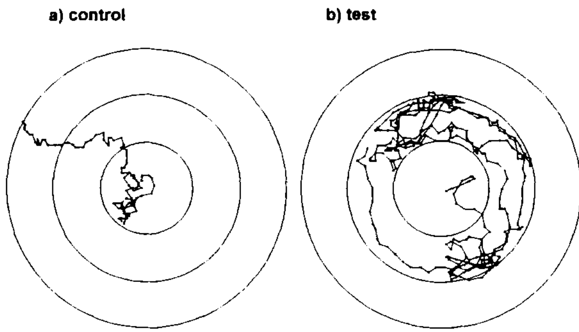

FIG. 1. *Varroa* tracks on a water-permeable biological membrane with test material applied between the inner and middle circles: (a) solvent alone (control) and (b) 30 μg n-$C_{21:0}/cm^2$ (test). Mites were released in the center and their paths drawn for the first 60 sec of the 5 min test or until the mite left the outer circle, which ever came first. On solvent control, the animal moved without return through the treated area in 5 sec. Once on the area treated with n-C_{210} (b) the mite moved for 147 sec on it, returning 61 times to the stimulus after contacting the border. Bar represents 10 mm.

($\geq$97% purity by GC, Sigma) were dissolved in hexane and tested at doses of 30 $\mu g/cm^2$ initially. Binary and ternary combinations of compounds of neighboring chain lengths were also tested at the same total amount. This quantity was visually similar to treating the membrane surface of the test arena with 12 leq of fraction F1. Additionally, doses of n-$C_{21:0}$ ranging from 1.5 to 15 $\mu g/cm^2$ were tested. A solution of the synthetic n-alkanes C_{21}, C_{23}, C_{25}, C_{27}, C_{29} at proportions (0.2 : 3 : 7 : 12 : 5) close to that found in cuticular extracts was tested at doses from 3 to 30 $\mu g/cm^2$ of treated area, corresponding to 2–24 leq n-alkanes. To test for any purely physical effect of the HCs on the arena surface, a comparison was made with Vaseline at 15 $\mu g/cm^2$ applied to the same surface. Two attractants for *Varroa*, i.e., palmitic acid (Rickli et al., 1992) and methyl palmitate (LeConte et al., 1989) were tested at doses of 3 and 1.5 $\mu g/cm^2$, respectively.

Test runs were recorded on VHS video from perpendicularly above the membrane, and the walking behavior was subsequently analyzed using a computer program linking the video observations with time (The Observer, Noldus Information Technology). In this manner, the time spent by a mite on the different zones of the arena and walks and stops were quantified. Once on the treated area: (1) the number of contacts made by the mite with the borders of the treated area, (2) returns at the border back towards the treated area, and (3) moves onto the untreated surface were quantified. The results from 671 runs on the solvent control were pooled and used to define a standard behavior for three

parameters of the walks, which showed highly asymmetric value distributions. In all, 95% of the mites in these controls: (1) moved for less than 41.0 sec on the test band (indexing for a general activity of the animal), (2) showed less than 2.34 returns/10 sec when moving on the test band (by contrast with chemostimulated mites that returned from test band borders much more often in the same time span), or (3) made fewer than four returns per run (border recognition). If an animal showed a value above any of the three 95% limits in a test situation, it was considered to react towards the solution applied. The number of runs with values above at least one of the 95% limits on a given test solution was compared to the number recorded for the other solutions tested on the same day with the Fisher exact test. A double check showed that none of the control groups was significantly different from the pool of control runs. This ruled out that relative activities of test solutions could be falsely judged considering the large day-to-day variations in the mites' walking behavior.

Additionally, the total time spent by the mites in contact with a solution (divided into moving and stopping periods) was analyzed. The contact time of each mite was plotted in notched box plots (McGill et al., 1978). If the 95% confidence intervals of the medians did not overlap between test and control, we considered the result significant. The notched box plot results are only stated if they differed from the results obtained by the method of 95% limits for the other parameters described above.

Analysis of Tracks. For this part of the study we made 10 tests each on stimulus doses corresponding to 0 (solvent control), 0.6, 1.2, 2.9, and 5.9 leq of the active nonpolar TLC fraction after removal of the wax esters (i.e., fraction F1A). All test runs on fraction F1A were done on the same day, i.e., with mites of the same lot. Tracks made by mites of a separate lot on n-$C_{21:0}$ at 30 $\mu g/cm^2$ (Figure 1) and on the corresponding solvent control (N = 10 mites per treatment) were also analyzed. Tests chosen for examination of *Varroa* tracks are representative of the mite's behavior to chemostimuli as observed using the bioassay system described above.

Track coordinates were obtained from an x,y grid laid on the video screen. A clock fed into the video image during recording permitted determination of frame number per second, which in 80% was 25 (video norm) and in 20% was 26 frames. Distances in x,y units were registered at a resolution of 0.34 mm (length of the mite 1 mm). For this part of the study, the position of the animal's pedipalps was noted every fifth frame, i.e., every 0.2 sec for the first minute of the run. *Varroa* have been suggested to bear chemosensitive sensilla on the tarsi of leg pair 1 (P1) (Ramm and Böckeler, 1989; Milani and Nanelli, 1988) and the pedipalps (Liu, 1990). For each x,y coordinate, an index was added to the records indicating whether the mite was fully on the treated area with all chemosensitive sites, in contact with the ink circle (i.e., on the border of the treated area), or outside the treated area. Statistical analysis was performed using

a post hoc ANOVA (Tukey HSD test) on distances covered and on angular data (see below) resulting in a matrix of pairwise comparisons between the treatments.

The analysis applied here was made on track segments where the mites walked fully on the treated area. (Tracks from the release point in the center of the arena to first contact with the stimulus, tracks after leaving the extract, and tracks made on the borders of the treated area are not dealt with because they reflect quite different situations for the mites.) The raw x,y coordinates with the origin ($x = 0$; $y = 0$) in the center of the arena were computerized and converted into real x,y distances in millimeters. The angle of each x,y pair to the 0° axis (the x axis here) of the coordinate system was calculated [$\arctan(y/x) * 180/\pi$; angles for y or $x = 0$ defined as 0°, ±90°, or ±180°]. With the formula $(\Delta x^2 + \Delta y^2)^{0.5}$, the distance covered was calculated, and with the formula $\arctan(\Delta y/\Delta x) * 180/\pi$ (angles for Δx or $\Delta y = 0$ defined as above) the direction moved per 0.2-sec interval (vector) was obtained. The mite's turn angle was calculated as the difference in displacement direction between two subsequent 0.2-sec vectors. However, the distribution of turn angles showed a bias for 90° and subdivisions thereof, which is an indication that the distance traveled by the mites (mean speed on solvent control for fraction F1A of 2.85 mm/sec) in the sampling interval chosen was inadequate to properly characterize the true angles described. Since *Varroa* is very quick in reacting to loss of stimulus at the border of the treated area (duration of one border contact followed by a return observed in one frame was 0.04 sec), we still decided to sample at 0.2-sec intervals rather than at longer ones. However, we calculated a running mean of the turn angles over triads of consecutive vectors, and natural logs of these means were used in the general path description. Duration and frequency of stops were not dealt with because mites walked for prolonged periods on the areas treated with 1.2, 2.9, 5.9 leq F1A and with 30 μg n-$C_{21:0}/cm^2$. Stops might therefore be due to exhaustion, something which has nothing to do with chemoreception.

We further broke the paths into segments between returns from the border onto the treated area to the next border contact. For these segments we calculated the walking speed (in millimeters per second) and angular velocity, i.e., the summed absolute turn angles per second. Statistical analysis was carried out on speed and angular velocity using the Tukey HSD test.

RESULTS

Behavior Responses to Cuticle Extract. The responses of the mites were tested to eight visible fractions including the nonmigrating material on the application band of the TLC plate employed to separate the cuticle extract. In addition, a stripe called F2, which revealed no material on charring between the

front-running fraction F1 (most apolar) and F3, was tested as the blank control. The mites only showed a response to TLC fraction F1, containing HCs and wax esters, to the same extent as they did to the cuticle extract (Table 1). A response was observed on the TLC fraction F5, containing fatty acid methyl and ethyl esters, in terms of increased duration of stops on the treated area from the outset of tests (notched box plots). The fraction containing the free fatty acids (F7) elicited a response no different to controls. After separating the active fraction F1 further into complex wax esters and simple HCs (fraction F1A), the latter was also as active as the total cuticle extract or F1. *Varroa*'s response to 0.6 leq of fraction F1A was significantly different than that shown to the solvent control (21% vs. 5%, respectively; $P < 0.05$) and on higher doses of F1A the difference to the control was more pronounced (33%, 50%, 49%, and 70% of

TABLE 1. RESPONSES OF *Varroa* TO WORKER HONEYBEE LARVA CUTICLE EXTRACT AND FRACTIONS THEREOF[a]

Extract tested	Amount (leq) applied/cm^2	No. Mites tested (N)	Runs (N) over at least one limit[b]	Mites reacting (%)[c]
Control		49	9	18a
Cutice extract	12	47	33	70b
TLC fractions				
Control		224	25	11a
F1 (apolar)	12	41	30	73b
F2 to F9	12	19-31	5[d]	16[d]a
$AgNO_3$				
Control		50	3	6a
Saturated HC's	12	30	15	50b
Alkenes	12	30	4	13a
Polar compounds	12	30	3	10a
Molecular sieve				
Control		82	6	7a
Saturated HC's	6	35	23	66b
Branched alkanes	16	29	5	17a

[a]For further explanation see text. TLC: thin-layer chromatography separation of extract into front running fraction (F1) and eight other bands (F2-F9). $AgNO_3$: Argentation TLC separation of saturated hydrocarbons (HC) from more polar constituents of the cuticle extract. Molecular sieve refers to separation (by adsorption) of straight-chain HCs from branched ones in the saturated HC fraction of the cuticle extract obtained by argentation TLC. Six larva equivalents (leq) of the saturated HCs weigh 12 μg whereas 16 leq of the branched alkanes are required to contain as much.

[b]See bioassay and data analysis section of Methods and Materials.

[c]Within test groups, percentages followed by different letters are significantly different at $P < 0.05$ (Fisher exact).

[d]Highest values obtained per TLC fraction F2-F9.

the mites responded to 1.2, 2.9, 5.9, and to 12 leq, respectively, $N > 21$). The wax esters were not active. Alkanes proved active, whereas alkenes (and alkadienes in the same fraction), on their own or together with all other more polar compounds such as fatty acids, fatty acid esters, or alcohols, were inactive in our bioassay.

The saturated HCs obtained by argentation TLC were further purified by removing *n*-alkanes with a molecular sieve. *br*-Alkanes remaining in solution were not active as judged by the absence of typical returning behavior at the borders of the treated area. However, the time the mites spent moving on the area treated with *br*-alkanes increased vis-à-vis the control (notched box plots).

Chemical Analysis of Cuticle Extract. GC-MS analysis showed that fraction F1, the only active fraction from the TLC analysis of cuticle extract (Table 1), contained *n*-alkanes, *br*-alkanes, alkenes (alkadienes only in minor quantities) and wax esters composed of $C_{16:0}$ or $C_{18:1}$ fatty acids esterified with even-numbered C_{24} to C_{32} alcohols. These wax esters were removed by running F1 again on an analytical TLC plate in hexane alone. Fraction F5 of the cuticle extract was shown by GC-MS to be composed mainly of nonanal, C_{12}–C_{18} even-numbered fatty acid methyl and ethyl esters, and to a lesser extent the corresponding fatty acids, possibly arising from the former via hydrolysis on the TLC-plates. Free $C_{16:0}$, $C_{18:0}$ and $C_{18:1}$ fatty acids and C_{24}–C_{30} even-numbered alcohols were identified in fraction F7.

GC-MS indicated that separation of cuticle extract by argentation TLC with hexane provided a front-running fraction that contained alkanes only, while the other constituents of the extract did not migrate. The branched HCs were harvested from this saturated HC fraction by using a molecular sieve that took up the *n*-alkanes to almost 100%.

Estimation by GC-FID of quantities of different types of HCs indicated that cuticle extract contains 2.67 μg hexane-soluble material/cm^2 (SD ± 0.40 μg) of larva. The extract was made up of 75% saturated and 25% unsaturated HCs (Figure 2). *n*-Alkanes were found to make up 64% of the saturated material and *br*-alkanes 36%. Heneicosane (n-$C_{21:0}$), active on its own, was present in all extracts at amounts of $\leq 1\%$ of cuticle extract or $\leq$ 0.03 $\mu g/cm^2$ of larva. Even-numbered *n*-alkanes also were present but at less than 1%.

Behavior Responses to Synthetics. A response by *Varroa* similar to that observed on cuticle extract was recorded on binary and ternary mixtures of odd-numbered $C_{19:0}$ to $C_{29:0}$ synthetic *n*-alkanes (Table 2) of neighboring chain lengths. Except for n-$C_{19:0}$ and n-$C_{21:0}$, none of these alkanes were active when tested singly. n-$C_{21:0}$ was active at doses of 6 $\mu g/cm^2$ (50% reacting mites vs. 15% on the control; Fisher exact, $P < 0.05$) or higher (Figure 1). A mixture of the *n*-alkanes C_{21}, C_{23}, C_{25}, C_{27}, and C_{29}, at the proportions (0.2 : 3 : 7 : 12 : 5) close to that found in the TLC fraction F1A, was active at a total dose of 6 $\mu g/cm^2$, which corresponds to 4.7 leq *n*-alkanes (25% reacting mites vs. 7% on

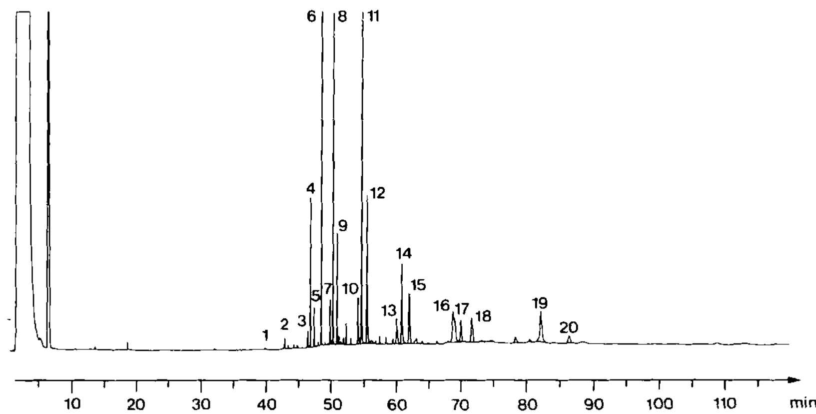

FIG. 2. Chromatogram (GC-FID) of cuticle extract of 8-day-old worker larvae on a nonpolar DB-5 high-resolution capillary column (see Methods and Materials). The numbered peaks (identified by GC-MS and retention time) show the following compounds: (1) n-$C_{19:0}$; (2) n-$C_{21:0}$; (3) n-$C_{23:1}$; (4) n-$C_{23:0}$; (5) br-$C_{23:0}$; (6) n-$C_{24:0}$ (internal standard); (7) n-$C_{25:1}$; (8) n-$C_{25:0}$; (9) br-$C_{25:0}$; (10) n-$C_{27:1}$; (11) n-$C_{27:0}$; (13) n-$C_{29:1}$; (14) n-$C_{29:0}$; (15) br-$C_{29:0}$; (16) n-$C_{31:1 or 2}$; (17) n-$C_{31:0}$; (18) br-$C_{31:0}$; (19) n-$C_{33:1 or 2}$; (20) br-$C_{33:0}$. Wax esters were only detected in GC-MS. In all, the n-alkanes (peaks 2, 4, 8, 11, 14, and 17) make up 48% of the products enumerated here. The extract was made up of 1.99 ($\pm$0.30) μg saturated and 0.69 ($\pm$0.12) μg unsaturated HCs. n-Alkanes were found to make up 1.27 ($\pm$0.24) $\mu g/cm^2$ and br-alkanes 0.73 ($\pm$0.09) $\mu g/cm^2$ of larva. n-$C_{19:0}$ was found below 1% and n-$C_{21:0}$, n-$C_{23:0}$, n-$C_{25:0}$, n-$C_{27:0}$, n-$C_{29:0}$, and n-$C_{31:0}$ were found in amounts of $\leq$ 0.03, 0.11 ($\pm$0.03), 0.35 ($\pm$0.15), 0.55 ($\pm$0.22), 0.21 ($\pm$0.09), and 0.09 ($\pm$0.6) $\mu g/cm^2$ of larva, respectively.

the control; $P < 0.05$, $N \geq 20$), or at higher amounts (27% and 41% reacting mites on 15 and 30 $\mu g/cm^2$, respectively). Addition of the attractant PA to the alkanes n-$C_{25:0}$ and n-$C_{27:0}$ either singly or to a mixture had no effect on the paraffins' activity, and PA alone was not active. This is consistent with the inactivity of TLC fraction F7 containing fatty acids. A mixture of three synthetic alkenes (n-$C_{19:1}$, n-$C_{21:1}$, and n-$C_{23:1}$), Vaseline, or a 12 leq dose of MP did not elicit a behavior response different from the solvent controls.

Track Analysis. The mean length per 0.2-sec vector (no-move vectors were excluded from calculations) increased with increasing doses of TLC fraction F1A on the treated area (Table 3), and a significant difference to the solvent control was observed at and above 1.2 leq (Tukey HSD, $P \leq 0.05$). The animals walked straighter on the treated area: the mean turn angle decreased significantly from 25° (log values reconverted into degrees) on controls to 19° on 5.9 leq. The analysis of parameters describing track segments from one border contact to the next confirmed the above results, i.e., mites increased walking speeds

TABLE 2. RESPONSE OF *Varroa* TO SYNTHETIC ODD-NUMBERED SATURATED AND UNSATURATED HYDROCARBONS (C_{19}–C_{29}) ON A CIRCULAR AREA[a]

Compounds	Amount (μg) applied/cm^2	Mites tested (N)	Runs (N) over at least one limit	Mites reacting (%)[b]
Single *n*-alkanes				
Control		34	7	21a
$C_{19:0}$	30	23	13	57b
$C_{21:0}$	30	21	15	71bc
$C_{23:0}$	30	23	5	22a
$C_{25:0}$	30	30	10	33ab
$C_{27:0}$	30	50	11	22a
$C_{29:0}$	30	20	2	10a
Binary mixtures				
Control		38	4	11a
$C_{19:0}$ and $C_{21:0}$	15 each	22	13	59bc
$C_{21:0}$ and $C_{23:0}$	15 each	21	16	76bc
$C_{23:0}$ and $C_{25:0}$	15 each	21	17	81bc
$C_{25:0}$ and $C_{27:0}$	15 each	41	30	76bc
$C_{27:0}$ and $C_{29:0}$	15 each	21	7	33b
Ternary mixtures				
Control		41	2	5a
Alkanes $C_{19:0}$–$C_{23:0}$	10 each	45	30	67b
Alkenes $C_{19:1}$–$C_{23:1}$	10 each	45	6	13a

[a] For explanation, see Methods and Materials.
[b] Within test groups, percentages followed by different letters are significantly different at $P < 0.05$ (Fisher exact).

from 2.85 ± 1.1 mm/sec on controls to 4.06 ± 0.9 mm/sec on 5.9 leq of fraction F1A and decreased their angular velocity from 201.7 ± 82.3°/sec on controls to 179.2 ± 61.0°/sec on 5.9 leq.

The mites behaved essentially the same way on a substrate treated with n-$C_{21:0}$ as on one treated with fraction F1A. Higher vector lengths (Tukey HSD, $P < 0.05$) and speeds (not significant) as well as lower turn angles ($P < 0.05$) and angular velocities ($P < 0.05$) were recorded on C_{21} compared to the solvent control (Table 3).

DISCUSSION

Varroa shows an arrestment response on a substrate treated with a cuticle extract of 8-day-old worker honeybee larvae at a stimulus density corresponding to 12 leq. Of 98 arbitrarily chosen responses on the active cuticle extract, only two

TABLE 3. TRACK ANALYSIS OF PATHS MADE BY *Varroa* ON ARENA TREATED WITH NONPOLAR TLC FRACTION OF HONEYBEE WORKER LARVA CUTICLE EXTRACT (F1A) AND ONE OF ITS CONSTITUENTS, n-$C_{21:0}$[a]

		0.2-sec vectors (mean ± SD)		Path segments from one border contact to the next (mean ± SD)	
Stimulus	Amount applied/cm^2	Vector length (mm)[b]	Log of turn angle[b,c]	Speed (mm/sec)[b]	Angular velocity (deg/sec)[b,d]
Control		0.53 ± 0.20a	3.23 ± 0.87a	2.85 ± 1.11a	201.7 ± 82.3ab
F1A	0.6 leq	0.60 ± 0.28a	3.25 ± 0.89ab	3.15 ± 1.14ab	231.1 ± 54.0ab
	1.2 leq	0.67 ± 0.32b	3.16 ± 0.95ab	3.66 ± 1.16b	227.4 ± 62.3a
	2.9 leq	0.70 ± 0.31b	3.01 ± 0.96b	3.65 ± 1.03b	193.8 ± 63.3ab
	5.9 leq	0.81 ± 0.33c	2.95 ± 1.02b	4.06 ± 0.90b	179.2 ± 61.0b
Control		0.87 ± 0.38a	3.10 ± 1.19a	4.81 ± 1.3a	272.5 ± 58.1a
n-$C_{21:0}$	30 μg	1.05 ± 0.41b	2.83 ± 1.25b	5.58 ± 1.1a	205.2 ± 68.2b

[a]The differences, especially in the walking speeds, between the solvent controls for F1A and for n-$C_{21:0}$ show that a considerable variation exists between mites from different lots. For this reason behavioral activities of test solutions are only compared to the solvent controls made with mites of the same lot.

[b]Within groups, values followed by different letters are significantly different at $P < 0.05$ by Tukey HSD. This test permitted comparison between all treatments even though the number of returns was low for controls and 0.6 leq. The differences attributed serve above all to underline trends with increasing dose.

[c]Turn angles were calculated from running means generated over triads of consecutive 0.2-sec vectors.

[d]Angular velocities were calculated as the sum of the absolute turn angles made on the track between one border contact and the next one on the treated area.

responses were designated positive solely for having moved longer than 41.0 sec on the treated area. The 96 others were considered positive due to the fact that the number of returns at the borders of the treated area (either per 10 sec walk or per run) were above the 95% limits. Border recognition is therefore an outstanding feature of the response. The mite makes decisions at the border of the treated area that permit it to stay on the stimulus. This results in arrestment on the treated area. The most apolar TLC fraction F1 as well as the purified HC fraction F1A (devoid of wax esters) give similar results at equivalent stimulus doses. For the latter, a dilution series has been tested, with amounts of 0.6 leq (1.6 μg HCs) or higher being behaviorally active. HC fractions of the extract contain, for the most part, odd-numbered branched and straight-chain saturated and unsaturated HCs; removing the unsaturated compounds did not change the fraction's activity.

Synthetic compounds were only active when saturated HCs were present. Heneicosane at 30 μg/cm^2 elicited a response similar to the cuticle extract (Fig-

ure 1), but the behavioral threshold for this compound was at 6 $\mu g/cm^2$ or some 200 leq. Since TLC fraction F1A of cuticle extract showed activity at a level some 300 times lower (0.6 leq containing 0.02 μg n-$C_{21:0}/cm^2$), the observed arrestment effect of the apolar fraction of cuticle extract is probably due a synergistic effect between the HCs it contains rather than to a single component. Heneicosane was active at much lower levels (0.04 μg) when presented in a mixture with odd-numbered C_{23}–C_{29} alkanes. Since Vaseline was not active, it shows that the arrestment behavior and border recognition is more specific than just to the fatty texture of the substrate. Despite the fact that individual HCs differ in their physical properties on the membrane (as seen by their capacity to reflect light on the treated surface), *Varroa* appeared to be able to discriminate between them independent of this, i.e., some were active on their own whereas others were not (Table 2).

The lowest active dose of a mixture of synthetic *n*-alkanes imitating the proportions found in TLC fraction F1A was 6 $\mu g/cm^2$, but a dose of 0.6 leq of TLC fraction F1A containing 0.76 μg *n*-alkanes was active. This suggests that the straight-chain alkanes do not alone account for the activity of the fraction, which also contains *br*-alkanes and alkenes. Both of the latter were inactive in terms of border recognition when tested alone, but notched box plot analysis showed that the *br*-alkanes significantly increased the duration of walking on the treated area compared to the solvent control due to the fact that *Varroa* often returned to the treated area after they had left it. Thus, apart from the presence of *n*-alkanes, the low behavioral threshold for fraction F1A depends, in addition, on the presence of *br*-alkanes. Monomethyl alkanes, which make up some 90% or more of the *br*-alkanes on adult bees, have been identified as mixtures of two or more 9-, 11-, 13- and 15-methyl alkanes (Francis et al., 1989), and our own GC-MS identification provided similar results for larval extracts. The straight-chain alkanes of our extracts could not be desorbed from the molecular sieve in sufficient purity (some branched material also migrated into the sieve) and were therefore not tested. TLC fraction F5 (fatty acid esters) prolonged the mites' stop times on the treated area (notched box plot analysis) suggesting that, in addition to the saturated HCs serving as cues for border recognition observed in our bioassay, further stimuli may control the mites' host recognition process.

Track analysis showed that *Varroa* walking on a homogeneously stimulating substrate move in a dose-dependent pattern. In general, the mites walk faster and straighter on higher doses of fraction F1A. Mites walking on 30 $\mu g/cm^2$ of heneicosane also walk faster and straighter than on the solvent control. Thus, synthetic n-$C_{21:0}$ elicits a similar response from the mites as the cuticle extract, a result that serves to confirm the importance of straight-chain odd-numbered alkanes as chemostimuli evoking the arrestment response in *Varroa*. These results indicate that chemostimuli from host cuticle not only influence the behavior of *Varroa* at the border of a treated area, but also affect *Varroa*'s walking behavior

on the treated substrate. Whereas 0.6 leq of fraction F1A was sufficient to evoke a response when considering criteria such as border recognition and the time spent walking on the treated area, 1.2 leq was necessary to evoke a significant response in walking speed and angular velocity.

Large day-to-day or lot-to-lot variability of mite behavior was observed throughout the study described here and was independent of the stimulus tested. A potential contamination of the membranes seems very unlikely considering the method of cleaning the Baudruche and treatment of the test area. Potential contamination by volatilization of some test compounds seems unlikely because day-to-day variations were observed even when the same batches of solutions were tested. The origin of the variations is most probably related to the mites' provenance.

Considering the extraction time of 15 min employed here, one may question the extent to which only compounds of purely cuticular origin were extracted. In other studies using the term "cuticular HCs", bees were extracted either for an unspecified time (McDaniel et al., 1984, Moritz et al., 1991, Nation et al., 1992) or for 10 min (Blomquist et al., 1980, Francis et al., 1989, Phelan et al., 1991). In this study, preliminary observations demonstrated that *Varroa* was arrested on a substrate rubbed with live larvae. In addition, the two major components of the extracts described here (n-$C_{25:0}$ and n-$C_{27:0}$) were also identified in volatiles from living larvae (Rickli et al., 1992), suggesting that the HCs originate from the exterior of worker larvae.

The tracks made by *Varroa* resemble those of other parasites and parasitoids, but with the difference that the mites increase speed and track straightness (doses $\geq$ 1.2 leq), while other arthropods usually decrease both in arrestment responses towards semiochemicals of their hosts (e.g., Waage, 1978) or conspecifics (Royalty et al., 1993). The walking pattern (fast and straight paths) and the net arrestment effect (arising from recognition of the border of the treated area and the return responses leading to highly increased periods of time spent on active substrates) seem to contradict each other. However, it might be explained by the behavioral context in which it operates. Two roles for contact chemoreception have been mentioned here for *Varroa*: for cell invasion and attachment to the bee larva during cocoon spinning. In both cases the success of the mite's response might depend on the speed of the reaction. The speed with which *Varroa* responds during cell invasion might contribute to avoiding detection by the host, which would lead to removal of the parasite from the colony, as in the case of the original Asian host, *Apis cerana* (Peng et al., 1987; Büchler et al., 1992). The function of speed to avoid being crushed between the larval body and cell wall during cocoon spinning by the bee is obvious.

Alkanes are widespread as chemostimuli in arthropods. In *Acarus immobilis* (Acarina) C_{13}, C_{25}, C_{27}, and C_{29} HCs are employed to attract females to the vicinity of males (Sato et al., 1993). It is significant that *Acarpis woodii*, a mite

which invades the tracheae of adult honeybees, shows an arrestment response on cuticular HCs of its host and, similar to *Varroa*, especially to alkanes (Phelan et al., 1991). Honeybees themselves can be trained to discriminate between $C_{23:0}$ and $C_{25:0}$ and even between their mixtures (Getz and Smith, 1987). Further, application of hexa- and octadecane increases aggressive behavior between hivemates (Breed and Stiller, 1992). Therefore, alkanes seem to be implicated in the nestmate-recognition process of bees, and the same is proposed for other social insects such as wasps, ants, and termites (Singer and Espelie, 1992, and references therein). Bumblebees, *Bombus terrestris*, mark visited flowers with a secretion from the tarsal glands containing C_{19}–C_{31} HCs; only when both saturated and unsaturated compounds were combined could a response similar to natural scent marks be observed, but alkanes alone could induce a part of the response (Schmitt et al., 1991). In the parasitoid *Trichogramma brassicae*, a blend of odd-numbered $C_{21:0}$–$C_{29:0}$ from host egg masses stimulates oviposition (Grenier et al., 1993).

Saturated and unsaturated HCs present on honeybees are also present on *Varroa* (Nation et al., 1992; our own unpublished results), a factor which may serve to reduce detection of the parasite via mimicry. The presence of these compounds to which *Varroa* is sensitive on the parasite's own cuticle may also contribute to the mutualistic relationship between members of the same and different families in single and multiinfested brood cells (Donzé and Guerin, 1994). *Varroa* and honeybees may, in fact, use the same compounds as semiochemicals, as demonstrated by the fact that fatty acid esters that attract *Varroa* to host larvae of a particular age are also employed to trigger cell capping in worker bees (LeConte et al., 1990). The same could be true for saturated HCs, i.e., alkanes are suggested to function as semiochemicals in bees for nestmate and age-recognition (Getz et al., 1989) and are simultaneously employed for host recognition by *Varroa*.

Straight-chain alkanes are ubiquitous within the hive, on adult bees (Francis et al., 1989; our own unpublished results) as well as on beeswax (Tulloch, 1980; our own unpublished results). Indeed, extracts of beeswax and adult bees cause arrestment of *Varroa* on the semipermeable membrane employed here (unpublished results). We must therefore conclude that *Varroa* can recognize bee larvae using HCs only if the relative proportions of saturated HCs employed are sufficiently specific to this lifestage, or in combination with other cues (chemical or otherwise) peculiar to the lifestage it parasitizes within brood cells. It is noteworthy that *br*-alkanes, which caused *Varroa* to walk for longer durations in our bioassay, make up 27% of the hexane-soluble material on larvae but constitute less than 7% of similar extracts from cell walls or from adult bees of two days or older (Francis et al., 1989, our own unpublished results).

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IMPORTANCE OF PHENOLIC GLUCOSIDES IN HOST SELECTION OF SHOOT GALLING SAWFLY, *Euura amerinae*, ON *Salix pentandra*

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Abstract—The effects of phenolic glucosides on the oviposition behavior of *Euura amerinae* L. (Hymenoptera: Tenthredinidae) were tested in multiple oviposition experiments using different shoot length categories of *Salix pentandra* L. (with different amounts of phenolic glucosides) and in experiments with pure phenolic glucosides (salidroside, arbutin, salicin, 90% salicortin, 90% 2'-*O*-acetylsalicortin) or composite total fractions of phenolic glucosides from three willow species (*S. pentandra*, *S. myrsinifolia* Salisb., *S. triandra* L.). This was the first time that the effects of pure phenolic glucosides on the oviposition behavior of sawfly species were tested. Total fraction of phenolic glucosides from *S. pentandra* and its main individual glucoside, 2'-*O*-acetylsalicortin, stimulated the strongest ovipositional behavior in *E. amerinae*. The results show clearly that females of *E. amerinae* can recognize and choose their host willow, *S. pentandra*, on the basis of phenolic glucosides. Moreover, they are probably able to use phenolic glucosides as a cue in shoot selection within host-plant individuals.

Key Words—Herbivory, host preference, host selection, oviposition, phenolic glucosides, willow, Salicaceae, *Salix*, galling sawfly, Hymenoptera, Tenthredinidae, *Euura amerinae*.

INTRODUCTION

Most of the important nutrients posses little or no taste or smell, but many plant secondary substances have characteristic odors or tastes. Many of these chem-

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icals, such as phenolics, alkaloids, and terpenoids, are known to be moderately poisonous to insects and microorganisms or harmful to the digestion of herbivores (Swain, 1979; Lindroth et al., 1988; Feeny, 1991; Lindroth, 1991; Kelly and Curry, 1991). Therefore, secondary substances are assumed to be important in the food selection of mammalian herbivores (Markham, 1971; Edwards, 1978; Palo, 1984; Palo et al., 1984; Tahvanainen et al., 1985a) as well as in host selection of herbivorous insects (Fraenkel, 1959).

Phenolic glucosides with low molecular weight, such as salicin and its derivatives (salicylates), are typical secondary substances of plants of the family Salicaceae. There are 24 native willow species in Finland (Hämet-Ahti and Kytövuori, 1984), which show considerable variation in composition and total concentration of phenolic glucosides between species and plant parts (Palo, 1984; Julkunen-Tiitto, 1985, 1986, 1989a,b). Salicin has been found in all species but only as a minor component. Salicortin is the most abundant salicylate in different tissues of most willow species, and 2′-*O*-acetylsalicortin is especially common in all tissues of *Salix pentandra*. The rarest phenolic glucosides in northern European willows seem to be salidroside, the characteristic component of *S. triandra*, and arbutin, which is found in *S. myrtilloides* only. Most studied willow species can be identified by phenolic glucosides (Julkunen-Tiitto, 1989a). Therefore, insect herbivores may use phenolic glucosides as specific cues to host-plant selection and also in recognition of specific plant parts within a host-plant individual. For example, longer shoots may be selected on the basis of correlated phenolic glucoside content.

Females of specialized sawflies are highly selective in their oviposition (Roininen and Tahvanainen, 1989; Roininen et al., 1992). Some specialist sawflies use only certain clones (Fritz et al., 1986, 1987; Fritz and Price, 1988) or long, fast-growing shoots of their host species (Craig et al., 1986, 1989; Price et al., 1987a,b; Roininen, 1991), but it is unknown how important phenolic glucosides are in clone or shoot selection by sawflies.

Many observations show that specialized insects prefer willow species with high concentrations of phenolic glucosides, but generalist species tend to avoid willows containing high amounts of these substances (Rowell-Rahier, 1984a–c; Tahvanainen et al., 1985b; Rowell-Rahier and Pasteels, 1986; Pasteels et al., 1988a,b). Rowell-Rahier et al. (1987) and Soetens et al. (1991) found that the abundance of the galls of the sawfly, *Pontania proxima*, was closely correlated with high phenolic glucoside content of the leaves of willows. Roininen and Tahvanainen (1989) reported that another sawfly *Nematus salicis* seems to be a phenolic glucoside specialist. It is well adapted in its oviposition behavior and in its larval physiology to the phenolic glucosides present in its main hosts. In contrast, a congener, *N. pavidus*, seems to be a generalist. It reacts quite erratically in its oviposition towards different arrays of phenolic glucosides and its larvae do best on willows with a low phenolic glucoside content (Roininen and

Tahvanainen, 1989). However, the phenolic levels of *Salix lasiolepis* were poor predictors of *Euura lasiolepis* attack in the study by Waring and Price (1988). Despite these findings, Waring and Price (1988) think that individual phenolic glucosides may play an important role in host-plant selection.

The aim of this study was to investigate the effects of phenolic glucosides on the oviposition behavior of *Euura amerinae*. It is a monophagous shoot galler on *S. pentandra* in Finland, although there are many other Salicaceae species available in its habitat. We did multiple oviposition experiments using different shoot length categories of *S. pentandra*, and we analyzed phenolic glucosides in the tips of the shoots in which eggs were laid. We also tested the effects of phenolic glucoside extracts from three willow species and the effects of five pure phenolic glucosides applied on the artificial substrate as oviposition stimulants. This was especially important because most of the studies about the effects of phenolic glucosides are based on correlative, indirect results (Rowell-Rahier and Pasteels, 1982; Smiley et al., 1985; Tahvanainen et al., 1985b; Rowell-Rahier et al., 1987; Roininen and Tahvanainen, 1989).

METHODS AND MATERIALS

Study Organisms. The sawfly species used in our experiments was *Euura amerinae* L. (Hymenoptera: Tenthredinidae). Tenthredinidae is a very large, cosmopolitan symphytan family containing more species than all other families in the suborder Symphyta combined (Gauld and Bolton, 1988). Galling sawflies (*Euura* and *Pontania* species) are usually strictly monophagous, and they show delicate control of their oviposition behavior (Benson, 1958; Vikberg, 1970). *Salix pentandra* is the most common host for *E. amerinae*, but it is seldom found on *Populus tremula* (Pschorn-Walcher, 1982). 2′-*O*-Acetylcortin is the main phenolic glucoside in *S. pentandra* and makes up 83% of the total glucoside yield in the fresh foliage (Julkunen-Tiitto, 1989a; Kolehmainen et al., 1994). Other glucosides, present only in low amounts, are salicin, acetylsalicin, and salicortin.

The adult *E. amerinae* sawflies emerge in late May and early June. They determine the location of the following larval generation through their oviposition preference. During the summer, larvae grow in galls, in which they also overwinter. The galls of *E. amerinae* for the experiments were collected from numerous clones of *S. pentandra* at Kempele, 15 km south from Oulu in western Finland. They were stored in a growth chamber at 6°C. At times they were moved to room temperature so that new individuals of *E. amerinae* emerged. Each individual was used in only one experiment.

Analysis of Phenolic Glucosides in Shoots of S. pentandra. Shoots in different length categories used in the oviposition tests with *E. amerinae* were

sampled for phenolic glucoside analysis from seven clones of *S. pentandra*. For the actual analysis, the very top of each shoot, 1–2 cm in length, with young leaves around the growing point was taken. This is the region of a shoot utilized by *E. amerinae* females during oviposition. The phenolic glucosides were extracted from the fresh shoot tips in a clipping homogenizer three times for 5 min with 20 ml of methanol, and purified on Bond Elut C_{18} octadecyl (500 mg) solid-phase extraction columns. The extracts were analyzed and identified using high-performance liquid chromatography (HPLC) with a photodiode array detector (HP 1040A Series) (Julkunen-Tiitto et al., 1989b). Amounts of total phenolic glucosides on different shoot length categories were analyzed by block design ANOVA, where the clones were blocks and the shoot length classes were the treatments (Sokal and Rohlf, 1981).

Extraction of Total Glucoside Fractions and Individual Phenolic Glucosides. The room-dried composite leaf samples of *S. myrsinifolia* and *S. pentandra* and composite twig samples of *S. triandra* (see Kolehmainen et al., 1994) were used to obtain total and individual glucoside fractions to be tested on the artificial substrate. Leaf and twig samples were extracted with methanol for 4 hr, methanol was concentrated, and the extract was dissolved in water. The extract was prepurified on a polyamid column (40 cm × 1.6 cm diameter) using distilled water as an eluent. The eluate was immediately freeze-dried. These were used later as water solutions to test the effects of total glucoside fractions on the oviposition behavior of *E. amerinae*. Besides phenolic glucosides, the total fraction contained some other, partly unknown components and simple sugars, viz., sucrose, glucose, and fructose. The relative amounts of compounds of total fractions from different willows are described in detail in Kolehmainen et al. (1994).

Salicortin (containing 10% of salicin), 2′-*O*-acetylsalicortin (containing 10% of acetylsalicin), and salidroside were further isolated from the total glucoside fraction using Sephadex LH 20 columns (40 cm high × 1.6 cm diameter). The elution with water resulted in clear separation for the main phenolic glucosides of each species from soluble carbohydrates, pigments, and other phenolics tested by HPLC, GC, and GC-MS. Salicin and arbutin, which were also used in the oviposition tests with *E. amerinae*, are commercially produced (Sigma Chemical Co.).

Experiments on Oviposition Behavior of Euura amerinae. Experimental sawflies were moved from the cool growth chamber (6°C) to the warmer laboratory a few hours before each experiment and placed in plastic chambers with males. With this schedule they had time to copulate, which probably stimulated oviposition.

We did multiple oviposition experiments with *E. amerinae* and three shoot length categories of *S. pentandra*: 1–4, 5–8, and 9–12 cm. These shoot length classes are normally available at the beginning of the *E. amerinae* flying period

in May and June. Different shoots for experiments were random samples from 13 individuals of *Salix pentandra*, so that each female was offered different shoot length categories from the same individual. This approach was used because we wanted to avoid the effects of variable phenolic glucoside levels among willow individuals.

E. amerinae lays eggs inside the very top of shoots. Therefore, we used only the tips of shoots from different shoot length classes in order to equalize the available resources for the oviposition. For the experiment, mated females were enclosed individually in 2-liter plastic chambers where shoot tips of three length categories were placed in random order. The experiments were carried out at 21–24°C and in 16-hour light period. The females were kept in the chambers from two to four days until their death. From the shoots we measured oviposition scars and eggs laid on different shoot categories. Results were analyzed by Friedman two-way ANOVA (Conover, 1980). A nonparametric test was used because variances were not normally distributed.

Next we tested the effects of five pure phenolic glucosides and the effects of total fractions extracted from the three willow species (see above). A piece of thin green paper (3 cm long × 5 cm wide) was dipped in the test solution (water as a solvent) or water (control). The concentration of the test solution was 4.5% by weight in the experiments with the total phenolic glucoside fractions and 0.45% or 4.5% in the experiments with pure phenolic glucosides. These concentrations are within the range of phenolic glucosides found in Finnish willow species (Julkunen-Tiitto, 1986, 1989a,b). The piece of treated, wet paper was placed in the middle of a Petri dish (9 cm diameter) and one mated female *E. amerinae* was added. The sawflies were observed for 20 min, and penetrations by the ovipositor into the test paper were recorded. These observations were repeated three times during 3 hr. The sum of penetrations by each female in these three measuring periods was used as an independent observation in statistical analysis. Numbers of females used in different tests varied from 18 to 38 (see Table 2 and 3 below). The differences between Petri dishes with phenolic glucoside solution and controls were tested using ANOVA. Each total fraction or individual glucoside was compared to the control Petri dishes in the same experiment.

RESULTS

Oviposition and Phenolic Glucosides in Different Categories of Shoot Length. In the multiple choice oviposition experiments, when females of *E. amerinae* had been offered shoots of three length categories, the females laid significantly more eggs (Friedman's two-way ANOVA, $P < 0.001$) and made more oviposition borings (Friedman's two-way ANOVA, $P < 0.001$) on the

longer shoot length classes (Figure 1). The shortest shoot length class had very few eggs or oviposition borings (Figure 1).

There was a significantly higher level of total phenolic glucosides in the longer shoots (Table 1). The clone effect on the total phenolic glucosides was also significant (Table 1). This means that different clones had different levels of phenolic glucosides, and they increased from short to long shoots.

Phenolic Glucosides in Artificial Oviposition Substrate. The total fraction of phenolic glucosides from *S. pentandra* was the best stimulant for oviposition of the tested total fractions (Figure 2A). The sawflies made significantly more penetrations on this fraction compared with the control dishes (Table 2). *S. myrsinifolia* extract also stimulated *E. amerinae* oviposition behavior significantly but *S. triandra* extract had no significant effect (Table 2).

The strongest phenolic glucoside oviposition stimulant for *E. amerinae* was 2′-*O*-acetylsalicortin, its effect was highly significant (Figure 2B, Table 3). Besides acetylsalicortin, salicortin and salidroside stimulated *E. amerinae* ovi-

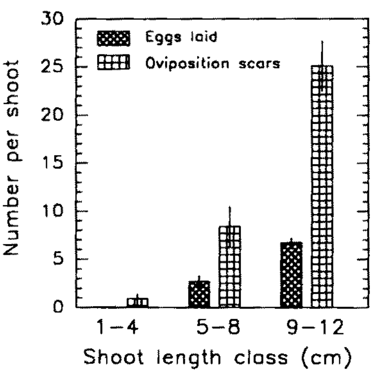

FIG. 1. Mean numbers (±SE) of oviposition scars and eggs laid by *Euura amerinae* per shoot among different shoot length categories.

TABLE 1. BLOCK DESIGN ANOVA FOR CONCENTRATION OF TOTAL PHENOLIC GLUCOSIDES IN DIFFERENT SHOOT LENGTH CATEGORIES

Source	*df*	Mean square	*F*	*P*
Clone	6	795.29	32.87	0.001
Shoot length	2	165.32	6.83	0.01
Error	12	24.20		

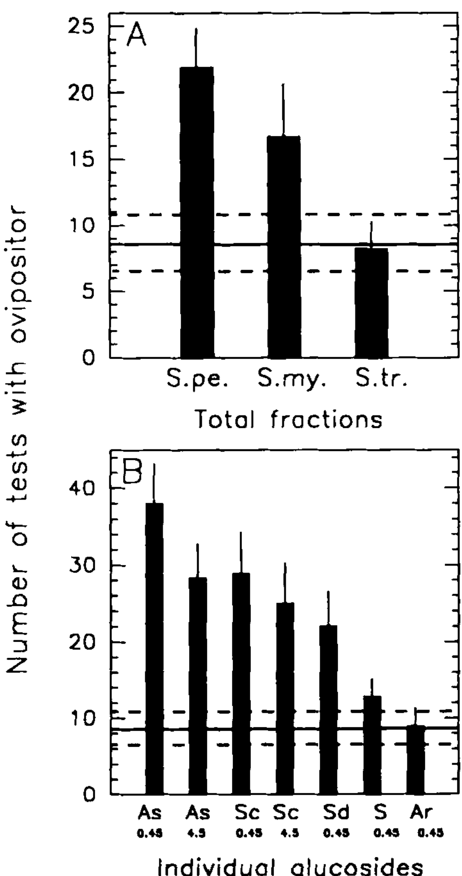

FIG. 2. Number (±SE) of penetrations with the ovipositor when the test paper had been dipped in (A) the total fraction of phenolic glucosides extracted from *S. pentandra* (S.pe.), *S. myrsinifolia* (S.my.), or *S. triandra* (S.tr.) or (B) 90% 2′-*O*-acetylsalicortin (As), 90% salicortin (Sc), salidroside (Sd), salicin (S), or arbutin (Ar). The solid line shows the mean of all control dishes, and the dashed lines show the 95% confidence limits of the mean. In the top graph (A) the concentration of each solution was 4.5% and in the bottom graph (B) the concentrations are indicated under each bar. For statistical analysis, see Tables 2 and 3.

TABLE 2. DIFFERENCES BETWEEN TOTAL FRACTIONS OF PHENOLIC GLUCOSIDES AND CONTROL TREATMENTS IN OVIPOSITION TESTS WITH *E. Amerinae*

Total fraction from[a]	*F*	*P*	*df*
S. pentandra	14.6182	0.0005	2, 38
S. myrsinifolia	3.9215	0.0632	2, 18
S. triandra	0.2941	0.5943	2, 18

[a]Each total fraction was compared to the control (water) treatment of the same experiment.

TABLE 3. DIFFERENCES BETWEEN INDIVIDUAL GLUCOSIDES AND CONTROL TREATMENTS IN OVIPOSITION TESTS WITH *E. Amerinae*

Individual glucoside[a]	Concentration (%)	*F*	*P*	*df*
90% 2′-*O*-acetylsalicortin	0.45	17.7023	0.0005	2, 18
90% 2′-*O*-acetylsalicortin	4.5	17.3786	0.0002	2, 38
90% salicortin	0.45	7.4147	0.0140	2, 18
90% salicortin	4.5	6.2985	0.0165	2, 38
Salidroside	0.45	8.8724	0.0081	2, 18
Salicin	0.45	0.1075	0.7514	2, 8
Arbutin	0.45	0.3417	0.5661	2, 18

[a]Each individual glucoside was compared to the control (water) treatment of the same experiment.

position, but the females did not respond to salicin and arbutin at a statistically significant level (Table 3).

DISCUSSION

Phenolic glucosides have an important effect on oviposition behavior of *E. amerinae*. This agrees well with earlier observational studies of Rowell-Rahier et al. (1987), Roininen and Tahvanainen (1989), and Soetens et al. (1991), which emphasized the importance of phenolic glucosides in host-plant selection of other willow-feeding sawflies. The total fraction of phenolic glucosides extracted from *S. pentandra* and its main individual glucoside, 2′-*O*-acetylsalicortin, were the best stimulants for *E. amerinae* (Figure 2). The stimulatory effects of *S. myrsinifolia* extract and salicortin were much weaker. This shows clearly that females of *E. amerinae* can recognize and select their host plant *S. pentandra* based on phenolic glucosides. They are probably also able

to use phenolic glucosides as a cue in shoot selection within host-plant individuals, because different shoot length categories have different levels of phenolic glucosides.

The role of salicin has been investigated and emphasized in many earlier studies on insect herbivores on Salicaceae, such as chrysomelid beetles (Rowell-Rahier and Pasteels, 1982; Rowell-Rahier, 1984a–c; Matsuda and Matsuo, 1985; Smiley et al. 1985; Kelly and Curry, 1991; Rank, 1992). However, in our study salicin did not seem to affect egg-laying behavior of *E. amerinae*. We have also found that salicin is a poor stimulant for the feeding of leaf beetles (Chrysomelidae) (Kolehmainen et al., 1994). This may be due to the fact that salicin is the most commonly found phenolic glucoside in Salicaceae species, and it is also found in certain members of the Rosaceae (Vickery and Vickery, 1981). In addition, salicin is present only in low concentrations in most willow species (Palo, 1984; Julkunen-Tiitto, 1986, 1989a,b). Therefore, salicin has apparently been a weak chemical cue in host recognition once insect herbivores have become adapted to Salicaceae host plants. However, salicin derivatives, such as salicortin, have so far been found only in salicaceous plants and vary greatly in concentration among different species (Julkunen-Tiitto, 1989b). Thus they can be extremely important factors in the physiological and behavioral differentiation of willow-feeding insects.

Individual phenolic glucosides seem to have a positive effect or no effect at all as oviposition stimulants. Salicin and arbutin did not stimulate egg-laying behavior of *E. amerinae* but we did not find any inhibitory effects either (Figure 2B). It is likely that herbivorous insects can easily become physiologically adapted to most phenolic glucosides present in willow species. Thus, specialization may take place largely at the behavioral level (Futuyma, 1983a,b; Futuyma et al., 1984). The inhibitory effects may arise later if herbivorous insects specialize more closely only to certain willow species and if the chemical evolution of willows progresses.

Both our multiple oviposition experiments using shoots of *S. pentandra* and experiments with pure phenolic glucosides show that phenolic glucosides offer a potent cue in the oviposition behavior of a specialized insect herbivore. Phenolic glucosides seem to form the specific signal pattern, the fingerprint, by which an ovipositing *E. amerinae* recognizes its host willow, *S. pentandra* (Figure 2A, Table 3). In addition to this, *E. amerinae* may choose the exact oviposition place within the host individual according to phenolic glucoside content of particular plant tissues or plant parts (Figure 1, Table 2).

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Book Review

Analysis of Phenolic Plant Metabolites. P.G. Waterman and S. Mole. Oxford: Blackwell Scientific Publications, 1994. £21.50 paper. ISBN 0-632-02969-2. Methods in Ecology Series. Series editors J.H. Lawton FRS and G.E. Likens

Phenolic compounds are a major group of plant secondary metabolites and are of great interest to ecologists because of their diverse and important roles in plant-animal, plant-pathogen, and plant-plant interactions. While our understanding of the role of these compounds in ecological processes has developed rapidly in recent years, this has often been despite rather than because of the methodologies used to measure phenolic compounds in ecological materials. There are many pitfalls to phenolic analysis into which ecologists without access to good procedural information have often fallen. Together with the problems associated with the wide variety of techniques available, their imprecise nature, and the lack of consistency and comparability between different methods and different laboratories, it is perhaps unsurprising that a large amount of data on phenolic contents of various ecological materials has been generated while our understanding and interpretation of these results has not advanced at the same pace!

This book is a timely and well-written assault on these difficulties: it brings together in one small volume a clear and concise analysis of the current methods for the measurement of phenolics and describes how best to carry out the recommended assays and the problems to look out for. It is a valuable book for experts and novices alike because it attempts to establish simple but accurate standard procedures, the use of which should dramatically improve comparability between different labs. If these uniform procedures are maintained for at least some time to come, the interpretation of future results should be enhanced. This is only one of the valuable aspects of this excellent book, but it could prove to be one of the most important in terms of increasing our understanding of the ecological role of these compounds.

This book is written for ecologists rather than chemists, and the nonspecialist should have no difficulty following either the theoretical or practical sections. Chapter 1 outlines the different types of phenolic compounds and their biosynthesis. Chapter 2 describes the distribution of these types among plant families, while Chapter 3 briefly reviews the various possible functions of phenolic compounds in ecology. While this section is a useful summary, it is forced by the size of the book to be rather superficial. Given the space constraints,

some of the biosynthetic detail could have been omitted as could the information on the Calvin cycle, especially since it appears in standard plant chemistry books. The section on alkaloids and terpenes is also a bit of a luxury. While it is certainly useful for ecologists to be aware of the different pathways that give rise to phenolic compounds, there it too much detail in the first two chapters, given that this is a book principally about methods of analysis (which we do not actually get to until p. 65). Curiously, despite the large amount of information on phenolic biosynthesis, there is no information on the methods available for measuring the activity of the main biosynthetic enzymes.

Chapters 4, 5, and 6 represent the "meat" of the book, describing methods for the extraction, biochemical, and in vivo analysis of phenolic compounds. These chapters are excellent. They are extremely well written, with the authors managing to be both informative and entertaining even when describing rather dull procedural details. All three chapters clearly describe laboratory procedures and any likely problems, and, while the authors are realistic about what can be achieved given the constraints of remote field sites, cost, and time, they demonstrate that precision and accuracy are absolute requirements that are obtainable in even the most basic laboratories. The famous Waterman vitriol descends upon inaccurate methods and those practitioners, (myself included!), who occasionally lapse from the standards required! The recommended methods are described clearly in schematic boxes that are particularly useful. The authors explain how they have arrived at these recommendations, selecting the most reliable repeatable techniques that are the least susceptible to interference. They then present ecologists with easy-to-follow standard procedures that should greatly improve within- and between-laboratory consistency. Chapter 4 is especially impressive, because it discusses in detail sample collection, preparation, and storage—aspects of chemical analysis that are usually virtually ignored. The only problem with this section of the book, which also occurs in the later chapters, is that, presumably to maximize appeal to nontechnical readers, the authors adopt an almost patronizing tone. They exhort us to think carefully, take notes, and not be afraid of chemistry, similar instruction to those we are given at school!

Chapter 7 addresses separation methods. Although this section was as well written as the rest of the book, I was disappointed at the relative amount of information on HPLC compared to PC/TLC. HPLC is one of the most widely used techniques for phenolic analysis in modern laboratories, and a lot of time is saved when using this technique if information on the most appropriate methodology is available in advance. However, the section on HPLC is only eight pages long and the most recent reference is 1989. Similarly, I thought the amount of space devoted to structural elucidation by NMR spectroscopy in Chapter 8 was excessive (18 pages) given that ecologists are rather more likely to encounter and use HPLC than NMR. Perhaps the balance here reflects the author's interests rather than general applicability!

However, these are very minor complaints about what is an outstanding book and an excellent and timely addition to the Methods in Ecology series. This book is a practical description of the best methods available for ecologists wishing to analyze phenolic compounds and is accessible to those ecologists with very limited chemical experience. The problems likely to be encountered in both the analyses and in their interpretation are dealt with clearly. This book is essential for anyone interested in phenolic compounds and is invaluable for even the most experienced chemical ecologists because of the standard procedures it outlines, which, if adopted widely, could provide a basis for future consistency between workers and thus improve our understanding of the ecological roles of these compounds. The preface to the Methods in Ecology series states that good methodology alone never solved any problems, but bad methodology can only make matters worse. This book is an inspiration for good methodology!

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EFFECT OF COUMARIN AND XANTHOTOXIN ON MITOCHONDRIAL STRUCTURE, OXYGEN UPTAKE, AND SUCCINATE DEHYDROGENASE ACTIVITY IN ONION ROOT CELLS

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Abstract—At concentrations in which they occur on the plant surface and retard mitosis, coumarin and xanthotoxin lowered uptake of oxygen (by 60 and 30%, respectively) by meristematic cells of *Allium cepa* root tips. They caused changes in the structure of the mitochondrial matrix to become dense, and protrusions of mitochondrial membranes were visible parallelling their hypertrophy, indicating alteration in the structure and physiology of these organelles. Coumarin and, to a lesser extent, xanthotoxin increased succinate dehydrogenase production in mitochondria and also in the cytoplasm, indicating changes in membrane permeability. Changes in oxygen uptake and mitochondrial structure, in addition to the retardation of mitosis, may be the reason these compounds act as allelochemicals after they have been removed from the plant surface and reach the root meristem.

Key Words—Coumarin, xanthotoxin, respiration, succinate dehydrogenase, mitochondrial structure, allelochemicals, *Allium cepa*.

INTRODUCTION

Coumarins play an ecologically important role for plant survival because they can be protective barriers, when located outside the plant (Zobel and Brown,

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1989), against ultraviolet radiation (Zobel and Brown, 1993; Zobel et al., 1994a) or against bacteria (Zobel and Brown, 1989; Zobel et al., 1994b). Several coumarins have been found on the surface of different plant species (Städler and Buser, 1984; Zobel et al., 1991b).

In plant–plant interactions these compounds may react as allelochemicals (Murray et al., 1982; Zobel et al., 1994a), and retard germination and growth of other plant species (Baskin et al., 1967; Friedman et al., 1982; Shimomura et al., 1982), as well as of the same species (Zobel et al., 1991a; Zobel and March, 1993). Some coumarins may retard growth in one part of the plant and stimulate it in another, which can depend on concentration (for review see Van Sumere et al., 1972; Murray et al., 1982). Coumarins may influence plant growth only after they enter the cells in solution (Kupidlowska et al., 1994). That fact has ecological importance in dry, as opposed to wet, surroundings (Zobel et al., 1994b).

After they have entered the cells, they must alter the cytophysiology of the tissue, but their influence on metabolic processes inside the plant cell has been little investigated. Glycolysis and oxidative phosphorylation were inhibited by coumarin (Stenlid and Saddik, 1962; Knypl, 1968, 1969). Some controversial results were obtained for xanthotoxin (Salet et al., 1982). We found that several coumarins (coumarin, 4- and 7-hydroxycoumarin, psoralen, and xanthotoxin) applied at concentrations in which they occur on plant surfaces inhibited or strongly retarded cell division (Zobel and Louis, 1990; Podbielkowska et al., 1994).

Inhibition of mitosis may be caused by a lack of intracellular energy or changes in mitochondrial structure (Podbielkowska et al., 1975, 1981; Podbielkowska and Kupidlowska, 1976). In the present paper we investigated the influence of coumarin, the most commonly occurring coumarin, and xanthotoxin, the most active furanocoumarin located on the surface of many plant species (Zobel and Brown, 1991), on the uptake of oxygen, the structure of mitochondria, and the activity of succinate dehydrogenase, an enzyme involved in electron transport.

METHODS AND MATERIALS

Experiments were performed on apical root meristems of *Allium cepa*, grown in aerated tap water. When the roots were 3 cm long, the onion bulbs were transferred into the following solutions: coumarin (200 ppm, 1.3 mM), or 8-methoxypsoralen (xanthotoxin, 100 ppm, 0.46 mM). The coumarins were obtained from Sigma Chemical Corp. (St. Louis, Missouri). Some bulbs were kept in water as controls. Incubation was allowed to proceed for 12 hr. In order to determine the rate of oxygen uptake, 50 fragments of 1-mm-long root tips,

including the apical meristem, were cut off and placed in 10 ml of Sorensen phosphate buffer, pH 6.6. The rate of oxygen uptake, measured as micrograms of oxygen uptake per gram fresh mass per minute, was determined with a Clark oxygen electrode (Oxi 39, MTW, G.M.B.H., D-812 Weilheim, Germany) at 21°C. For each treatment, measurements were taken three times.

The localization of active succinate dehydrogenase was determined in cells of periderm belonging to the promeristem of root tips, after they had been incubated in coumarin, xanthotoxin, or water as a control. Root fragments 500 μm thick were taken from the meristematic zone after the root cap had been excised. Such specimens were fixed in a mixture of 1% glutaraldehyde and 4% formalin (Podbielkowska and Waleza, 1987). After fixation for 20 min, specimens were rinsed in Sorensen's buffer of pH 6.6 for 10 min. Then, to locate active succinate dehydrogenase, samples were placed for 30 min at 37°C in a reaction medium containing: 0.2M phosphate buffer, pH 6.6; 20 μm sodium succinate; 50 mM in Na/K tartrate; 5 mM in copper sulfate; 200 mM in sucrose; 1.5 mM in potassium ferricyanide. The reaction product of active succinate dehydrogenase was the electron-dense granular precipitate of copper ferrocyanide, so-called Hatchett's brown.

Samples were then rinsed in phosphate buffer and postfixed in 1% osmium tetroxide in 0.1 M cacodylate buffer, pH 7.2, for 12 hr at 4°C. The fixed material was dehydrated in a series of increasing alcohol concentrations, acetone, and propylene oxide before embedding in Epon 812. Ultrathin sections were contrasted according to Reynold's method (1963) without sodium hydroxide washing. Specimens were examined with a Jeol electron microscope JEM 100B.

RESULTS

Coumarin and xanthotoxin reduced the rate of oxygen uptake by about 60 and 30%, respectively (Figure 1). In the control group the structure of mitochondria was typical of their "phosphorylated" orthodox state (Figure 2). The reaction product of active succinate dehydrogenase (electron-dense granular precipitate of copper ferrocyanide, Hatchett's brown) occurred in a few mitochondria (ca. 5%) on the outer surface of cristae that faced the matrix (Figure 3).

Coumarin induced changes in the structure of the mitochondria (Figure 4 and 5); most of them became condensed with electron-dense matrix and extended intracristal spaces. In about 10% of mitochondria protrusions containing fragments of membranes were formed as the result of hypertrophy of mitochondrial membranes (Figure 4, arrow). The succinate dehydrogenase reaction product occurred much more frequently in cells treated with coumarin (in ca. 60% of mitochondria) than in the control (in ca. 30% of mitochondria). The precipitate of copper ferrocyanide was chiefly formed in condensed mitochondria, but in

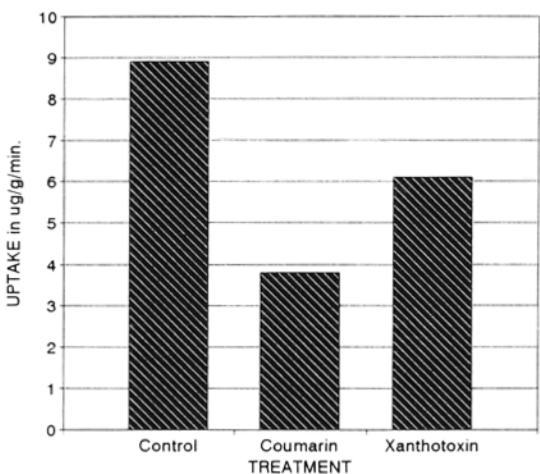

FIG. 1. Influence of coumarin and xanthotoxin on oxygen uptake.

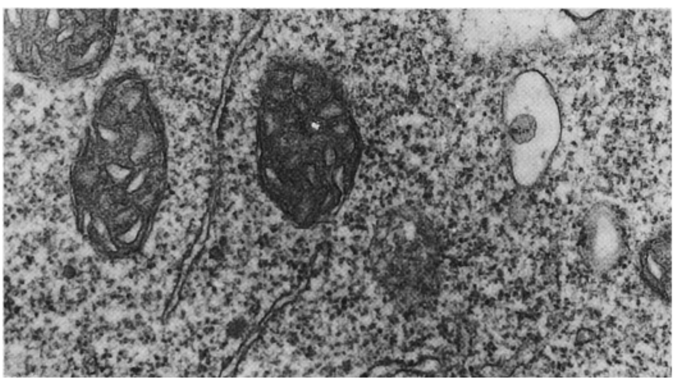

FIG. 2. Control promeristem cell. Typical structure of mitochondria in their orthodox form, ca. 20,000×.

cells with degraded protoplast, when ground cytoplasm appeared to be precipitated and contained fewer ribosomes, it was also observed in such ground cytoplasm, and in the intracristal space of mitochondria (Figure 5, white arrows).

The ground cytoplasm of cells treated with xanthotoxin did not degenerate to the same degree as those treated with coumarin, and still contained numerous ribosomes (Figures 7 and 8). Mitochondria occurred only in the condensed form (Figure 7), and only a few of them (ca. 5%) showed hypertrophy of the outer

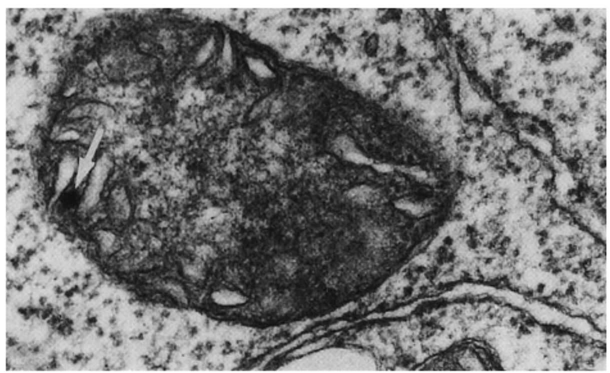

FIG. 3. Control. Mitochondrion in its orthodox form. Arrow indicates reaction product of active succinate dehydrogenase, ca. 30,000×.

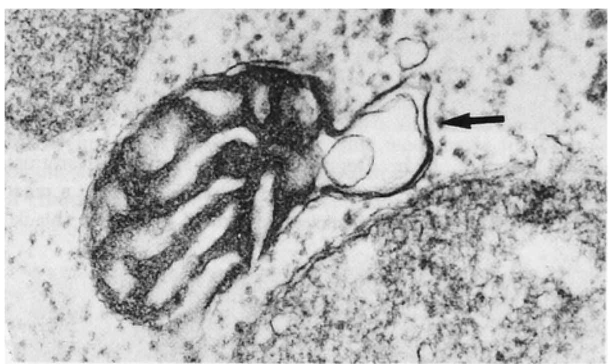

FIG. 4. Coumarin, 200 ppm, 12 hr. Condensed form of mitochondrion. Protrusion of mitochondrial membranes (arrow), ca. 40,000×.

membrane (Figure 7, arrowhead). Mitochondria containing the reaction product (Figures 6 and 8) were present in smaller quantities treated with xanthotoxin (ca 40%) in comparison to coumarin-treated cells. Electron-dense granules were observed mostly on the outer surface of cristae (Figure 9, arrowhead).

DISCUSSION

For growth all cells need energy, most of which is provided through respiration. Our analysis of mitochondrial structure and the location of active succinate dehydrogenase indicate that coumarin and xanthotoxin affect respiration

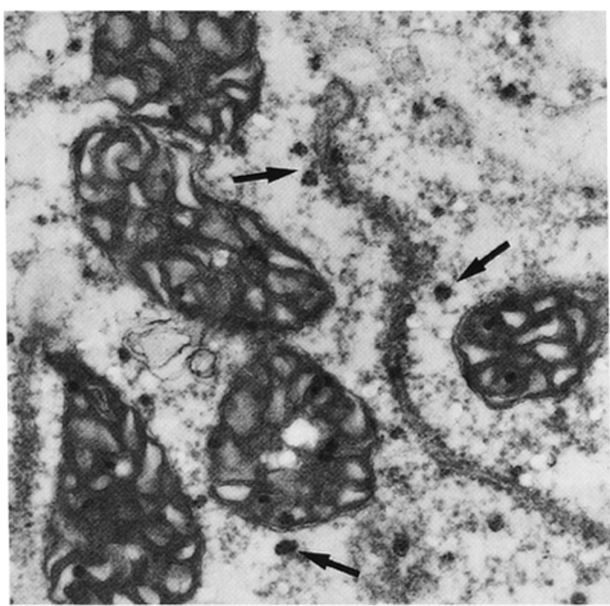

FIG. 5. Coumarin, 200 ppm, 12 hr. Degradation of ribosomes and protrusion of mitochondrial membranes (arrowhead). Condensed mitochondria contain a reaction product in cristae (white arrows); reaction also present in ground cytoplasm (black arrow), ca. 30,000×.

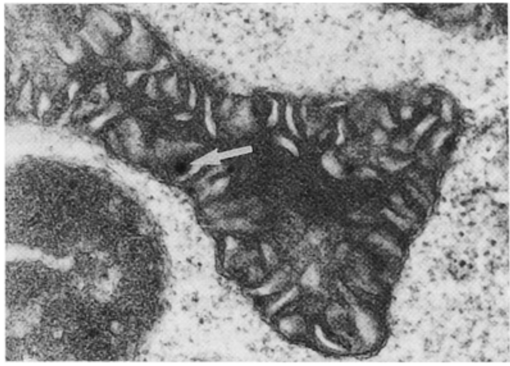

FIG. 6. Xanthotoxin, 100 ppm, 12 hr. Electron-dense matrix with reaction products of dehydrogenase (arrow). Elaborate cristae, ca. 25,000×.

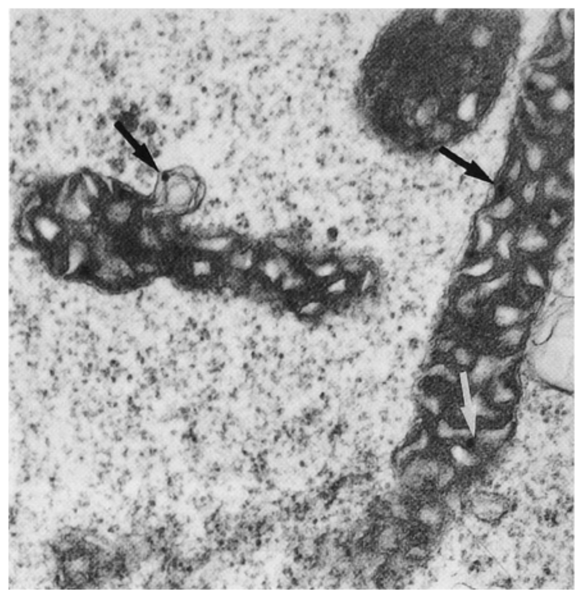

FIG. 7. Xanthotoxin, 100 ppm, 12 hr. Protruding membranes from mitochondrion (arrowhead) contain dehydrogenase. Numerous dilated cristae (white arrow). Numerous ribosomes in ground cytoplasm, ca. 25,000×.

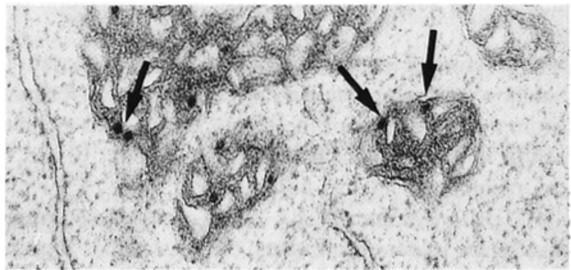

FIG. 8. Xanthotoxin, 100 ppm, 12 hr. Reaction product of dehydrogenase in mitochondrial matrix (arrowhead), ca. 25,000×.

in root meristematic cells by lowering oxygen uptake. Similar inhibition was observed in mitochondria of red liver cells (Murray et al., 1982). Xanthotoxin inhibited respiration in the mouse fibroblast (Gawron, 1992).

In the presence of uncoupling factors, the efficiency of staining for succinate dehydrogenase is maximal and limited only by the amount of substrate available.

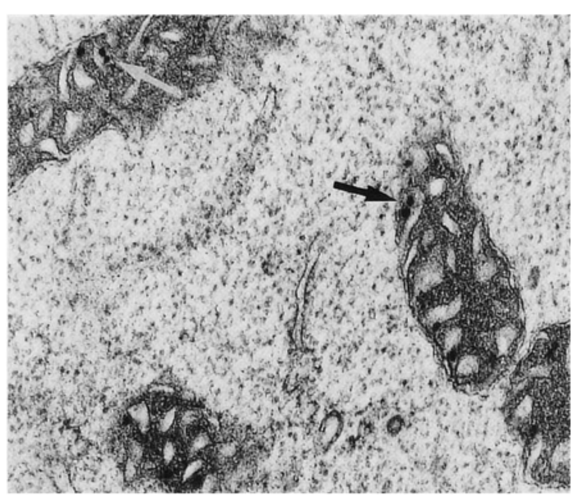

FIG. 9. Xanthotoxin, 100 ppm, 12 hr. Localization of double deposits of dehydrogenase reaction products in mitochondrial matrix (arrowhead), ca. 25,000×.

However, the rate of reaction is limited by phosphorylation (Lewis and Knight, 1986). Our results suggest that coumarin and, to a lesser extent, xanthotoxin may account for the stronger precipitation of copper ferrocyanide in cells treated with coumarin, and they suggest that oxidative phosphorylation is uncoupled to some degree.

Analysis of structure of ground cytoplasm and mitochondria indicated that coumarin and xanthotoxin might interfere with energy metabolism, which probably resulted from the interaction of these compounds with membranes, changing their structure and, possibly, permeability. Similarly, the structure and permeability of membranes in the fibroblasts of mice (Gawron, 1992) and lymphoblasts HUT 102 (Malinin et al., 1990) were altered after xanthotoxin and psoralen treatment. In our experiments, the presence of copper ferrocyanide granules after coumarin treatment, located outside the mitochondria, also indicated that permeability of mitochondrial membranes was altered. Similar results were obtained when staining was carried out under conditions that increased membrane permeability (Kerpel-Fronius and Hajos, 1970).

Disturbance of the system of cell membranes by both lipophilic and hydrophilic coumarins seems to be associated with the ability of these compounds to react with the unsaturated fatty acids or proteins of the membrane, respectively (Zboril and Dadak, 1973; Laskin and Laskin, 1988; Döring et al., 1992). Binding of these compounds to proteins and lipids in membranes must have changed

membrane permeability and may thus be responsible for additional extrusion of coumarins to the surface and then to the environment.

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2-(*E*)-Nonen-1-ol: MALE ATTRACTANT FOR CHAFERS *Anomala vitis* FABR. and *A. dubia* SCOP. (COLEOPTERA: SCARABAEIDAE)[1]

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[1]*The present paper is dedicated to the memory of our coauthor, friend and colleague Mátyás Lesznyák who died a premature and tragic death in late 1993.*

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Abstract—Traps baited with 2-(*E*)-nonen-1-ol alone or in combination with other compounds caught large numbers of males of both the vine chafer, *Anomala vitis* Fabr. and the margined vine chafer, *A. dubia* Scop. (Coleoptera: Scarabaeidae), vineyard and orchard pests. In a dosage test, the largest numbers were caught by traps baited with 10 mg of 2-(*E*)-nonen-1-ol, which was the highest dosage tested. This is the first report on male attractants for chafer species occurring in Europe.

Key Words—*Anomala vitis*, *Anomala dubia*, chafer, Coleoptera, Scarabaeidae, Melolonthidae, Rutelinae, attractant, 2-(*E*)-nonen-1-ol.

INTRODUCTION

Among the more than one thousand species of the genus *Anomala* [Coleoptera: Scarabaeidae (Melolonthidae), Rutelinae] worldwide there are widespread defoliator pests of many agricultural plants (Endrödi, 1956). In temperate and espe-

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cially in warmer regions of Europe, the vine chafer, *Anomala vitis* Fabr. and the margined vine chafer, *A. dubia* Scop. are regularly reported to cause leaf damage in vineyards, and, occasionally, in various orchard trees (Hurpin, 1962). In Hungary, *A. vitis* clearly prefers the regions with chalky sand of the Great Plain area, while *A. dubia*, although in many cases cooccurring with *A. vitis*, can also be found on areas with more acidic sand (Endrödi, 1956; Bognár and Huzián, 1974; Koppányi, 1988). The species have regular mass outbreaks a couple of years apart; in such years in heavily damaged areas vineyards can be totally defoliated (Homonnay and H. Csehi, 1990).

In order to search for an attractant that could later be used in traps for the monitoring and forecast of these pests, several compounds, described as pheromone components from other species of Scarabaeidae were screened at several sites in Hungary. The compounds screened included all compounds identified so far from *Anomala* spp. in Japan, namely, methyl (*Z*)-tetradec-5-enoate (Tamaki et al., 1985); (*R*,*Z*)-5-(−)-(oct-1-enyl)oxacyclopentan-2-one (Leal, 1991), 2-(*E*)-nonen-1-ol (Leal et al., 1992a), as well as (*R*,*Z*)-5-(−)-(dec-1-enyl)oxacyclopentan-2-one, the pheromone of *Popillia japonica* Newman (Tumlinson et al., 1977), and the major and minor components of the pheromone of *Holotrichia parallela* Motschulsky [L-isoleucine methyl ester (Leal et al., 1992b), (*R*)-(−)-linalool (Leal et al., 1993a)]. Some binary mixtures of the above compounds were also tested. In the present paper we describe the discovery of a male attractant for both *A. vitis* and *A. dubia*.

METHODS AND MATERIALS

Synthesis. Methyl (*Z*)-tetradec-5-enoate was synthesized starting by the coupling of 1-decyne with 1-bromo-3-chloropropane by Wittig reaction. The Grignard reaction of the product, 1-chloro-4-tridecyne, with methyl chloroformate gave methyl tetradec-5-ynoate. This was reduced by Lindlar catalyst to give an isomeric mixture containing 98% of the desired *Z* product. 2-(*E*)-Nonen-1-ol and L-isoleucine methyl ester were synthesized as previously described (Leal et al., 1992a,b). (*R*,*Z*)-5-(−)-(oct-1-enyl)oxacyclopentan-2-one was prepared as previously reported (Leal, 1991). (*R*,*Z*)-5-(−)-dec-1-enyl)-oxacyclopentan-2-one was obtained starting from D-ribose (Koseki et al., 1993).

The optical purity of all lactones was determined to be >97% ee by chiral chromatography (Leal, 1991). *R*-(−)-Linalool, kindly provided by Fuji Flavor Co. (Tokyo, Japan), was determined to be ca 100% ee by a previously described method (Leal et al., 1993b).

Trapping Tests. Dispensers for the tests were prepared by using pieces of rubber tubing (Taurus, Budapest, Hungary; No. MSZ 969 1/6; extracted three

times in boiling ethanol for 10 min, then also three times in methylene chloride overnight, prior to usage). For making up the baits the required amounts of compounds were administered to the surface of the dispensers in hexane (Merck) solutions. In the case of L-isoleucine methyl ester, instead of hexane dichloromethane (Merck) was used. Prepared dispensers were stored at −65°C until use. Field tests were conducted at several sites in Hungary. Trap types used included a funnel trap (Japan Tobacco Inc., Tokyo, Japan), made from green plastic (later in the paper referred to as JT), and a home-made funnel trap similar in shape and size to the JT traps, but hand made from transparent plastic bottles (later in the paper referred to as HM). Traps were suspended from branches of vine or trees at a height of 0.5–1.0 m above ground. Traps containing different baits were set up along a straight line. The distance between traps within a line was 10 m. The distance between lines ranged between 100 and 1000 m. Traps were moved one position forward within a line, and the trap positioned at the end of the line was returned to the beginning position at each occasion when the traps were inspected. At the same time, the number of captured beetles was recorded and traps were emptied. In screening tests, one replicate of each bait per trap type was set up, while in dosage tests three replicates were used.

RESULTS AND DISCUSSION

In the screening tests, *A. vitis* and *A. dubia* male beetles were captured in traps baited with 2-(*E*)-nonen-1-ol, or with mixtures containing this compound (Table 1). No other compound showed any attractive activity for these beetles. *A. vitis* was captured in very high numbers at Kunbaracs, where *A. dubia* was missing; on the other hand, at Ladánybene, and Józsa, where *A. dubia* was abundant, there were only low catches of *A. vitis* recorded. However, at Gödöllö, both species were present, albeit in moderate numbers. Of the ca. 100 randomly selected beetles of the catch, all were found to be males.

In a preliminary dosage test of 2-(*E*)-nonen-1-ol for *A. vitis*, traps baited with 10 mg, the highest dosage tested, caught significantly more beetles than lower dosages at Kunbaracs, where population density was high (Table 2). The catches of the two lower doses did not differ. In the case of *A. dubia* catches by 10 or 1 mg were not different, while catches by 0.1 mg were significantly lower (Table 2).

2-(*E*)-Nonen-1-ol has been identified as the sole component of the female-produced pheromone of *Anomala schonfeldti* Ohaus (Leal et al., 1992a) and as the major constituent of the *A. daimiana* Harold sex pheromone (Leal et al., 1993a). It has also been found that this compound is a potent attractant for the rose chafer *Macrodactylus subspinosus* F., a serious pest of fruit crops, ornamentals, and flowers in eastern North America (Williams et al., 1993). Although

TABLE 1. CAPTURES OF MALE *A. vitis* AND *A. dubia* IN TRAPS BAITED WITH COMPOUNDS PREVIOUSLY IDENTIFIED AS PHEROMONE COMPONENTS IN SCARABAEIDAE AND THEIR BINARY MIXTURES IN 1993[a]

Bait (mg)						Total number of male *Anomala vitis* caught				Total number of male *Anomala dubia* caught			
						Gödöllö		Kunbaracs		Gödöllö		Józsa,	Ladánybene,
Methyl(*Z*)-tetradec-5-enoate	(*R*, *Z*)-5-(−)-(Dec-1-enyl)-oxacyclopentan-2-one	(*R*, *Z*)-5-(−)-(Oct-1-enyl)-oxacyclopentan-2-one	(*R*)-(−)-Linalool	2-(*E*)-Nonen-1-ol	L-Isoleucine methyl ester	JT trap	HM trap	JT trap	HM trap	JT trap	HM trap	JT trap	HM trap
1						0	0	0	0	0	0	0	0
	1					0	0	0	0	0	0	0	0
		1				0	0	1	0	0	0	1	0
			1			0	0	0	0	0	0	0	0
				1		4	1	760	68	4	1	317	57
					10	0	0	0	0	0	0	0	0
		1		1		8	0	1110	180	10	12	184	34
	1	1				0	0	2	0	0	0	0	0
1	1					0	0	1	0	0	0	0	0
	1			1		12	3	705	268	15	15	138	76
1				1		9	6	325	101	11	7	59	77
1		1				0	0	1	0	0	0	0	0

[a]Gödöllö, Pest County, April 22–July 9, nursery with *Pinus nigra* trees; Kunbaracs, Bács-Kiskun County, HM trap type May 4–June 28; JT trap type June 8–July 12; commercial vineyard; Józsa, Hajdú-Bihar County, June 2–30, backyard gardens with small vineyards and mixed orchard trees; Ladańybene, Pest County, April 21–June 28; *Pinus nigra* stands. Traps were inspected twice weekly, baits were renewed fortnightly, except with methyl (*Z*)-tetradec-5-enoate, which was replaced weekly. At each site one replicate was set up of each bait variation per trap type.

TABLE 2. CAPTURES OF MALE *A. vitis* AND *A. dubia* IN TRAPS BAITED WITH DIFFERENT AMOUNTS OF 2-(*E*)-NONEN-1-OL[a]

	Mean catch		
	Anomala vitis		*Anomala dubia*,
Dose of 2-(*E*)-nonen-1-ol (mg)	Ladánybene	Kunbaracs	Ladánybene
0.1	0.13 a	24.27 a	0.60 a
1	0.60 ab	34.40 a	2.67 ab
10	1.13 b	72.13 b	4.20 b

[a] Ladánybene, Pest County, and Kunbaracs, Bács-Kiskun County; June 10–28, 1993; at each site three replicates were set up of each dose, using JT traps. Traps were inspected twice weekly. In statistical analyses, catches recorded at an inspection were regarded as replicates. Capture data were transformed to $\sqrt{x} + 0.5$ and differences between means were tested for significance by ANOVA followed by Duncan's new multiple-range test (NMRT) Means followed by same letter within one column are not significantly different at $P = 5\%$.

at present it is not known whether this compound is also produced by females of *A. vitis* or *A. dubia*, results of the present study suggest that 2-(*E*)-nonen-1-ol can be one of the key pheromone structures in this genus. Chemically identified pheromones for phytophagous Scarabaeidae (Melolonthidae) have been described so far almost exclusively for species originating in the Pacific region: in Japan (Tumlinson et al., 1977; Tamaki et al., 1985; Leal, 1991, 1993; Leal et al., 1992a,b, 1993a,b) or in New Zealand (Henzell and Lowe, 1970). This is the first report of male attractant compounds for species occurring in Europe.

Although biological evidence is known concerning the cross-attractancy of natural pheromones in Scarabaeidae (Leal et al., 1993b), it is still surprising that males of both *A. vitis* and *A. dubia* were so strongly attracted to a single compound in the present study. The geographical distribution of the two species and their seasonal occurrence pattern broadly overlaps (Endrödi, 1956; Hurpin, 1962; Homonnay and H. Csehi, 1990). What is more, in the course of the present study, males of both species were equally frequent in approaching baits in the same period of the day, i.e., late morning (9–12 AM) (Tóth and Szöcs, unpublished). Consequently, it is highly probable that reproductive isolation between the two species is achieved by the production of secondary components in each natural pheromone. This clearly emphasizes the importance of future analyses of female-produced pheromone and the study of the influence of other synthetic pheromonal compounds added in differing ratios.

Due to the preliminary nature of the screening test in this study, we did not try to establish the influence of other compounds added to 2-(*E*)-nonen-1-

ol on captures. However, there have been some cases described where the addition of minor amounts of a second component had a striking effect on captures of male scarabs (Leal et al., 1993a).

For the time being, baits with 1–10 mg of 2-(*E*)-nonen-1-ol yield a suitable basis for the development of monitoring traps for both *A. vitis* and *A. dubia*. From the practical point of view, the mutual attraction of both species is not harmful, since both species damage the same cultures in the same period of the year. As most damage is caused by the adults (Homonnay and Csehi, 1990), trapping out of a significant part of the beetle population may have a direct diminishing effect on damage, provided the capacity and number of traps is high enough.

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CHEMICAL CHARACTERIZATION OF URINARY VOLATILE COMPOUNDS OF *Peromyscus californicus*, A MONOGAMOUS BIPARENTAL RODENT

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Abstract—The urinary profiles of adult female and male California mice were examined to determine the volatile compounds that may affect reproduction. The urinary volatiles identified by gas chromatography-mass spectrometry (GC-MS) include ketones, pyrazines, alkanes, nitrile, and aldehyde. None of volatile compounds was specific to males or females. The concentration of urinary volatiles in pregnant and lactating females was significantly higher than in virgin females. Male urinary volatile concentrations were similar to those of pregnant and lactating females. The GC profiles of both sexes were distinguished by a high percentage (36%) of pyrazine derivatives that were also in unusually high concentrations. These compounds may play a wide-ranging role in the control of reproduction in the California mouse.

Key Words—*Peromyscus*, reproduction, reproductive inhibition, urine, volatiles.

INTRODUCTION

The social conditions that affect sexual maturation and reproduction in rodents have been well documented, particularly in the house mouse, *Mus musculus* (Bronson and Rissman, 1986; Drickamer, 1981). In general, sexual maturation

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of juvenile females is accelerated by exposure to a male or his urine, the urine of females in estrus, and the urine of pregnant or lactating females (Castro 1967; Drickamer, 1982; Drickamer and Hoover, 1979; Teague and Bradley, 1978; Vandenbergh, 1967). The urinary chemosignals from pregnant/lactating female *M. musculus* accelerate puberty in juvenile females regardless of whether the females are related or unrelated to the urine donors (Drickamer, 1984). These puberty-accelerating urinary volatiles have been chemically characterized in house mice (Jemiolo et al., 1989).

In other rodents, however, reproduction in young females is suppressed by the presence of family members, e.g., deermice, *Peromyscus maniculatus*, (Haigh, 1983b; Hill, 1974) white-footed mice, *P. leucopus* (Haigh, 1987) cactus mice, *P. eremicus* (Skryja, 1978), California mice, *P. californicus* (Gubernick and Nordby, 1992), gerbils, *Meriones unguiculatus* (Payman and Swanson, 1980), and prairie voles, *Microtus ochrogaster* (Hasler and Nalbandov, 1974). This reproductive suppression is apparently mediated by urinary chemosignals because exposure to urine from an adult female delays puberty in juvenile females, e.g., prairie deermice, *P. maniculatus bairdii* (Lombardi and Whitsett, 1980), and because removal of the adult female leads to rapid reproduction in previously inhibited juvenile females, e.g., *P. leucopus* (Haigh 1983a, 1987). The delay of reproductive maturation by urinary chemosignals in *Peromyscus* may play a significant role in the sociobiology of reproduction and population growth (Terman 1979, 1980, 1987). However, the urinary chemosignals that delay sexual maturation in *Peromyscus* have not yet been chemically characterized.

The California mouse, *P. californicus*, offers a model system for investigation of urinary chemosignals that may affect social and reproductive behavior. This species is exclusively monogamous in the wild (Ribble, 1991; 1992b) and pairs remain together permanently unless the mate dies (McCabe and Blanchard, 1950; Ribble and Salvioni, 1990). Thus, male and females (and their urinary chemosignals) may affect their partner's social and reproductive behavior. Male *P. californicus* exhibit all the components of parental care displayed by mothers, and to the same extent, except for nursing (Dudley, 1974; Gubernick and Alberts, 1987). Juveniles emerge from their nest at about 35 days of age and disperse at about 75–80 days of age (Ribble, 1992a). Thus juveniles continue to interact with their parents after emergence and prior to dispersal and their sexual development may be influenced by their parents.

Sexual maturation of juvenile *P. californicus* females is delayed if females remain housed with both parents, and this delay is the result of contact with the mother (Gubernick and Nordby, 1992). Fathers have no apparent affect on their daughter's sexual maturation. Puberty also is delayed by exposure to a strange adult virgin female. Contrary to other rodents (Drickamer, 1986; Levin and Johnston, 1986; Vandenbergh, 1983), strange adult males did not accelerate

puberty in female *P. californicus* (Gubernick and Nordby, 1992). These findings suggest a possible female-specific cue(s) given by pregnant/lactating females and virgin females that delays first estrus.

In *P. californicus* urinary chemosignals are involved in the maintenance of male parental care. Fathers kept only with their mate or exposed to their mate's urine for the first three days after birth were more likely to act parental than males separated from their mate on the day of birth (Gubernick and Alberts, 1989; Gubernick, 1990). The volatile fraction of the maternal urinary chemosignals is sufficient to maintain paternal responsiveness postpartum (Gubernick, 1990).

Most research on rodent urinary chemosignals is limited to investigations of polygamous species (Levin and Johnson, 1986; Sawrey and Dewsbury, 1991). To investigate the functional significance of such urinary chemosignals and whether they are related to mating systems, it is necessary to examine rodent species that differ in their mating and parental rearing patterns.

The present experiment examined the urinary profiles of adult female and male California mice and characterized the composition of their urinary volatile compounds. This study represents our first step in identifying the critical components of urinary chemosignals potentially involved in the delay of sexual maturation and the maintenance of paternal behavior. As such, it provides a necessary foundation for subsequent biobehavioral analyses. This study also adds to the growing comparative database on rodent urinary chemosignals that may help provide insight into the adaptive significance and evolution of such chemosignals.

METHODS AND MATERIAL

Animals used in this study were sixth-generation descendants of mice originally captured in the Santa Monica Mountains, northeast of Los Angeles, California. The virgin females and males were housed individually in a standard mouse cage measuring 28 × 19 × 13 cm and fitted with a wire lid. The experienced pregnant and lactating females were housed with their mates throughout the experiment in a standard mouse maternity cage measuring 45 × 24 × 13 cm. The colony room was kept at 22–24°C and maintained on a 16:8 hr light–dark cycle initiated at 0700 hr. Cages and bedding were changed weekly. Purina Mouse Chow and water were provided ad libitum.

Urine samples used for chemical analyses were obtained by holding a mouse over a glass container and gently squeezing the abdomen and flanks. Immediately after collection, samples were stored at −20°C and analyzed within one to two months. All urine samples were analyzed three times by using a 1-ml sample each time.

Urine from virgin females ($N = 9$) was collected throughout all stages of their estrous cycle, which lasted from 6 to 14 days. Urine collected from pregnant females ($N = 26$) began on day 23–25 of gestation and continued until the end of pregnancy (day 31–33) (Gubernick, 1988). Lactating female urine ($N = 29$) was collected from the first to the eighth day of lactation. Seven adult virgin males also served as urine donors. All urine samples were collected daily and then pooled within groups to avoid the individual variation in the concentration of urinary compounds. Pooled urine was divided into three 1-ml samples ready for chemical analysis.

The urinary volatiles were analyzed using the headspace technique, employing Tenax GC (a porous polymer) as the adsorption medium (Novotny et al., 1974). The volatiles were sparged from 1-ml urine samples at room temperature with purified helium gas at a flow rate of 100 ml/min and adsorbed onto a precolumn packed with 4 mg of Tenax GC. The sample was subsequently desorbed in the heated injection port (220–240°C) of a gas chromatograph (Perkin-Elmer, 3920 instrument, Norwalk, Connecticut) equipped with a flame ionization detector, and retrapped into a cooled section of a glass capillary column. The analytical column was a glass capillary (60 m × 0.25 mm ID) of a soda-lime type, coated statically with UCON 50-HB-2000 (Schwende et al., 1984a,b).

Quantitative comparisons were obtained through a peak integration routine (Sigma 10, Perkin-Elmer), whereas identification of the individual profile constituents was established through a combined GS-MS (Hewlett-Packard, 5981 dodecapole mass spectrometer, Palo Alto, California) using electron impact ionization at 70 eV.

Statistical comparisons of the concentration of excreted volatiles were performed using a one-way analysis of variance (ANOVA; F at $P < 0.05$) with post-hoc comparisons by Tukey test ($P < 0.02$). Because of the heteroscedastic data, the inequality of variances was "corrected" by logarithmic transformation of the original values prior to analysis (Zar, 1984).

RESULTS

The capillary gas chromatograms shown in Figure 1 representative of California mouse volatile profiles obtained from pregnant female urine (Figure 1A) and virgin male urine (Figure 1B). Eighteen volatile compounds were positively identified by combined capillary GC-MS and retention measurements. Identified substances included ketones, pyrazines, alkanes, nitrile, and aldehyde (Table 1).

Through careful visual inspection of the profiles and statistical comparison of integrated peak areas of urinary volatiles obtained from the four experimental groups, we established that 11 constituents exhibited significant differences in

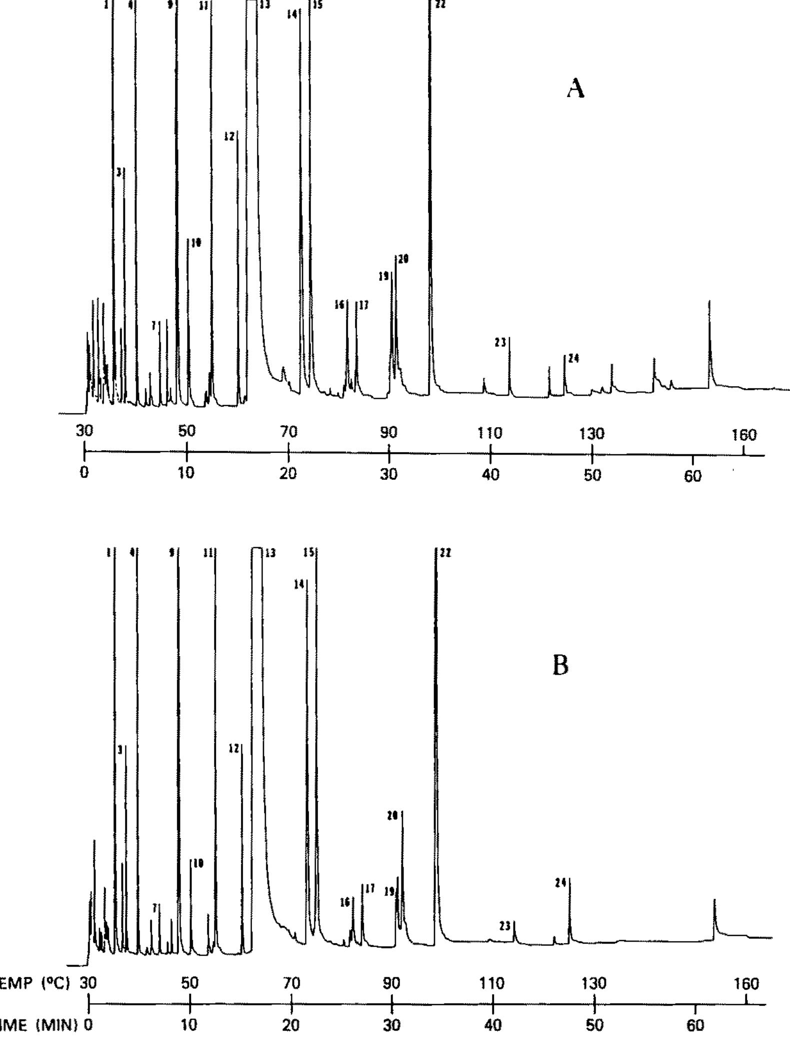

FIG. 1. GC-MS chromatographic profiles of the volatile compounds in urine from (A) pregnant female and (B) virgin male of *P. californicus*.

their chromatographic peak areas (concentrations) as a function of gender and reproductive status of an animal (Table 1). The concentrations of the remaining seven compounds listed in Table 1 did not differ significantly between groups.

Volatile Profiles of Female California Mouse Urine. A qualitative com-

TABLE 1. CLASS AND STRUCTURE OF VOLATILE COMPOUNDS OCCURRING IN CONSISTENTLY MEASURED QUANTITIES IN URINE OF FEMALE AND MALE CALIFORNIA MICE

Class and structure of urinary volatiles	Peak code	Concentration changes of volatiles in four investigated types of urine F^a	P
Ketones			
3-Pentanone	1	85.2	<0.0005
3-Methyl-2-pentanone	3	1.2	NS
3-Hexanone	4	1.9	NS
3-Methy-2-hexanone	7	7.5	<0.01
3-Heptanone	9	4.5	<0.05
2-Heptanone	10	105.4	<0.0005
6-Methyl-3-heptanone	11	3.1	NS
3-Octanone	12	7.4	<0.01
$C_6H_8O_2$	19	1.4	NS
Acetophenone	22	1.5	NS
Pyrazine			
2,5-Dimethylpyrazine	13	9.1	<0.005
2-Ethyl,5-methylpyrazine	14	4.8	<0.05
2,3,5-Trimethylpyrazine	15	10.3	<0.005
2-ethyldimethylpyrazine	17	16.8	<0.001
Alkane			
Alkane	23	27.1	<0.0005
Alkane	24	1.1	NS
Nitrile			
Benzonitrile	20	9.8	<0.005
Aldehyde			
Benzaldehyde	16	0.4	NS

[a] $F(df) = 3,8$

parison of volatiles from pregnant, lactating, and virgin female urine revealed that each type of urine can be characterized by the presence of a specific volatile compound(s). All three types of urine contained volatiles from the same structural classes. However, a quantitative comparison of urinary volatiles showed marked concentration differences among the three groups of female urine. Each type of female urine can be characterized by the presence of one or a few volatiles in high concentration not found in the other urine samples (Table 2). For example, the urine of virgin females contained only one compound in significantly higher concentration than in the urine of pregnant and lactating females, i.e., the first selected urinary alkane (peak 23). 2-Heptanone (peak 10),

TABLE 2. MEAN VALUES (SEM) OF PEAK AREAS (TRANSFORMED TO LOG) OF CALIFORNIA MOUSE URINARY VOLATILES EXHIBITING SIGNIFICANT CONCENTRATION CHANGES AS A FUNCTION OF GENDER OR REPRODUCTIVE STATUS OF TESTED ANIMALS

	Type of urine[a]			
Class/peak code	Pregnant females	Lactating females	Virgin females	Virgin males
Ketones				
1	2.43 (0.03)	2.42 (0.06)	1.43 (0.01)a	2.32 (0.04)
7	1.30 (0.04)	1.20 (0.02)	0.91 (0.07)a	0.92 (0.06)a
9	2.53 (0.04)	2.44 (0.06)	2.06 (0.00)a	2.44 (0.00)
10	1.41 (0.03)c	0.73 (0.02)a	0.76 (0.03)a	1.23 (0.03)b
12	1.83 (0.03)b	1.40 (0.11)a	1.34 (0.04)a	1.33 (0.03)a
Pyrazine				
13	4.06 (0.02)	4.10 (0.02)	3.72 (0.08)a	4.00 (0.04)
14	2.21 (0.05)	2.24 (0.02)	2.00 (0.02)a	2.19 (0.00)
15	2.09 (0.06)	2.10 (0.04)	1.72 (0.05)a	2.11 (0.04)
17	1.45 (0.01)c	1.01 (0.08)b	0.49 (0.01)a	1.05 (0.03)b
Alkane				
23	1.67 (0.09)b	1.51 (0.03)b	2.10 (0.03)c	0.51 (0.18)a
Nitrile				
20	1.19 (0.03)b	1.59 (0.13)c	0.66 (0.14)a	1.27 (0.05)b

[a] a, b, c: means in a given row that are not marked or are marked with the same letter do not differ significantly from one other.

3-octanone (peak 12), and 2-ethyldimethylpyrazine (peak 17) were found in significantly higher concentration only in the urine of pregnant females. Lactating female urine contained only benzonitrile (peak 20) in significantly higher concentration than the urine of virgin and pregnant females.

The urine of virgin females had the lowest levels of almost all investigated volatiles. More than half of their ketones (peaks 1, 7, 9), all pyrazines (peaks 13, 14, 15, 17), and nitrile (peak 20) were significantly lower in concentration than in the urine of pregnant or lactating females (Table 2).

Volatile Profile of Male California Mouse Urine. A qualitative comparison of urinary profiles revealed that both males and females excreted the same urinary volatiles (Figure 1). The concentration of several ketones [3-pentanone (peak 1), 3-heptanone (peak 9), 2-heptanone (peak 10)] in virgin male urine was significantly higher than in the urine of virgin females, whereas the other ketone concentrations [3-methyl-2-hexanone (peak 7) and 3-octanone (peak 12)] did not differ between virgin males and virgin females (Table 2). Several ketone concentrations in male urine were similar to those in pregnant female urine (peaks 1, 9) and lactating female urine (peaks 1, 9, 12), whereas other ketones

were significantly lower than in pregnant female urine (peaks 7, 10, 12) (Table 1).

The first selected urinary alkane (peak 23) was the only compound in significantly lower concentration in male urine compared to the urine of all investigated females. Male urine did not contain a single compound in higher concentration than in the urine of any of the female groups.

DISCUSSION

A typical gas-chromatographic profile of California mouse urine contained relatively few constituents, around 40, as compared with the volatile-rich house mouse urine, which contains well over 100 compounds (Schwende et al., 1986). A similar low number of volatiles, about 30, was found in the urine of pine voles, *Microtus pinetorum* (Boyer et al., 1989).

Many of the compounds present in California mouse urine have been found in the urine of house mice and pine voles. 2-Heptanone, 2,5-dimethylpyrazine, and benzaldehyde are common to all three species. 3-Hexanone, 3-heptanone, 6-methyl-3-heptanone, and acetophenone are found both in house mouse and in California mouse urine. 2,3,5-Trimethylpyrazine and benzonitrile are present in California mouse and pine vole urine. Such characteristic male mouse urinary compounds as α- and β-farnesene, dehydro-*exo*-brevicomin, and 2-*sec*-4,5-dihydrothiazole, known to elicit territorial marking and male aggression, (Jemiolo et al., 1989; Novotny et al., 1990) were not found in the urine of male California mice. In addition, the urine of California mice did not contain any lactol derivatives that are typically found in pine vole urine (Boyer et al., 1989). Comparative behavioral studies are needed to determine the significance of these interspices differences in the urinary compounds.

Comparison of the urinary profiles from female and male California mice indicates that there are no identifiable female-specific or male-specific volatiles and that the concentration of urinary volatiles of pregnant/lactating females differs from that of virgin females. Pregnant and lactating female rodents typically ingest more food than virgin females or males (Kaczmarski, 1966; Migula, 1969; Stebbins, 1977; Marsteller and Lynch, 1987a,b). Thus, it is possible that the qualitative differences between pregnant/lactating female and virgin female urinary volatiles in the present study are the result of differences in the quantity of food intake and excretion of metabolites in urine. Although we cannot rule out this possibility, the fact that virgin males eat less than pregnant/lactating females and yet the concentration of most urinary volatiles of males was similar to that of pregnant and lactating females suggests that differences in food intake cannot alone account for the differences between groups in urinary volatiles.

Of particular interest is the finding that California mouse urine contained a relatively large number of pyrazines and in high concentrations in comparison to pyrazines in the urine of pine voles and house mice. Pyrazine derivatives accounted for 36% of all selected compounds in California mouse urine (in both sexes), whereas pyrazines accounted for only 8% of the constituents in pine vole urine (both sexes) (Boyer et al., 1989) and 4% of the compounds in house mouse urine (Andreolini et al., 1987).

In the house mouse, urine from group-housed females delays sexual maturation of juvenile females (Drickamer, 1977). 2,5-Dimethylpyrazine was present only in the urine of nonreproductive female house mice and at high concentrations in the urine of females kept in crowded conditions (Novotny et al., 1986). A solution of synthetic 2,5-dimethylpyrazine delayed first vaginal estrous (Novotny et al., 1986) and pubertal ovulation (Jemiolo and Novotny, 1994). In addition, long-term exposure of female mice to 2,5-dimethylpyrazine inhibited their overall reproductive fitness (Jemiolo and Novotny, 1993).

Pyrazines also may affect sexual maturation and reproduction in the California mouse. Both male and female California mice excreted in their urine as many as four (2,5-dimethylpyrazine, 2-ethyl-5-methylpyrazine, 2,3,5-trimethylpyrazine, and 2-ethyldimethylpyrazine) pyrazine derivatives in unusually high concentrations. However, in *P. californicus* both adult virgin females and mothers delay sexual maturation of juvenile females (Gubernick and Nordby, 1992), but lactating females and virgin females differ in their concentrations of pyrazines (this study). This suggests that pyrazines may be acting in concert with other urinary volatiles and/or nonvolatiles to delay puberty in *P. californicus*.

In *P. californicus*, a maternal urinary chemosignal maintains male paternal care postpartum, whereas virgin female urine does not maintain parental behavior (Gubernick and Alberts 1989; Gubernick 1990). Because lactating females differed from virgin females in most urinary ketones and all pyrazines, it is possible that these compounds acting alone or in combination may affect male maintenance of paternal behavior postpartum. However, the extent to which the specific volatiles identified in this study affect sexual maturation, paternal behavior, or other aspects of reproduction in the California mouse remains to be determined.

This study focused upon chemical characterization of the volatile compounds of *P. californicus* urine. However, the nonvolatile fraction of rodent urinary chemosignals may also affect various aspects of social and reproductive behavior (Vandenbergh 1983; Vandenbergh et al., 1976; Dluzen et al., 1992; Price and Vandenbergh 1992). Comparative biobehavioral analyses of volatile and nonvolatile fractions of urine both within and between species are needed to address the functional significance and evolution of rodent chemosignals.

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SEX PHEROMONE COMPONENTS OF THE SPRING HEMLOCK LOOPER, *Lambdina athasaria* (WALKER) (LEPIDOPTERA: GEOMETRIDAE)

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Abstract—Two methylated hydrocarbons, 7-methylheptadecane (7) and 7,11-dimethylheptadecane (7,11), are female sex pheromone components of the spring hemlock looper (SHL), *Lambdina athasaria* (Walker). Compounds extracted from female pheromone glands were identified by coupled gas chromatographic-electroantennographic detection (GC-EAD) and coupled GC-mass spectrometry (GC-MS) in selected ion monitoring mode. In field trapping experiments, (7) and (7,11) by themselves were behaviorally inactive, but in combination attracted numerous male moths. (5,11)-Dimethylheptadecane (5,11) was detected in female SHL pheromone gland extracts, but did not enhance attraction to the binary blend of (7) and (7,11). The sex pheromone of SHL is related to that of congeneric eastern hemlock looper (EHL), *Lambdina fiscellaria fiscellaria* (Guen.) [(5,11) and 2,5-dimethylheptadecane (2,5)] and western hemlock looper (WHL), *L.f. lugubrosa* (Hulst) [(5,11), (2,5) and (7)]. Specificity of the pheromonal blend, spatial separation of coseasonal EHL and WHL, and temporal separation of sympatric EHL and SHL contribute to reproductive isolation.

Key Words—Lepidoptera, Geometridae, *Lambdina athasaria*, *Lambdina fiscellaria fiscellaria*, *Lambdina fiscellaria lugubrosa*, sex pheromone, syner-

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[4]Dedicated to my father, Johannes Dinter, in honor of his 73th birthday.

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gism, 7,11-dimethylheptadecane, 7-methylheptadecane, 5,11-dimethylheptadecane.

INTRODUCTION

In northeastern America the spring hemlock looper (SHL), *Lambdina athasaria* (Walker), has sporadic outbreaks during which it feeds primarily on hemlock, *Tsuga canadensis* (L.) Carr. (Houser, 1927; Cameron and Mastro, 1975). Unlike the congeneric eastern hemlock looper (EHL), *Lambdina fiscellaria fiscellaria* (Guen.), and western hemlock looper (WHL), *L.f. lugubrosa* (Hulst), which both reproduce in the fall and overwinter as eggs, SHL flies in the spring and overwinters as a pupa (Maier et al., 1993). In 1992, SHL moderately to severely defoliated 5000 acres of hemlock forest in Connecticut and additional acreage in other New England states (Maier et al., 1993). The high-density populations present in Connecticut provided an opportunity to study the sex pheromone of this geometrid moth. We report the identification and field testing of sex pheromone components of SHL.

METHODS AND MATERIALS

Laboratory Analyses. Pupae were field collected at Devil's Hopyard State Park, near East Haddam, Middlesex County, Connecticut, and overwintered outdoors in screened boxes filled with hemlock forest duff. In February, pupae were sent to Simon Fraser University, placed into filter paper-lined Petri dishes and gradually warmed up (0–20°C at 1°C/day) over a period of 20 days under a 14L:10D photoperiod. Emergent moths were separated to avoid mating. Because mating of SHL females has been observed to occur around midnight (Maier, unpublished), pheromone glands of 1- to 2-day-old virgin females were removed 3–4 hr into the scotophase and extracted in hexane for 5 min. Aliquots of one female equivalent (1 FE) of pheromone extract were subjected to gas chromatographic analyses with both flame ionization and electroantennographic detection (GC-EAD) (Arn et al., 1975) on two fused silica columns (DB-210, DB-23, 30 m × 0.25 or 0.32 mm ID; J&W Scientific, Folsom, California). Male SHL antennae were also used in GC-EAD analyses of female EHL pheromone gland extracts, stored in our laboratory from previous research.

Coupled GC–mass spectrometry (GC-MS) (Hewlett Packard 5985B, fitted with a 30-m × 0.25-mm-ID, DB-210 coated column) in full-scan and selected ion monitoring (SIM) mode was conducted to confirm the presence of candidate pheromone components in gland extracts. For GC-MS-SIM, full-scan electron impact spectra of synthetic compounds were obtained to select ions indicative of methyl branch positions (Pomonis et al., 1980; Gries et al., 1993a,b). In

sequence, 200 pg of synthetic compounds, a hexane blank, and a concentrated pheromone gland extract (36 FE) were then chromatographed, scanning for the diagnostic ions.

Synthesis. Candidate pheromone components, 7-methylheptadecane (7), and 5,11-dimethylheptadecane (5,11) were synthesized as previously described (Gries et al., 1991a). For synthesis of candidate pheromone component 7,11-dimethylheptadecane (Scheme 1), practical grade glutaraldehyde **1** (Eastman Kodak Company, Rochester, New York) was dissolved in ether, the solution washed twice with saturated NaCl, and dried over anhydrous Na_2SO_4. Following ether evaporation, dried **1** was obtained by distillation under vacuum. Dried **1** in ether was then added at 0°C to an ether solution of three equivalents of hexyl magnesium bromide **2,** obtained from magnesium and 1-bromohexane. Normal workup of this reaction afforded 7,11-heptadecanediol **3,** which was dissolved in boiling hexane and crystallized out at room temperature (melting point: 112.8–114.0°C). Compound **3** was then oxidized with pyridinium chlorochromate (PCC) in CH_2Cl_2 to 7,11-heptadecanedione **4,** which was purified as above (melting point: 76.5–77.6°C). Addition of **4** at room temperature to a THF solution of methylenetriphenylphosphorane, obtained from methyltriphenylphosphonium bromide and butyl lithium, resulted in crude 7,11-dimethyleneheptadecane **5,** which was purified by silica gel column chromatography with hexane as eluent. Hydrogenation of **5** in hexane under H_2 with 5%Pd/C as catalyst yielded 7,11-dimethylheptadecane **6.** Yields of synthetic intermediates and final product approximated 80%. Structures of these compounds have not

OCH CHO (1) + MgBr (2)

1) Ether; 2) H^+ → HO, OH (3)

PCC, CH_2Cl_2 → O, O (4)

CH_2=PPh_3, THF → CH_2, CH_2 (5)

H_2, Pd/C → 7,11-dimethylheptadecane (6)

SCHEME 1. Scheme for the synthesis of 7,11-dimethylheptadecane

been previously reported and were fully consistent with their mass spectra (MS), nuclear magnetic resonance (^{1}H NMR), and infrared (IR) spectra. MS were obtained on a Hewlett Packard 5895B mass spectrometer equipped with a fused silica column (30 m × 0.25 mm ID) coated with DB-1. NMR spectra (Bruker WU-400 spectrometer) were taken in $CDCl_3$ at 400 MHz (J values in hertz). IR spectra were obtained from a Perkin–Elmer 599B spectrophotometer.

7,11-Heptadecanediol. EI-MS, m/z (% relative abundance): 229 (M^+-43, 5), 187 (10), 169 (100), 151 (45), 109 (25), 97 (25), 95 (55), 83 (25), 81 (25), 69 (25), 55 (30), 43 (15); ^{1}H NMR ($CDCl_3$): d: 3.61 (2H, m), 1.61–1.35 (14H, m), 1.28 (14H, m), 0.88 (6H, t, J = 7.0 Hz); IR (KBr): 3313, 2956, 2923, 2853, 1468, 1459, 1136, 1112, 1087, 1030, 905, 862 cm^{-1}.

7,11-Heptadecanedione. EI-MS, m/z (%): 268 (M^+, 5), 211 (11), 198 (48), 183 (45), 165 (10), 155 (65), 141 (50), 128 (100), 113 (60), 95 (15), 85 (30), 71 (15), 55 (20), 43 (30); ^{1}H NMR ($CDCl_3$): d: 2.42 (4H, t, J = 7.1), 2.37 (4H, t, J = 7.6), 1.82 (2H, p, J = 7.1), 1.54 (4H, m), 1.26 (12H, m), 0.87 (6H, t, J = 7.0 Hz); IR (KBr): 2955, 2932, 2850, 1699, 1470, 1452, 1421, 1385, 1287, 1250, 1128, 1075, 1005, 789, 722 cm^{-1}.

7,11-Dimethyleneheptadecane. EI-MS, m/z (%): 236 (M^+-28, 5), 179 (60), 166 (25), 151 (45), 109 (25), 95 (40), 81 (50), 68 (100), 55 (35), 43 (15), 41 (30); ^{1}H NMR ($CDCl_3$): d: 4.70 (4H, s), 2.00 (8H, t, J = 7.4), 1.55 (2H, m), 1.40 (4H, m), 1.28 (12H, m), 0.88 (6H, t, J = 7.0 Hz); IR (film): 3072, 2928, 2856, 1644, 1458, 1378, 889 cm^{-1}.

7,11-Dimethylheptadecane. EI-MS: Figure 2 below; ^{1}H NMR ($CDCl_3$): d: 1.26 (24H, m), 1.07 (4H, m), 0.88 (6H, t, J = 6.8 Hz), 0.84 (6H, d, J = 6.5 Hz); IR (film): 2956, 2924, 2855, 1462, 1377 cm^{-1}.

Each of racemic (7), (7,11) and (5,11) was >98% chemically pure. None of the chemical contaminants elicited antennal responses in GC-EAD recordings.

Field Bioassay. Field experiments (June 1–18, 1993) at Devil's Hopyard State Park, East Haddam, and Peoples State Forest, Barkhamsted, Litchfield County, Connecticut, were set up in randomized, complete blocks with traps and blocks at 15 to 20-m intervals. Green Unitraps (Phero Tech Inc., Delta, British Columbia, V4G 1E9) were suspended from hemlock trees 1.5–2 m above ground 2–5 m within the forest margin and baited with rubber septa (Sigma Chemical Co., St. Louis, Missouri) impregnated with candidate pheromone components in HPLC-grade hexane. A Dichlorvos (Bio-strip Inc., Reno, Nevada) cube was placed in the bottom of each trap to ensure rapid death of captured moths and potential insect predators. Captured males were recorded at the end of each experiment. The first experiment tested the three candidate pheromone components (7), (7,11), and (5,11) alone at 100 μg each and in ternary combination. The second experiment tested the three components in ternary and all binary combinations. A final experiment tested (7) and (7,11) alone and in binary combination versus virgin SHL females. One- to 2-day-old

virgin females kept in wire mesh cages below the lid of Dichlorvos-containing Unitraps were provided with a 10% sucrose solution in a 9 × 30-mm shell vial with cotton wick.

Statistical Analysis. Despite transformation, data of all three field experiments were not normally distributed and were therefore subjected to nonparametric analysis of variance by ranks (Friedman's test) (Zar, 1984; SAS/STAT User's Guide, 1988, release 6.03 edition, SAS Institute Inc., Cary, NC 27513).

RESULTS

GC-EAD analyses of female SHL pheromone gland extract revealed three EAD-active compounds (Figure 1). DB-23 column retention indices of compounds 1 (~50 pg/FE) and 3 (<1 pg/FE) were identical to those of (7) and (5,11). GC-EAD responses by male SHL antennae to female SHL pheromone extract, female EHL pheromone extract, and synthetic (7) and (5,11) coincided on DB-210 and DB-23 columns. The molecular structure of the second, strongly EAD-active compound (~10 pg/FE) was hypothesized to be (7,11) (Figure 2) based on its retention index and the methyl branching of candidate pheromone

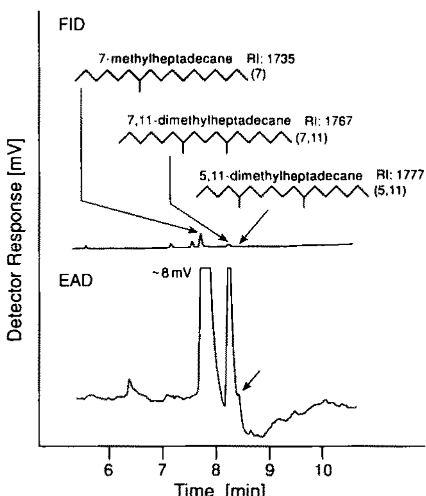

FIG. 1. Representative recording ($N = 9$) of flame ionization detector (FID) and electroantennographic detector (EAD) (male moth antenna) responses to one female equivalent of female *L. athasaria* pheromone gland extract. Antennal response to (5,11) was small but reproducible. Chromatography: splitless injection; injector temperature: 240°C; FID temperature: 240°C; DB-23 column; 1 min at 70°C, 20°C/min to 120°C, then 2°C to 200°C. RI = retention index.

components (7) and (5,11) (Figure 3). Ten picograms of synthetic (7) and (7,11) elicited very strong antennal responses (>5 mV each), whereas 10 pg of (5,11) was only moderately EAD-active.

GC-MS-SIM of pheromone gland extract and synthetic candidate pheromone components, scanning for ions indicative of methyl branch positions in

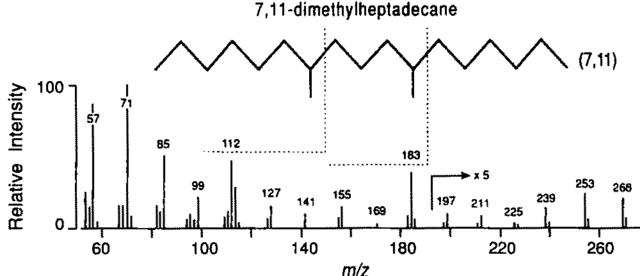

FIG. 2. Electron impact (70 eV) mass spectrum of synthetic 7,11-dimethylheptadecane (7,11). Ions *m/z* 112 and *m/z* 183 are diagnostic of methyl branch positions and were used for selected ion monitoring to confirm the presence of (7,11) in female pheromone gland extracts.

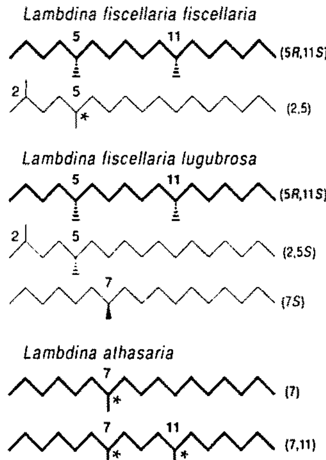

FIG. 3. Pheromone components of eastern hemlock looper (EHL), *Lambdina fiscellaria fiscellaria*, western hemlock looper (WHL), *Lambdina fiscellaria lugubrosa*, and spring hemlock looper (SHL), *Lambdina athasaria* (Gries et al., 1991a,b, 1993a; Li et al., 1993a,b). Boldface molecules are essential for attraction of males. *Pheromone chirality has not yet been determined.

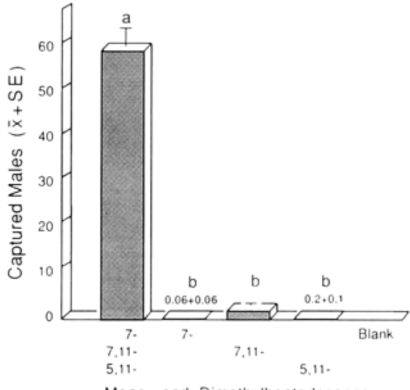

FIG. 4. Captures of SHL in Unitraps baited with candidate pheromone components alone at 100 μg each and in ternary combination (at 100 μg each). East Haddam, Connecticut, June 1-8, 1993; $N = 17$. Bars superscripted by the same letter are not significantly different, $P < 0.05$.

(7), (5,11), and (7,11), resulted in ion ratio and retention time matches of synthetic versus female-produced compounds: [synthetic (7): *m/z* 112 (100) and *m/z* 168 (83), gland extract: *m/z* 112 (100) and *m/z* 168 (81); synthetic (7,11): *m/z* 112 (100) and *m/z* 183 (94), gland extract: *m/z* 112 (100) and *m/z* 183 (97); synthetic (5,11): *m/z* 183 (100) and *m/z* 211 (65), gland extract: *m/z* 183 (100) and *m/z* 211 (67). These results confirmed the presence of (7), (7,11), and (5,11) in female SHL pheromone gland extracts.

Field-Trapping Experiments. The ternary blend of (7), (7,11), and (5,11) was most attractive, whereas none of the three compounds tested individually attracted significant numbers of male SHL (Figure 4). Compound (7) in combination with (7,11), but not with (5,11), was as attractive as the ternary blend (Figure 5). A final experiment confirmed synergistic attraction of (7) and (7,11) (Figure 6), but failed to demonstrate attraction of males to virgin females.

DISCUSSION

Sex pheromones of SHL, EHL, and WHL are related. All three *Lambdina* moths utilize a pheromone blend of mono- and/or dimethylheptadecanes (Figure 3). Compounds (5,11) and 2,5-dimethylheptadecane (2,5) comprise the sex pheromone of EHL (Gries et al., 1991a,b). The same two compounds plus (7) are sex pheromone components of WHL (Gries et al., 1993a), and (7) and (7,11) constitute the sex pheromone of SHL.

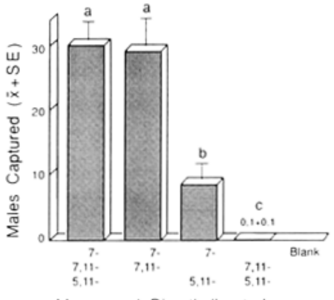

FIG. 5. Captures of SHL in Unitraps baited with candidate pheromone components (100 μg each) in ternary and all binary combinations. East Haddam, Connecticut, June 8–11, 1993; $N = 8$. Bars superscripted by the same letter are not significantly different, $P < 0.05$.

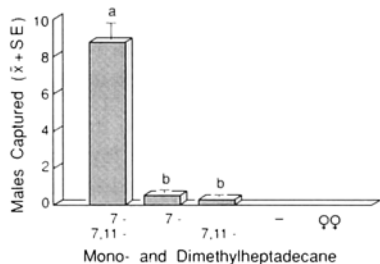

FIG. 6. Captures of SHL in Unitraps baited with candidate pheromone components (7) and (7,11) alone at 100 μg each, in binary combination, or with virgin females. Barkhamsted, Connecticut, June 15–18, 1993; $N = 10$. Bars superscripted by the same letter are not significantly different, $P < 0.05$.

Molecular structure and composition of pheromone components are similar in these three *Lambdina* moths, but sex pheromones of EHL and WHL are most closely related. Compound (5,11) is the major pheromone component of both EHL and WHL. The stereoisomer (5*R*, 11*S*) elicits antennal and behavioral responses in male EHL and WHL (Li et al., 1993a). Although present in female SHL pheromone gland extracts, (5,11) is apparently not a sex pheromone component in SHL. Compound (2,5) enhances attraction of (5,11) in both EHL and WHL, but has not been detected in pheromone glands of SHL. Compound (7) is a common, synergistic pheromone component in both WHL and SHL, but has different, species-specific functional roles. Compound (7) in combination with (7,11) is essential for attraction of males in SHL (Figure 6), whereas it enhances attraction to (5,11) in WHL.

In SHL, attraction of males resides with two compounds: (7) and (7,11) (Figures 5 and 6), but neither compound is attractive by itself (Figures 4 and 6). This type of interaction between pheromone components has rarely been demonstrated. (*Z*7,*Z*11)-Hexadecadienyl acetate and (*Z*7,*E*11)-hexadecadienyl acetate in combination attracted male pink bollworm, *Pectinophora gossypiella* (Saunders), but either diene acetate alone was not attractive (Hummel et al., 1973). A combination of (*Z*,*E*)-9,12-tetradecadien-1-ol and (*E*,*E*)-10,12-tetradecadien-1-ol strongly attracted male white rice stem borer, *Maliarpha separatella* Ragonot (from Sierra Leone), while either alcohol by itself was not attractive (Cork et al., 1991). Similarly, both enantiomers of (3Z,9Z)-*cis*-6,7-epoxy-eicosadiene attracted males of the noctuid *Bleptina caradrinalis* (Guen.), while either enantiomer alone was behaviorally inactive (Millar et al., 1991). Strong synergism in these moths is due to two geometrical or optical isomers of pheromone components. In SHL, the presence of two different molecules, a mono- and a dimethylheptadecane, is required for attraction of male moths.

Identification of (7,11) in SHL was based on retention index calculations of authentic methylated hydrocarbons. This previously unknown lepidopteran sex pheromone component eluted too late to be a monomethylheptadecane and too early to be a monomethyloctadecane. With a retention index similar to (5,11) and known methyl branches 2, 5, 7 and 11 in *Lambdina* sex pheromone components (Figure 3), the unknown was hypothesized to be (7,11). GC-MS of synthetic (7,11) revealed enhanced fragmentation ions *m/z* 112 and 183, indicative of methyl branch positions 7 and 11. In contrast to other mono- and dimethylated isomers, 7-methyloctadecane gave a mass spectrum similar to (7,11), but on a DB-210 column eluted >40 retention index units later than (7,11). Scanning for *m/z* 112 and 183 in GC-MS-SIM on a DB-210 column resulted in retention time and ion ratio matches of synthetic (7,11) and the female-produced compound. Comparable antennal activity of (coinciding) synthetic (7,11) and the female-produced compound further supported correct structural identification of the candidate pheromone component.

The two coeluting enantiomers and one *meso* compound of synthetic (7,11) and the two coeluting enantiomers of (7) did not allow determination of the optical isomer(s) eliciting antennal and behavioral responses. However, strong synergistic attraction in field experiments of synthetic (7,11) and (7) suggests that nonnatural isomers of pheromone components are behaviorally benign, as demonstrated in both EHL and WHL (Li et al., 1993a,b).

As in female EHL (Gries et al., 1991a,b) and WHL (Gries et al., 1993a), sex pheromone titers in female SHL pheromone glands were exceedingly small. Female EHL in Newfoundland and WHL in British Columbia weakly attracted conspecific males (Ostaff et al., 1974; Otvos, 1972). When tested in comparison to synthetic pheromone, however, female EHL (Gries et al., 1991a,b) and SHL (Figure 6) failed to attract males. Calling behavior of caged female SHL may

have been adversely affected by Dichlorvos on trap bottoms, but one virgin female in each of two adhesive, nonpoisonous delta traps (Phero Tech. Inc.), field tested apart from the synthetic pheromone experiment, also attracted only two males (0,2). Superior attraction of synthetic pheromone may therefore be attributed to release rates exceeding those of virgin females.

The structure and composition of sex pheromones of EHL, WHL, and SHL support taxonomic classification of these three geometrids as congeners. Spatial separation of coseasonal EHL and WHL, temporal separation of sympatric EHL and SHL, and specificity of pheromonal blends contribute to reproductive isolation.

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RELEASE OF ETHYLENE FROM PRUNED OLIVE LOGS: INFLUENCE ON ATTACK BY BARK BEETLES (COLEOPTERA, SCOLYTIDAE)

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Abstract—In recently pruned olive logs, an increase in ethylene release has been observed between 48 and 72 hr after pruning. The values reached, as well as the duration of ethylene release, varied greatly from one log to another. Pioneer *Phloeotribus scarabaeoides* females have shown a preference for logs in which ethylene emission was higher. In logs treated with ethrel, a significant increase in ethylene emission was observed, together with a greater period of release. Therefore, the use of logs treated with ethrel could be of great importance in the control of this pest of olive trees.

Key Words—Semiochemicals, kairomones, ethylene, ethrel, *Olea europaea*, olive tree, *Phloeotribus scarabaeoides*, Coleoptera, Scolytidae.

INTRODUCTION

Phloeotribus scarabaeoides (Coleoptera, Scolyitidae), the olive bark beetle, is a pest of olive trees and causes serious damage to certain areas of olive production (Russo, 1938). Olive bark beetles reproduce in pruned logs, and the next generation flies back to the olive trees to feed on the young frutiferous branches and to overwinter. The attraction of the logs has been explained by nonspecific emitted odors (Neuenschwander and Alexandrakis, 1982; Arambourg, 1986), which are detected by the pioneer females. Byers (1992) has indicated that the so-called "secondary" bark beetle species, those that colonize dying or decaying trees, use an aggregation pheromone less often, but rather are attracted to host volatiles.

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Ethylene is a plant hormone whose levels undergo various quantitative oscillations during the ontogeny of the different plant organs (Abeles, 1973; Sánchez Raya, 1983). In olive trees, the release of ethylene from leaves and fruits has been correlated and takes place even when environmental conditions produce stress situations in the plant (Sánchez Raya, 1990).

Apart from a normal situation, any additional stress in a plant, organ or tissue, due to harm or to such traumatic conditions as insect damage, extreme temperature variation, drought (Stumpff and Johnson, 1987), disease, mechanical or chemical treatment, can induce a sharp and quick increase of ethylene production. If the injury is extreme and the tissue dies, ethylene emission ceases (Abeles, 1973).

Ethylene and ethrel [2-(chloroethyl)phosphonic acid], a formulation that releases ethylene, have been shown to be attractive to both sexes of *P. scarabaeoides* in a laboratory bioassay with a glass olfactometer (Campos and Peña, 1994). In addition, an application of ethrel in the field induced a one-day advance in the attack of the olive bark beetles to the olive logs (González, 1990).

Ethylene is involved in some insect–plant relationships. It has been reported (Raina et al., 1992) that *Heliothis zea* females recognize the presence of ethylene, which in turn regulates insect pheromone production. Likewise, conifers seem to release ethylene together with monoterpenes (Popp and Johnson, 1990) as a defense mechanism against bark beetle attack and the fungi they vector.

For *P. scarabaeoides*, the olive bark beetle, which reproduces in olive logs due to pruning, it has been observed that some logs, among the huge number available, are preferred for gallery construction (González, 1990). The present paper deals with the role of ethylene in host choice, through the study of ethylene production in pruned olive logs, and its possible application in the integrated control of scolytids attacking olive trees.

METHODS AND MATERIALS

Apparatus. A Carlo Erba Fractovap 2350 gas chromatograph equipped with a flame ionization detector and a 2-m Porapack Q column was employed. Injector and column temperatures were 150 and 60°C, respectively.

Ethylene Measurements. In every case, logs with similar characteristics were selected: Marteño variety, mean length, 17.7 cm and mean diameter, 6.5 cm. Pruning dates are reported for each experiment. Logs were placed in the open air in conditions similar to those existing in the olive grove. Logs were introduced daily into glass bottles of 2.2-liter capacity, which had a hole in their top cover sealed with a rubber stopper. The bottles were tightly closed for 3 hr at room temperature (approximately 22°C). Then the rubber stopper was pierced with a gas-tight syringe and 3 ml were taken from the inner bottle atmosphere,

2 ml of which were injected in the gas chromatograph. Logs were then returned to the open air. Results were adjusted for the volume of each log. Injections were made once a day for each sample. With each set of samples, different ethylene standards at different concentrations were injected to correct the possible changes in the chromatographic runs. Ethylene was measured until its concentration was negligible. Results are expressed as picoliters of ethylene per gram of plant material per hour.

Ethylene Curve in Olive Logs. Logs pruned on April 6, 1993, were used. The first measurement was carried out the following day.

Ethylene Curve in Olive Logs Attacked by Phloeotribus scarabaeoides. Logs pruned on April 13, 1993, were placed in the olive grove and observed weekly until the bark beetle attacks had started, one week after the pruning. Five logs each, with and without bark beetle attack, were selected at random and brought to the laboratory to measure ethylene production.

Logs pruned on March 28, 1993, were placed in the olive grove until the attack by pioneer females was observed. This attack started two weeks afterwards in some of the logs. In this case, the pioneer beetles were still walking on the logs or just starting their reproduction galleries. Females were brushed off from three logs, which were brought to the lab to measure their ethylene release.

Barkless Log and Bark. To study the varying capacity for ethylene production, the logs with the bark removed and their corresponding bark, both divided longitudinally into small strips, were analyzed separately. Mean weight was 10.1 g for bark and 8.8 g for logs without bark. In this case, smaller glass vials with screw caps and septa of about 30 ml capacity were used. The vials were filled with either plant sample, and ethylene was determined following the same methodology described earlier. Pruning was done on June 1, 1993, and measurement was started the following day.

Ethylene Curve in Olive Logs Treated with Ethrel. Ethrel 48 is a formulation that contains a 48% aqueous solution of etephon, 2-(chloroethyl)phosphonic acid, which releases ethylene. Logs pruned on April 6, 1993, were sprayed to runoff on April 7, 1993, with a 1% solution of ethrel 48. Ethylene release was measured starting the day following this spraying.

Ethylene Released from Inactivated Logs. Six logs were placed in an oven at 60°C for six days to render them physiologically inactive. Three of them were used as controls and treated only with water. The other three were sprayed to runoff with a 1% solution of ethrel 48, and the ethylene release was measured as previously indicated. In addition, washed quartz sand, desiccated at 60°C, was also included as an inert support and added with the same solution of ethrel in approximately the same volume used to treat the logs (30 ml). Measurements were started the day following the spraying. Different ethylene production rates from logs and controls were compared using t tests.

RESULTS AND DISCUSSION

Ethylene Curve in Olive Logs. Logs that had been cut the preceding day produced ethylene for 22 days (Figure 1B) and after 48 hr, ethylene production was at its highest level, which could be attributed to the pruning itself. During the first week, the average ethylene release was eight times higher than during the rest of the measuring period. Ethylene production leveled off in the second and third weeks and decreased afterwards.

A very high variability in ethylene emission among the logs has been observed (Figure 1A) (mean relative standard deviation of 43%, ranging from 97 to 18%), even when the five logs were selected from a woodpile, according to criteria of maximal homogeneity (form, size, roughness, etc.). Therefore, this variability lay in the differences among the logs coming from different branches of different trees, which implies differences in water availability, phenology, orientation, number of secondary branches, nutrition, tissue hydration at the time of pruning, etc. Although only one injection was made in the chromatograph, previous analytical data were obtained with mean relative standard deviations of 4.7% for three injections.

Ethylene Curve in Olive Logs Attacked by Phloeotribus scarabaeoides. It has already been established that this scolytid is attracted to ethylene (Campos and Peña, 1994) and selects certain logs to start its reproduction galleries (González, 1990). Hence, differences in ethylene production in pruned logs with or without attack of *P. scarabaeoides* have been studied. Figure 2 shows that in logs in which bark beetle attack had taken place (average number of penetrations, 6/log), the ethylene production was higher and the period of ethylene release was extended. The difference was more evident in the first week ($P = 0.0502$, t test). These differences could be explained in two ways: First, the attacked logs may have been selected by the pioneer females due to an initially higher ethylene emission or, second, logs were selected at random and wounds produced by the maternal galleries would be responsible. In order to clear up this aspect, logs on which *P. scarabaeoides* females were walking and initiating galleries were brought to the laboratory, after brushing the beetles off. The values measured of approximately 2000 pl ethylene/g/hr (Figure 3) were very high, considering that two weeks had elapsed from the pruning date, when compared to the ethylene curve in Figure 1. These ethylene levels in the logs may play a decisive role in a first-attack phase in which the host plant is selected.

Barkless Log and Bark. Results have shown (Figure 4) that the log with the bark removed released more ethylene than the bark during the first two weeks, with a significant difference in the first week ($P < 0.01$). After the third week, the opposite occurred ($P < 0.01$). The division of bark and log without bark into strips to obtain pieces of similar size produced many wounds. This

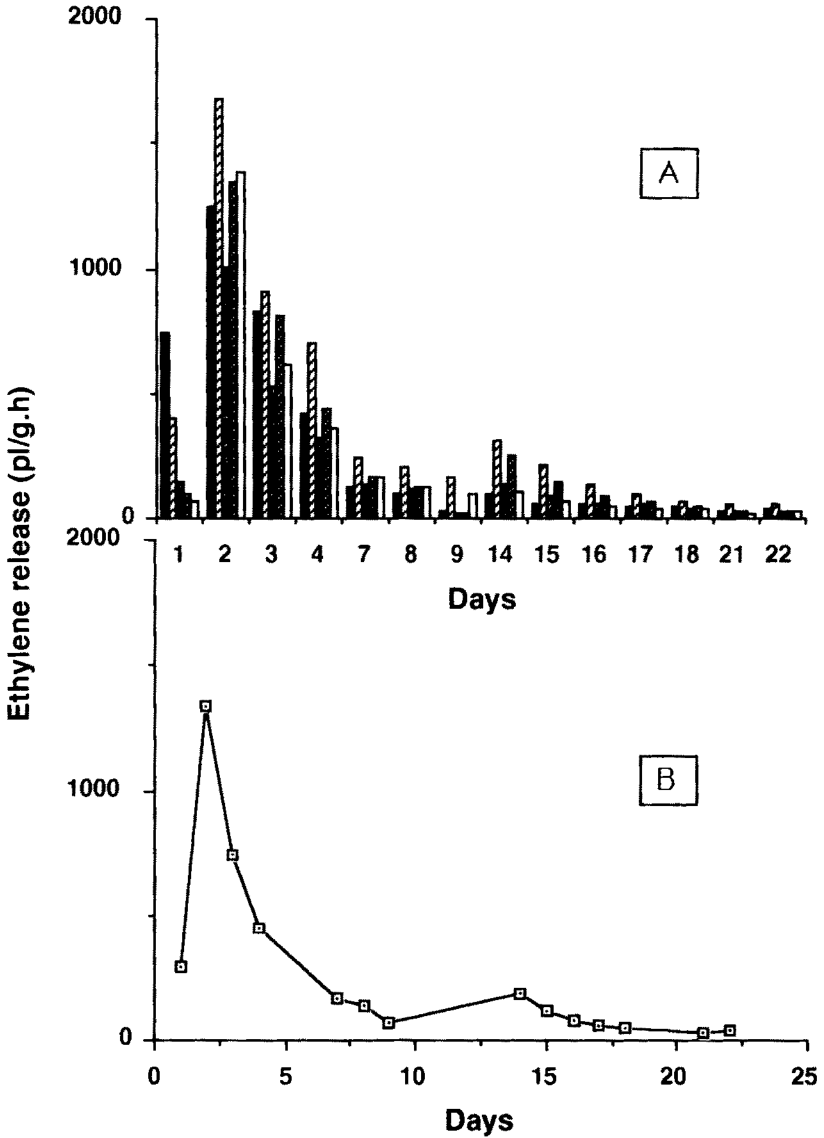

FIG. 1. Individual (A) and mean (B) ethylene release in olive logs recently pruned ($N = 5$).

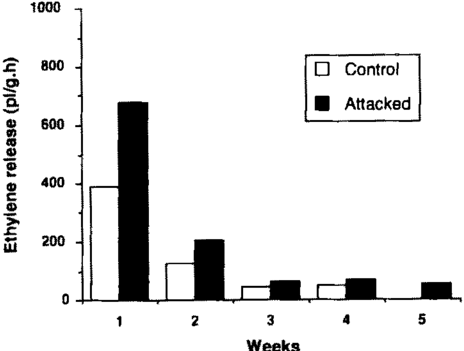

FIG. 2. Ethylene release in control logs and in logs in which attack by *Phloeotribus scarabaeoides* has already started ($N = 5$). For statistical significance see text.

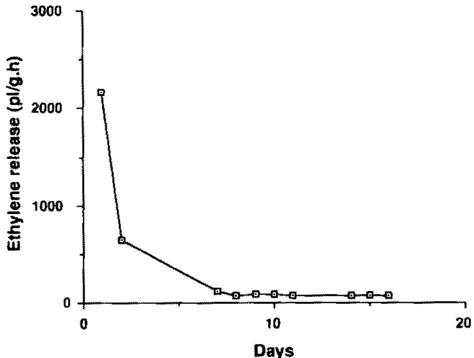

FIG. 3. Ethylene release in pruned olive logs in which attack by *Phloeotribus scarabaeoides* females has just started ($N = 3$).

explains the tremendous values of ethylene emission measured (ca. 35000 pl/g/hr), which declined very quickly.

Effect of Ethrel Treatment on Pruned Olive Logs. In the logs treated with ethrel, an enhancement of ethylene production during the first week (Figure 5) was observed [2.6 times higher than the control logs (Figure 1)], increasing up to 23 times higher than the control during the second week ($P < 0.01$). No inhibition of ethylene production in the first 48 hr was observed, as has been described for olive leaf and fruit (Goren et al., 1988). In addition, ethylene was released in control logs for about four weeks, while in the treated ones this period was extended to 14 weeks, which was significantly different from the control ($P < 0.01$).

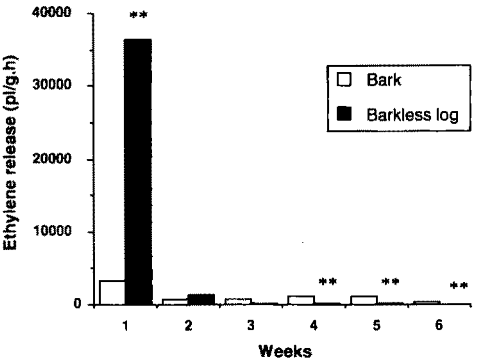

FIG. 4. Ethylene release in wood without bark and the corresponding bark of pruned olive logs ($N = 4$; t test; $^{**}P < 0.01$).

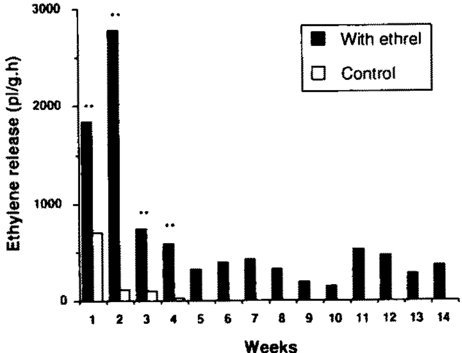

FIG. 5. Ethylene release in pruned olive logs sprayed with a 1% aqueous ethrel 48 solution ($N = 5$; t test, $^{**}P < 0.01$).

The enhancement of ethylene release in recently pruned logs sprayed with ethrel, together with the increase in the period of release, is a physiological response, since for inactive logs, as well as for the quartz sand (Table 1), treatment with ethrel does not manifest a noticeable ethylene release. This behavior cannot be explained as a slow release of an exogenous ethylene trapped in the wood tissue, as has been reported for olive fruit (Goren et al., 1988), but rather, as the authors themselves indicate for leaves, as an autoenhancement of ethylene production.

The higher attraction of the logs treated with ethrel to adult populations of *P. scarabaeoides* (González, 1990) could be partially explained by this increase, with values similar to those measured in logs in which bark beetle attack had

TABLE 1. ETHYLENE RELEASE BY PHYSIOLOGICALLY INACTIVATED LOGS (60°C) AND AN INERT SUPPORT

	Ethylene release (pl/g/hr)[a]
Control logs	67.5 ± 20.3
Treated logs	249.7 ± 90.7
Quartz sand	34.7 ± 22.8

[a] Mean release for three treatments and five measurements each ± standard deviation.

just begun. The results obtained point to the use of ethylene in the control of these scolytids using ethrel-treated woodpiles as a lure.

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SEQUESTRATION OF HOST-PLANT-DERIVED FLAVONOIDS BY LYCAENID BUTTERFLY *Polyommatus icarus*

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Abstract—Larvae of the lycaenid butterfly *Polyommatus icarus* were reared on inflorescences of *Coronilla varia* and *Medicago sativa*, which are rich in flavonoids. Twelve different flavonoids (five compounds from the former and nine from the latter), including aglycones and *O*-glycosides of kaempferol, quercetin, and myricetin were isolated and identified by spectroscopic means. NMR and MS data for the new acylated glycoside kaempferol 3-*O*-6″-(3-hydroxy-3-methylglutaroyl)-β-D-glucopyranoside are reported. Comparative HPLC analysis of the respective host plants and of larvae, pupae, and imagines of *P. icarus* indicated selective uptake and accumulation of kaempferol vs. quercetin and myricetin derivatives. The latter were excreted largely unchanged through the feces. Irrespective of the larval host plant kaempferol 3-*O*-glucoside was found as the major flavonoid in larvae, pupae, and imagines of *P. icarus*, accounting for approximately 83–92% of all soluble flavonoids in adult butterflies. Within the imagines, approximately 80% of all flavonoids are stored in the wings (especially in the orange submarginal lunules), whereas the remaining 20% reside in the bodies. Feeding experiments with artificial diet demonstrated that the insects are able to form kaempferol

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3-*O*-glucoside by glucosylation of dietary kaempferol. Possible functions of the sequestered flavonoids, especially for mate recognition of *P. icarus*, are discussed.

Key Words—*Polyommatus icarus*, Lepidoptera, Lycaenidae, *Coronilla varia*, *Medicago sativa*, Fabaceae, flavonoids, sequestration, plant–insect interactions.

INTRODUCTION

Since flavonoids are found ubiquitously in higher plants, any herbivorous insect will come in contact with these secondary compounds, at least during its larval stages, and, as such, flavonoids may cause a multitude of behavioral responses in insects. Flavonoids may be deterrent to herbivorous insects, as demonstrated for several methylated flavonol aglycones, such as quercetin 3,7-dimethyl ether, which is present as a constituent of the leaf resin of *Larrea tridentata* (Zygophyllaceae) and deters feeding by grasshoppers and geometrid moth larvae (Rhoades, 1977). In contrast, other flavonoids serve as insect attractants, such as morin and isoquercitrin, which help to attract the silkworm *Bombix mori* to its host plant *Morus nigra* (Hamamura et al., 1962; Harborne, 1991).

Butterflies from the families Lycaenidae, Papilionidae, and Nymphalidae, however, are exceptional in this context as they frequently sequester flavonoids (Morris and Thomson, 1963; Wilson, 1985, 1987; Harborne, 1991). Members of these families are active during daytime as adults, and it has been suggested that flavonoids may contribute to the wing patterns of these butterflies. Indeed, flavonoids are mainly, albeit not exclusively, allocated to the butterfly wings during development. As flavonoids absorb UV light, these plant-derived compounds could play a role in intraspecific visual communication. In a number of butterfly species (Silberglied, 1979, 1984), including some members of the family Lycaenidae (Douwes, 1976; Bernard and Remington, 1991), patterns of UV reflectance and/or absorbance are important in the location of conspecific males or females. In addition, UV-reflectance patterns are often highly species-specific and allow discrimination even among closely related species (e.g., Meyer-Rochow, 1991). Sequestration of flavonoids appears to be much less common in night-flying Lepidoptera ("moths") (Harborne, 1991), corroborating the hypothesis that flavonoids in butterflies may be related to visually mediated behavior.

Flavonoids sequestered by butterflies, however, have so far mainly been studied using imagines caught in the wild with uncertain feeding histories (e.g., Wilson, 1985, 1986, 1987). This is an obvious obstacle to any comparison of flavonoid profiles from butterflies and host plants. We have investigated the uptake and sequestration of flavonoids from different host plants by a European Lycaenid butterfly, *Polyommatus icarus* Rott., a species already known to con-

tain flavonoids as adult butterfly (Wilson, 1987). Lycaenid butterflies are suitable organisms for such a study as information on the host plant relationships is available for a large number of species (Fiedler, 1991).

Starting from the quantitative documentation of flavonoid patterns of two different legume host plants (*Coronilla varia* L. and *Medicago sativa* L.), we addressed the following questions: Which flavonoids are taken up by the caterpillars and which compounds are excreted with the feces? Are the flavonoids processed by the insects? Are there instar-specific differences between the flavonoid patterns of larvae, pupae, and adult butterflies? How do different host plants affect the flavonoid content and pattern of the butterflies? To which parts of the body do the insects allocate flavonoids? In this paper we prove for the first time a selective uptake of flavonoids from larval food sources by a lepidopteran and provide experimental evidence for a bioconversion of host plant flavonols in butterflies.

METHODS AND MATERIALS

Insects. All butterflies were reared from eggs laid by field-caught or laboratory-bred females (origin: Würzburg, northern Bavaria) of *P. icarus*. Eggs, larvae, and pupae were kept in an environmental chamber (25°C, 16L:8D). After hatching from the eggs, groups of caterpillars were transferred into closed translucent plastic vials (125 ml) lined with moist filter paper. Cut inflorescences of the host plants were used as larval food and were changed every one to two days. The filter paper was exchanged daily, and the frass was removed. Caterpillars were assigned to three food treatments. One group was reared on inflorescences of *Coronilla varia* L. (Fabaceae) from the eggs until pupation and another group was fed on inflorescences of *Medicago sativa* (Fabaceae).

A third group was initially reared on *M. sativa* inflorescences and was transferred to an artificial diet at the beginning of the third (prefinal) instar (fresh weight <10 mg). This transfer was necessary as first-instar larvae rejected the artificial diet. Performance of larvae on this diet was comparable to plant-fed control larvae, if the transfer to the diet took place in the third or fourth instar (Burghardt and Fiedler, unpublished results). The diet consisted of split peas, wheat germ agar, and a mixture of minerals and vitamins. Under the above conditions, larval development took an average of 20 days and the caterpillars pupated at fresh weights of 90 mg (on *M. sativa*) or 75 mg (on *C. varia* or on artificial diet), respectively.

Larvae of various sizes (mainly fourth = final instars), pupae, and adults were killed by freezing (−20°C). Larvae were kept without food for at least 2 hr prior to freezing to ensure that their alimentary canal was empty. Adults were frozen as soon as they had excreted their meconium (i.e., within 1–2 hr after

eclosion without access to any food). In addition, larval feces from several individuals were sampled, pooled, and stored at −20°C. Animals and frass samples were freeze-dried as soon as possible to prevent decomposition.

Food plants. Inflorescences of *M. sativa* and *C. varia* were collected and fed to the caterpillars as necessary. Both plant species serve as natural hosts of *P. icarus*. All plants originated from the same locality (Würzburg) throughout the study period. Samples of inflorescences, as well as of the artificial diet, were stored at −20°C for analysis.

Flavonoid Analysis. For preparative isolation of flavonoids, freeze-dried inflorescences of *C. varia* or *M. sativa* were ground and extracted with MeOH. The extract was concentrated and partitioned between H_2O/*n*-hexane, H_2O/ethyl acetate, and H_2O/*n*-butanol. All fractions were monitored for flavonoids by HPLC. Flavonoid-bearing fractions were subjected to column chromatography on Polyamide SC-6 (Macherey & Nagel, Germany, Düren) using H_2O, H_2O/MeOH (50:50), or MeOH as eluents followed by column chromatography on Sephadex LH-20 (Sigma, Germany, Deisenhofen) with MeOH as eluent. Where necessary the final purification was achieved by preparative TLC on cellulose (Macherey & Nagel) with a mixture of H_2O/acetic acid (85:15) as eluent. Detection was usually under $UV_{365\,nm}$. Following lyophilization insects (larvae, pupae and imagines) were weighed, extracted with MeOH, and directly subjected to HPLC analysis. Aliquots of artificial diet were also freeze-dried prior to extraction with MeOH.

The HPLC system was from Pharmacia (Sweden, Uppsala) equipped with a photodiode array detector (Waters, USA, Milford). Samples were injected on a Nova-Pak C_{18} column (Waters) (150 × 3.9 mm, 4 μm pore size) and separated using a linear gradient from 100% A (10% MeOH, 90% H_2O adjusted to pH 2 with orthophosphoric acid) to 100% B (MeOH) in 50 min. Flavonoids were quantified either by the internal or external standard method using kaempferol (Roth, Germany, Karlsruhe) as standard.

NMR spectra were recorded at room temperature on a Bruker AM 300 spectrometer and negative-ion fast atom bombardment mass spectra on Finnigan MAT 8430 mass spectrometer using glycerol as matrix: 5, ^{1}H NMR (CD_3OD) δ = 8.07 ["d," H-2′/6′, J(2′-3′) + (2′-5′) 8.9], 6.91 ["d," H-3′/5′], 6.45 [d, H-8, J(6-8) 1.8], 6.25 [d, H-6], 5.23 [d, H-1″, J(1″-2″) 7.3], 4.24 [dd, H-6″A, J(6″A-5″) 1.7, J(6″A-6″B) 12.0], 4.09 [dd, H-6″B, J(6″B-5″) 5.2], 3.50–3.30 [m, H-2″-5″], 2.52 [d, H-2‴A, J(2‴A-2‴B)15.5], 2.49 [s, H-4‴], 2.40 [d, H-2‴B], 1.24 [s, H-6‴]. ^{13}C NMR (CD_3OD) δ = 179.58 (s, C-4), 172.50 (s, C-1‴), 166.26 (s, C-7), 163.22, 161.82 (sx2, C-5, C-4′), 159.67, 158.68 (sx2, C-2, C-9), 135.51 (s, C-3), 132.47 (d, C-2′/6′), 122.86 (s, C-1′), 116.31 (d, C-3′/5′), 105.87 (s, C-10), 104.47 (d, C-1″), 100.21 (d, C-6), 95.08 (d, C-8), 78.07 (d, C-3″), 75.87, 75.80 (dx2, C-2″, C-5″), 71.45 (d, C-4″), 70.95 (s, C-3″), 64.44 (t, C-6″), 47.12, 46.80 (tx2, C-2‴, C-4‴), 27.85 (q, C-6‴).

The signal of C-5‴ was either broadened and occurred at 177.7, or overlapped with C-1‴ at 172.50 ppm. FAB MS: *m/z* 591 $[M-H]^-$.

RESULTS

Flavonoid Analysis of Flowers from Coronilla varia *and* Medicago sativa. Freeze-dried flowers of *C. varia* yielded five major flavonol glycosides including kaempferol 3-*O*-glucoside (**2**, Figure 1), kaempferol 3-*O*-galactoside (**3**), kaempferol 3-*O*-(6″-malonyl)glucoside (**4**), quercetin 3-*O*-glucoside (**9**), as well a quercetin 3-*O*-galactoside (**10**). All compounds were readily identified from their spectral data (UV, NMR, and MS), as well as from a comparison with published data. Kaempferol 3-*O*-glucoside (**2**) was present as the major flavonol, accounting for 47% of all flower flavonoids (total amount ca. 30 mg/g dry wt, Table 1), as indicated by HPLC analysis (Figure 2).

Nine different flavonoids were isolated from lyophilized flowers of *M. sativa* including kaempferol (**1**), **2**, and **4**, kaempferol 3-*O*-6″-(3-hydroxy-3-methylglutaryl)-β-D-glucoside (**5**), kaempferol 3, 7-di-*O*-glucoside (**6**), kaempferol 3-*O*-6″(malonyl)-glucoside, the 7-*O*-glucoside (**7**), quercetin (**8**), myricetin (**11**), as well as 3′-Me-myricetin (**12**) (Figure 1). Compound **5** was a new flavonoid derivative, even though the analogous quercetin derivative has been found previously (Wald et al., 1986). Kaempferol (**1**), as well as the kaempferol glycosides **4** and **5**, accounted for more than two thirds of all soluble flower flavonoids (total amounts ca. 5.6 mg/g dry wt, Table 2), as indicated by HPLC analysis (Figure 3).

Flavonoid Analysis of Insects Feeding on Flowers of Coronilla varia. HPLC analysis of feces from larvae of *P. icarus* (Figure 2) revealed a similar pattern of flavonoids as compared to the flowers of *C. varia* with compound **2** as the predominant constituent (ca. 57%) followed by **4** (ca. 24%) (Table 1).

Larvae were also found to contain the complete set of flower flavonoids, with **2** again being present as major component (Figure 2, Table 1). In addition to the host-plant-derived flavonols, kaempferol 3,7-*O*-diglucoside (**6**) was detected as a biotransformation product in larvae of *P. icarus* from a comparison (coelution and on-line UV absorbance) with authentic material isolated from flowers of *M. sativa*. Compound **4**, kaempferol 3-*O*-6″-(malonyl)-glucoside was only found as a minor constituent in the larvae, whereas it was present in considerable quantities in the larval feces (Figure 2).

HPLC analysis of pupae of *P. icarus* revealed a simplified flavonol pattern, compared to those of larvae or flowers of *C. varia* (Figure 2). Kaempferol 3-*O*-glucoside (**2**) formed the major flavonol of pupae (61.3%), whereas the quercetin glycosides **9** and **10** were detected only in minor amounts (7% in total) (Table 1). An additional flavonoid, which eluted between the quercetin glyco-

1 $R_{1,2}$ = H
2 R_1 = β-D-glucose, R_2 = H
3 R_1 = β-D-galactose, R_2 = H
4 R_1 = (6''-malonylglucoside), R_2 = H
5 R_1 = [structure], R_2 = H
6 $R_{1,2}$ = β-D-glucose
7 R_1 = (6''-malonylglucoside), R_2 = β-D-glucose

8 R = H
9 R = β-D-glucose
10 R = β-D-galactose

11 R = H
12 R = Me

FIG. 1. Structures of flavonoids identified in this study.

TABLE 1. DISTRIBUTION, CONCENTRATION, AND ABSOLUTE AMOUNTS OF FLAVONOIDS IN *P. icarus* AND IN LARVAL HOST PLANT *C. varia*

	Abundance of flavonoids (%) in					
					Imagines	
Compound	Flowers of *C. varia*	Larval feces	Larvae (N = 5)	Pupae (N = 4)	Female (N = 5)	Male (N = 4)
1		2.4				
2	47.2	57.6	42.2	61.3	83.0	85.0
3	14.7	3.9	1.3			
4	28.0	23.4	5.3			
6			18.4	tr[a]		
9	5.2	7.5	2.7	3.0	5.0	3.0
10	4.9	5.2	2.0	4.0	tr	tr
Unknowns			28.1	31.7	12.0	12.0
Conc. (mg/g dry wt)	30	ND	3.6 ± 0.1	2.6 ± 0.2	3.2 ± 0.5	2.7 ± 0.3
Absolute amounts (μg/individual)			52.0 ± 6.4	42.1 ± 4.9	45.0 ± 10.3	35.6 ± 6.5

[a] tr = trace amount detectable; ND = not determined; numbers of compounds refer to Figure 1.

sides (**9** and **10**) and the main constituent **2** (Figure 2) accounted for the remaining 31.7% of the soluble pupal flavonols (Table 1). Based on the on-line UV-absorption spectrum, this unknown pupal flavonoid was tentatively identified as an additional kaempferol glycoside that apparently originated from biotransformation within the insects, as it was not detected among the host-plant flavonoids (Figure 2).

Imagines of *P. icarus* showed the simplest flavonoid pattern, which was composed of only three compounds (Figure 2), with kaempferol 3-*O*-glucoside (**2**) as major constituent (83–85%) accompanied by minor amounts of quercetin 3-*O*-glucoside (**9**) and the unknown kaempferol glycoside already detected in pupae of *P. icarus* (Figure 2).

The mean amounts of flavonoids detected in larvae, pupae, and imagines of *P. icarus* ranged from ca. 52 μg/individual for larvae to ca. 35–45 μg/individual for pupae and imagines (Table 1). No pronounced qualitative or quantitative differences with regard to flavonoids were observed between male and female imagines (Table 1). The larger amounts of flavonoids present in larvae (when compared to pupae or imagines) may be due to host plant tissue remaining in the alimentary system of the larvae at the time of extraction.

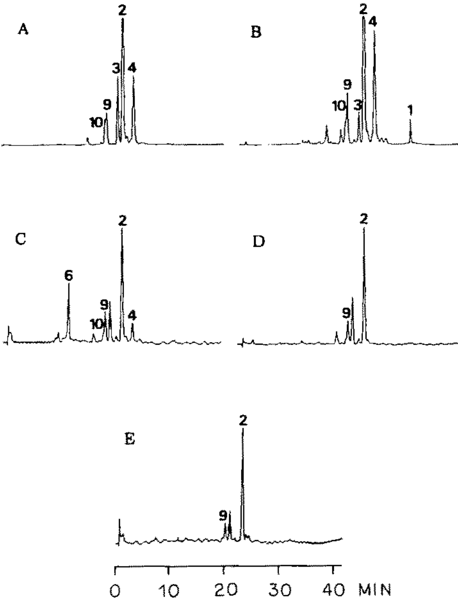

FIG. 2. Comparative HPLC analysis of flavonoids from flowers of *C. varia* (A) and from larval feces (B), larvae (C), pupae (D), and imagines (E) of *P. icarus*. Numbers of compounds follow Figure 1. The peak eluting in front of **2** was an unknown flavonoid metabolite originating through biotransformation by the insects.

Flavonoid Analysis of Insects Feeding on Flowers of Medicago sativa. HPLC analysis of feces from larvae feeding on flowers of *M. sativa* revealed a similar pattern of flavonoids to that of the flowers of *M. sativa* (Figure 3). The acylated kaempferol glucosides **4** and **5** formed the main constituents in both samples followed by kaempferol (**1**) (Table 2).

Larvae of *P. icarus* exhibited a simpler flavonoid pattern than that observed in the host plant or in the larval feces (Figure 3). Kaempferol 3-*O*-glucoside (**2**) formed the major constituent (60%), followed by the unknown kaempferol glycoside (Figure 3), that had already been detected as biotransformation product in specimens of *P. icarus* feeding on flowers of *C. varia* (Figure 2). Kaempferol 3,7,-*O*-diglucoside (**6**) was detected in minor amounts (3.4%) in larvae of *P. icarus* feeding on flowers of *M. sativa* (Figure 3). Quercetin or myricetin derivatives, that are present in flowers of *M. sativa*, were not detected in larvae of *P. icarus* (Figure 3).

Pupae revealed a simpler flavonoid pattern than larvae (Figure 3). The major pupal flavonoid was again kaempferol 3-*O*-glucoside (83%), followed by minor amounts of the unknown kaempferol glycoside (17%) (Figure 3). Kaemp-

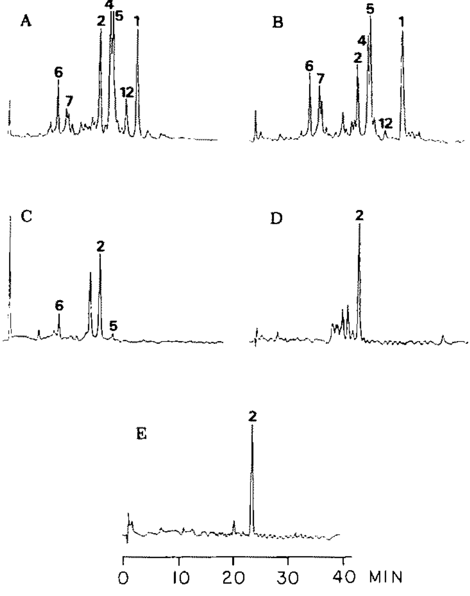

FIG. 3. Comparative HPLC analysis of flavonoids from flowers of *M. sativa* (A) and from larval feces (B), larvae (C), pupae (D), and imagines (E) of *P. icarus*. Numbers of compounds follow Figure 1. The peak eluting in front of **2** was an unknown flavonoid metabolite originating through biotransformation by the insects.

ferol 3,7-*O*-diglucoside **(6)** was not detected in pupae (Figure 3). Imagines of *P. icarus* contained almost exclusively kaempferol 3-*O*-glucoside **(2)** that amounted to 90–91.8% of all flavonoids extracted (Figure 3, Table 2).

The mean amounts of flavonoids (in micrograms per individual), extracted from specimens of *P. icarus* feeding on flowers of *M. sativa*, were 34.4 μg for larvae, 32.2 μg for pupae, and 34.5–42.6 μg for male and female imagines respectively (Table 2).

Female adults of *P. icarus* tended to have somewhat higher flavonoid concentrations (expressed in micrograms per individual or in milligrams per gram dry weight) than males (Tables 1 and 2), irrespective of the larval food source. However, the limited number of specimens available (usually $N = 5$) prohibited evaluation of the data statistically.

Localization of Flavonoids in Imagines of Polyommatus icarus. When imagines of *P. icarus* were dissected into wings and bodies and analyzed for flavonoids by HPLC, no differences were observed with regard to the flavonoid patterns. Kaempferol 3-*O*-glucoside **(2)** was the major flavonoid in the bodies as well as in the wings (data not shown). Quantitative measurements, however,

TABLE 2. DISTRIBUTION, CONCENTRATION, AND ABSOLUTE AMOUNTS OF FLAVONOIDS IN *P. icarus* AND IN LARVAL HOST PLANTS *M. sativa*

	Abundance of flavonoids (%) in					
					Imagines	
Compound	Flowers of *M. sativa*	Larval feces	Larvae (N = 6)	Pupae (N = 5)	Female (N = 5)	Male (N = 5)
1	21.6	25.8				
2	15.8	12.4	60.0	83.0	91.8	90.0
4	22.5	14.3	0.9			
5	23.1	18.7				
6	6.0	9.4	3.4	tr[a]		
7	4.8	8.9				
8	1.1	1.8				
11	1.9	2.2				
12	3.2	1.8				
Unknowns		4.7	35.7	17.0	8.2	10.0
Conc. (mg/g dry wt)	5.6	ND	2.4 ± 0.5	2.2 ± 0.2	3.0 ± 0.4	2.8 ± 0.3
Absolute amounts (μg/individual)			34.4 ± 8.9	32.2 ± 3.4	42.6 ± 9.3	34.5 ± 5.0

[a] tr = trace amount detectable; ND = not determined; numbers of compounds refer to Figure 1.

revealed pronounced differences in flavonoid concentrations of the bodies and wings (Table 3). Approximately 80% of the total flavonoids present in male or female imagines of *P. icarus* were found to be confined to the wings, whereas the remaining 20% resided in the bodies (Table 3). Within the wings, the highest concentrations of flavonoids (up to 118 ng/mm^2) were detected in the distinct orange submarginal lunules that account for approximately 10% of the total wing area (Figure 4, Table 4). The black-and-white colored discal, postdiscal, and submarginal ocelli, that account for 28–33% of the wing area, contained ca. 70–80 ng of flavonoids/mm^2. Both the orange lunules, as well as the black ocelli present on the undersides of wings, are strongly UV-absorbing when viewed under $UV_{365\,nm}$ (data not shown). The flavonoid concentration in the remaining parts of the wings (ca. 60% of the total wing area) was 34–49 ng/mm^2 (Figure 4, Table 4). Kaempferol 3-*O*-glucoside **(2)** formed the major flavonoid in all wing segments analyzed (data not shown).

Biotransformation of dietary kaempferol ***(1)*** *in* Polyommatus icarus. The artificial diet used for rearing larvae of *P. icarus* was devoid of any flavonols as indicated by HPLC analysis. Imagines (N = 3) originating from larvae reared

TABLE 3. QUANTITATIVE ANALYSIS OF FLAVONOIDS FROM BODIES AND WINGS OF MALE AND FEMALE IMAGINES OF *P. icarus*[a]

	Female		Male	
	Body	Wings	Body	Wings
Dry wt (mg)	11.3	2.4	9.4	2.7
Absolute amounts of flavonoids (μg)	7.5	27.4	5.2	22.3
Conc. of flavonoids (μg/mg dry wt)	0.6	9.7	0.5	6.9

[a] Imagines used for the experiment were from a culture reared on flowers of *M. sativa*. Data reported are means of three male and female imagines that were pooled for flavonoid analysis. No qualitative differences with regard to flavonoid patterns were observed for bodies and wings.

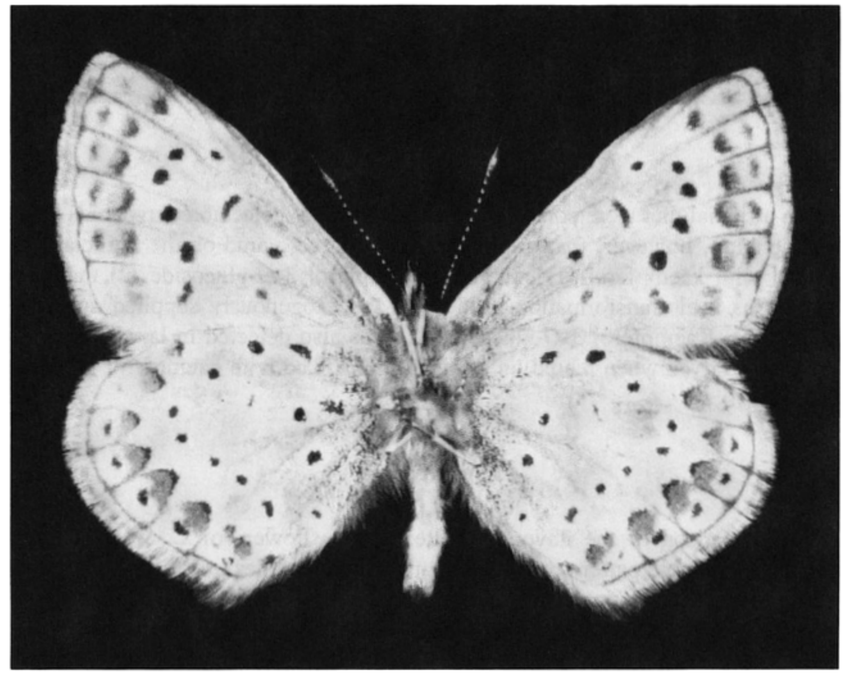

FIG. 4. Photographs of female (next page) and male (above) imago of *P. icarus* (viewed from the undersides).

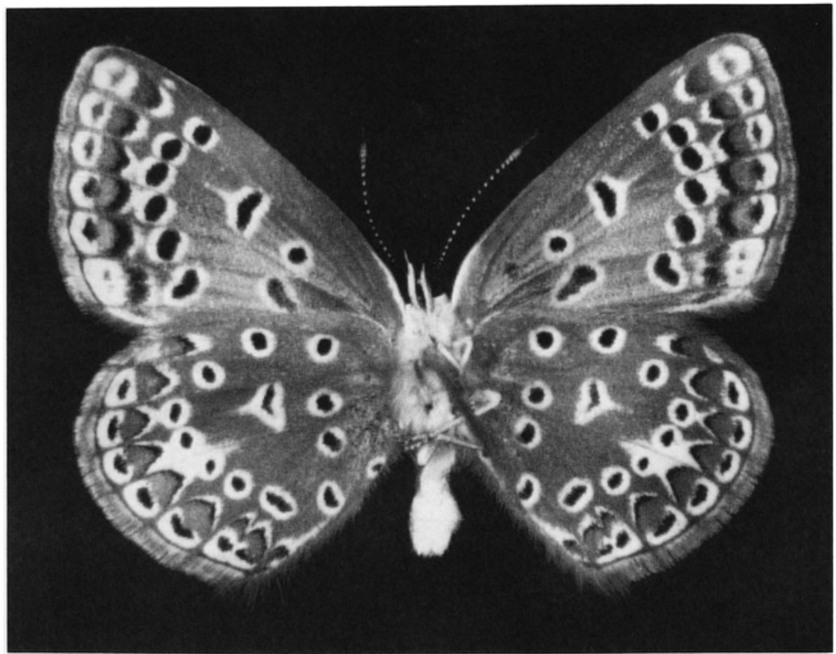

FIG. 4. Continued.

on an artificial diet were consequently devoid of any detectable flavonoids. One female adult, however, that originated from a larva reared on the artificial diet spiked with kaempferol **(1)** contained kaempferol 3-*O*-glucoside **(2)** that was formed as a biotransformation product of the exogenously supplied aglycone (Figure 5). Kaempferol 3-*O*-glucoside **(2)** was also detected in larval feces, as well as in larvae when reared on artificial diet spiked with kaempferol **(1)** (data not shown).

DISCUSSION

Comparison of the flavonoid patterns from flowers of *C. varia* and *M. sativa* to flavonoid patterns of larvae, pupae, and imagines of *P. icarus* indicates a selective sequestration of host plant flavonols of the kaempferol **(1)** type—especially kaempferol 3-*O*-glucoside **(2)**—vs. those of the quercetin **(8)** or myricetin **(11)** type (Figures 2 and 3). This selectivity is most striking for pupae and imagines that show almost superimposable flavonoid patterns (with compound **2** as major component) irrespective of the larval food plant (Figures 2

TABLE 4. DISTRIBUTION OF FLAVONOIDS IN DIFFERENT WING SEGMENTS OF MALE AND FEMALE IMAGINES OF *P. icarus*[a]

	Orange submarginal lunules		Wing segments (fore- and hindwings combined)			
			Black/white colored ocelli		Brown-colored area	
	Female	Male	Female	Male	Female	Male
Area of segment (%) compared to total wing area	10.0	6.0	28.0	33.0	62.0	61.0
Absolute amounts of flavonoids in different wing segments (ng/mm^2)	113.0	118.0	80.0	69.0	49.0	34.0
Distribution of flavonoids (in %) in different wing segments (total amount of flavonoids in the wings set at 100%)	47.0	53.0	33.0	31.0	20.0	17.0

[a]Wings of three male and female imagines from a culture of *P. icarus* reared on flowers of *M. sativa* were pooled for the analysis. Data reported are means from these individuals. For the assignment of the different wing segments analysed see Figure 4.

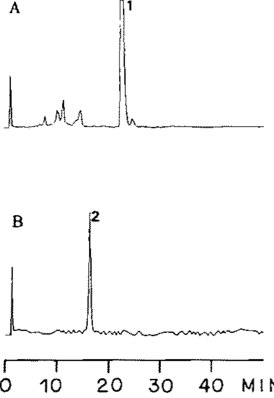

FIG. 5. HPLC profiles of artificial diet spiked with kaempferol (**1**) (A) and of imagines (B) originating from larvae reared on the treated diet. Numbers of compounds follow Figure 1.

and 3), whereas the flavonoid patterns of larvae still show a certain similarity to the pattern of the respective host plant (Figures 2 and 3).

The exact origin of the major pupal and imaginal flavonoid **2,** however, is still unclear. Flowers of *C. varia*, as well as those of *M. sativa*, are rich in kaempferol 3-*O*-glucoside **(2)** as well as in acylated derivatives of kaempferol 3-*O*-glucoside (e.g., **4** and **5**) (Figures 2 and 3), which may easily be converted into the precursor kaempferol 3-*O*-glucoside **(2)** by esterases within the larval gut (Ahmad et al., 1986). It is therefore possible that the insects sequester dietary kaempferol 3-*O*-glucoside. On the other hand, feeding experiments with artificial diet unambiguously proved the ability of the insects to sequester and to glucosylate the flavonol aglycone kaempferol **(1)** (Figure 5). Hence, it is possible that all or at least a significant portion of kaempferol 3-*O*-glucoside **(2)** detected in *P. icarus* arises through resorption and glucosylation of dietary kaempferol, which may be generated from various kaempferol glycosides through β-glucosidases in the larval gut (Ahmad et al., 1986). Feeding experiments with labeled precursors (e.g., kaempferol 3-*O*-glucoside labeled specifically in the glucose moiety) would be necessary to clarify whether the insects are able to sequester kaempferol 3-*O*-glucoside **(2)** or whether this compound is exclusively resynthesized by the insects following resorption of the aglycone. Glucosylation of dietary flavonol aglycones (e.g., quercetin) has recently also been proven for the band-winged grasshopper, *Dissoteira carolina* (Hopkins and Ahmad, 1991).

The demonstrated preference of *P. icarus* for kaempferol derivatives (Figures 2 and 3) is corroborated by a previous comparative study of the flavonoid patterns of wild imagines of *P. icarus* from Oxfordshire, England, and of several of their recorded larval host plants (Wilson, 1987). Despite the wide range of flavonoid aglycones (e.g., kaempferol, quercetin, apigenin, chrysoeriol, or genistein) present in host plants, such as *Lotus corniculatus*, *Medicago*, or *Trifolium* spp., the wild-caught imagines were found to contain mainly kaempferol 7-rhamnoside, kaempferol 3-rhamnoside, as well as isorhamnetin 3-glucoside as judged by the spot size on paper chromatograms (Wilson, 1987). Using our HPLC-method, we were able to detect kaempferol 3-O–glucoside **(2)** also in various wild-caught imagines of *P. icarus*, indicating that sequestration of this particular flavonol glycoside is not restricted to individuals that feed on flowers of *C. varia* or *M. sativa* but occurs also under natural conditions where the larvae have the choice between a plethora of suitable host plants (Fiedler, 1991).

Approximately 80% of all flavonoids present in male or female imagines of *P. icarus* analyzed in the study are accumulated in the wings (Table 3). The occurrence of relatively large quantities of flavonoids in clearly defined UV-absorbing areas (Table 4) on the undersides of wings (Figure 4) argues for a precise control of flavonoid deposition, as well as for specific functions of these pigments in *P. icarus*. Even though the biological roles of flavonoids sequestered by *P. icarus* are still unclear, it is possible that the distinct wing pattern of *P.*

icarus, which is composed of UV-absorbing as well as UV-reflecting areas, may be important for species recognition and mate selection (Silberglied, 1979, 1984). Support for this assumption is provided by early observations of Ford (1941) that flavonoids are usually found only in day-flying Lepidoptera. Investigations to elucidate the role of flavonoid pigments in *P. icarus* are planned.

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HOMARINE AS A FEEDING DETERRENT IN COMMON SHALLOW-WATER ANTARCTIC LAMELLARIAN GASTROPOD *Marseniopsis mollis*: A RARE EXAMPLE OF CHEMICAL DEFENSE IN A MARINE PROSOBRANCH

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Abstract—The common bright yellow antarctic lamellarian gastropod *Marseniopsis mollis* was examined for the presence of defensive chemistry. Proton nuclear magnetic resonance (NMR) spectroscopy indicated that a major component of ethanolic extracts purified by reversed-phase column chromatography was homarine. Further high-performance liquid chromatography (HPLC) analysis of the mantle, foot, and viscera verified the presence of homarine in all body tissues at concentrations ranging from 6 to 24 mg/g dry tissue. A conspicuous macroinvertebrate predator of the shallow antarctic benthos, the sea star *Odontaster validus*, always rejected live individuals of *M. mollis*, while readily feeding on pieces of fish tail muscle. Filter paper disks treated with shrimp elicited a broad range of feeding behaviors in the sea star *O. validus* (movement of disc to mouth, extrusion of cardiac stomach, humped feeding posture). Shrimp disks treated with homarine (0.4 and 4 mg/disk) were rejected by *O. validus* significantly more frequently than control disks treated with solvent carrier and shrimp or shrimp alone. The highest concentration of homarine tested not only caused feeding deterrence, but in several sea stars a flight response was noted. Homarine was not detected in the tunic

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of the antarctic ascidian *Cnemidocarpa verrucosa*, a presumed primary prey of *M. mollis*. Nonetheless, crude extracts of the epizooites that foul the tunic (primarily the bryozoans and hydroids) contain homarine, suggesting *M. mollis* may ingest and derive its chemistry from these organisms. This appears to be only the third example of chemical defense in a member of the Order Mesogastropoda. As the vestigial internalized shell of *M. mollis* is considered a primitive condition, the findings of this study lend support to the hypothesis that chemical defense evolved prior to shell loss in shell-less gastropods.

Key Words—*Marseniopis mollis*, Mesogastropoda, homarine, defense, sea star, *Odontaster validus*.

INTRODUCTION

Below the zone of ice scour and anchor ice (33 m depth) (Dayton et al., 1969), the shallow antarctic benthos is characterized as environmentally stable and the abundant faunal community considered to be structured primarily by biological factors such as competition and predation (Dayton et al., 1974). Such conditions are appropriate for the evolution of chemical defense mechanisms. In contrast to temperate and especially tropical marine benthos (Bakus et al., 1986), the most conspicuous predators of benthic macroinvertebrates in the antarctic benthos are not fish (Eastman, 1993), but rather other macroinvertebrates such as sea stars (Dayton et al., 1974; Dearborn, 1977; McClintock, 1994). A number of studies have recently examined the chemically mediated feeding-deterrent properties of a diverse group of antarctic marine invertebrates including nemerteans (Heine et al., 1990), brachiopods (McClintock et al., 1993), echinoderms (McClintock, 1989), soft corals (Slattery et al., 1990), sponges (McClintock et al., 1994), tunicates (McClintock et al., 1992a), and gastropods (McClintock and Janssen, 1990; McClintock et al., 1992b). Relatively few studies have investigated aspects of the secondary metabolite chemistry of antarctic marine invertebrates (Eggersdorfer et al., 1982; Seldes et al., 1986; Molinski and Faulkner, 1987, 1988; Davies-Coleman and Faulkner, 1991; Blunt et al., 1990; Kong et al., 1992; Baker et al., 1993; Perry et al., 1994; Trimurtulu et al., 1994).

The antarctic lamellarian gastropod *Marseniopsis mollis* is abundant in shallow (>33 m depth) coastal waters of Ross Island, McMurdo Sound, Antarctica (Dayton et al., 1974). As in all lamellarians, the shell is vestigial and internalized, and the conspicuous bright yellow mantle tissues are exposed to potential predators. Whether this gastropod is chemically defended is of particular evolutionary significance, as it would lend support to the theory postulated by Faulkner and Ghiselin (1983) that chemical defense in "shell-less" gastropods is a preadaptation to shell loss, which permitted individuals to survive without a shell. In a detailed analysis of the macroinvertebrate predators of

McMurdo Sound, Dayton et al. (1974) noted that *M. mollis* appears to be the only predator of the large solitary ascidian *Cnemidocarpa verrucosa*. Moreover, several individuals were noted to bore in to the tunic of *C. verrucosa* to deposit their eggs. Although there are a number of potential predators of *M. mollis*, including sea stars and sea anemones, no reports of predation on this lamellarian have been made (Dayton et al., 1974; Slattery and Heine, personal observation).

The purpose of the present study was to determine whether the conspicuous antarctic mesogastropod *M. mollis* contains secondary metabolites that serve as defensive compounds. Moreover, an examination of the chemistry of the tunic and the associated epizooites on the tunic surface of the ascidian *C. verrucosa* provided an opportunity to assess whether dietary derivation of defensive chemistry may occur in this marine prosobranch.

METHODS AND MATERIALS

Chemical Analysis. Marseniopsis mollis were collected from the shallow benthos near McMurdo Station, Antarctica between 30 and 35 m depth in October–December 1992. Thirty individuals were lyophilized and then exhaustively extracted in ethanol. Partially purified homarine (Figure 1) was obtained by reversed-phase vacuum chromatography eluted with water, yielding 60 g of salts and other water-soluble compounds. The ^{1}H NMR spectrum indicated the presence of homarine, which was confirmed by HPLC analysis (10 mm × 20 cm Waters μbondapak RCM, 0.04 M KH_2PO_4, pH 3.0, 2.0 ml/min) in comparison to synthetically prepared homarine (see below).

In an attempt to deduce body components in which homarine is sequestered, three individuals of *M. mollis* collected in October 1993 from near McMurdo Station were dissected into the viscera (all internal organs), mantle, and foot. Body components were pooled and extracted thrice for 24 hr in MeOH and the combined extracts concentrated. Homarine in the extracts was quantified by HPLC as above.

The reported food item of *M. mollis*, the solitary antarctic ascidian *C. verrucosa*, was investigated for the presence of homarine to establish the presence or absence of dietary derivation of chemical protection. The tunics of three individuals were lyophilized and cleaned of fouling organisms, then collectively

+N CO₂
CH₃

FIG. 1. Structure of homarine.

extracted thrice in MeOH for 24 hr each. The extract was concentrated and analyzed by ^{1}H NMR for the presence of homarine.

C. verrucosa is significantly fouled by a complex community of bryozoa, hydroids, and other epibionts, leading to the speculation that *M. mollis* may well be grazing these epibionts rather than feeding directly on the tunic. The epibionts removed from the tunic of the above specimens of *C. verrucosa* were extracted thrice in MeOH for 24 hr each, and these combined and concentrated extracts analyzed by ^{1}H NMR for the presence of homarine, which was quantified by NMR by integration of the signal before and after addition of a standard solution of synthetic homarine.

Homarine was prepared from picolinic acid (Aldrich) for use as a standard. Picolinic acid (1.23 g, 0.001 mol) was refluxed overnight in ethanol with 2.82 g (0.02 mol) methyl iodide. Homarine was recrystallized from methanol to yield 1.28 g (0.93 mol, 93%).

Feeding-Deterrent Analysis. The sea star *Odantaster validus* is a conspicuous predator of sessile and mobile macroinvertebrates in McMurdo Sound (Pearse, 1965, 1969; Dayton et al., 1974; McClintock, 1994). *O. validus* moves rapidly enough to capture the sluggish *M. mollis* (McClintock, laboratory observations) and has been observed to include some gastropods in its omnivorous diet (Pearse, 1965, 1969). No observations of predation on *M. mollis* by *O. validus* have been made (Dayton et al., 1974; Slattery and Heine, personal observation). Individuals of *M. mollis* (mean $\pm$ 1 SD = 5.6 $\pm$ 1.5 cm; N = 3) and *O. validus* (mean $\pm$ 1 SD R = 44.5 $\pm$ 5.3 mm; N = 30) were collected either from Hut Point or Danger Slopes in September and October 1993. The lamellarian gastropods were held in a shallow (10 cm depth) sea water table with ambient running sea water pumped directly from the sea (−1.5°C). Sea stars were held in a large circular tank (2 m diameter) also equipped with running sea water.

In one series of feeding assays, an individual of *O. validus* was either placed directly on top of the dorsum of an individual *M. mollis*, or alternatively, on top of a 2 × 2 × 2-cm piece of tail muscle of the antarctic pup fish *Rhigophila dearborni*. The time each sea star remained in contact with the lamellarian gastropod or fish tissue, as well as any stereotypic feeding behavior, were recorded (N = 10 trials for gastropods and fish, respectively). Each sea star was tested only once, while the three lamellarian gastropods were repeatedly rotated through the assays. The order of presentation of gastropods or fish was haphazard.

The feeding deterrent characteristics of homarine were examined in a second series of feeding assays in the antarctic sea star *O. validus*, perhaps one of the most likely potential predators of *M. mollis*. Individuals employed in the assay were those found on the sides of the tank at the air–water interface, each with the oral sides of one or two arms extended parallel to the water surface.

Circular filter paper disks (5 mm diameter BBL Blank Paper Discs; 10 mg dry wt, 50 mg wet wt) were soaked 24 hr in a 1 : 6 w/v ratio of ground dried shrimp mixed with sea water. Disks were dried under air. Preliminary trials indicated that *O. validus* showed stereotypic feeding behaviors when presented shrimp-treated disks (movement of disk towards the mouth, extrusion of the cardiac stomach, humped feeding posture). Experimental shrimp disks were prepared at one of three concentrations of homarine (0.04, 0.4, and 4 mg/disk using 20 μl methanol as a carrier). Homarine employed in these bioassays was obtained by synthesis in the laboratory (see above). Controls consisted of disks treated with 20 μl of methanol and shrimp or shrimp alone. Ten replicate assays per treatment were conducted by placing a disk equidistant between the arm tip and oral opening of an individual *O. validus* and observing the response over a 60-min period. When individuals dropped a disk, it was considered a rejection response. In instances where the disk was carried to the oral opening by the tube feet, the time required to do was recorded. Disks were considered accepted when an individual showed stereotypic feeding behaviors. Disks were presented in a haphazard sequence and the 30 sea stars were never tested more than once per day. No individual sea star was employed more than twice over the duration of all the feeding assays, preventing any possibility of a conditioned response.

All experiments were statistically analyzed using a contingency analysis. Acceptable probability levels were calculated using the Bonferroni adjusted probability correction to avoid a type II error. Comparisons were considered statistically significant if alpha was less than or equal to 0.05.

RESULTS

Chemical Analysis. The presence of homarine was verified using different extraction and isolation techniques on individuals collected in both the austral summers of 1992 and 1993. The results of HPLC analysis of individuals dissected into the primary body components indicated that the mantle, foot, and viscera contained 6.3, 23.8, and 11.3 mg homarine/g dry tissue, respectively. When calculated on a wet tissue weight basis for each body component (wet-dry tissue ratios taken from McClintock et al., 1992a), values were determined to be 0.52, 2.91, and 2.37 mg/g wet tissue for the mantle, foot, and viscera, respectively.

Analysis of homarine in three pooled specimens of *C. verrucosa* (51.7 g dry weight) and their pooled epibionts (9.7 g dry wt) revealed homarine only in the epibionts. Quantification by NMR revealed homarine to constitute 7.5% of the methanol extract on a dry weight basis, or 1.7% of the organism dry weight.

Feeding Deterrent Analysis. The sea star *O. validus* rejected individual *M.*

mollis significantly ($P < 0.05$) more frequently than similarly presented pieces of fish tail muscle. All sea stars placed onto fish pieces remained in a feeding posture for the entire 60-min period. In contrast, all sea stars moved off of *M. mollis*, with a mean ± 1 SD time spent in contact of 12.45 ± 5.39 min (N = 10).

O. validus presented with shrimp-treated filter paper disks dosed with three concentrations of homarine showed significant rates of rejection when compared to disks treated with shrimp and solvent carrier or shrimp alone ($P < 0.05$; Figure 2). Significant feeding deterrence was detected for disks containing 4 mg ($P < 0.05$) and 0.4 mg ($P < 0.05$) homarine, but not for those containing 0.04 mg homarine ($P = 0.1967$). There was no significant difference between levels of rejection between those shrimp disks containing 4 and 0.4 mg homarine ($P = 0.0603$), but there was a significant decrease in levels of rejection when comparing shrimp disks containing 4 and 0.04 mg homarine ($P < 0.05$).

DISCUSSION

The antarctic lamellarian gastropod *Marseniopsis mollis* appears to be chemically defended. When the common sea star *Odontaster validus*, a voracious omnivorous predator (Pearse, 1965; Dearborn, 1977; McClintock, 1994), is placed on to the dorsum of *M. mollis*, it invariably results in the movement of the sea star off the gastropod. This sea star is quite capable of capturing and feeding upon this gastropod. *M. mollis* contacted by *O. validus* display a "defensive" mushrooming of the mantle tissue, with the edge of the mantle pushed down to the substrate to provide a seal against access to the soft visceral tissues.

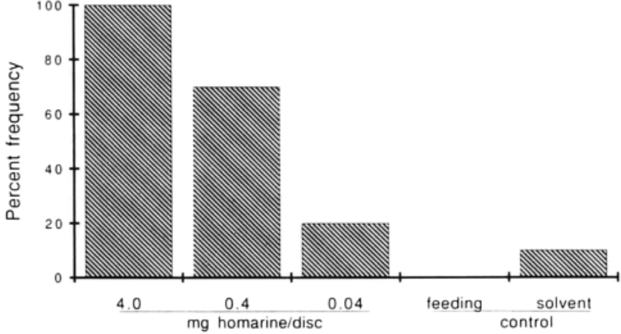

FIG. 2. Percent frequency of rejection of shrimp disks treated with three concentrations of homarine and presented to the antarctic sea star *Odontaster validus*. Also presented are the rejection frequencies for feeding (shrimp alone) and solvent (shrimp with methanol carrier alone) controls. N = 10 for all treatments.

Although flight is an expected molluscan response to contact with a predatory sea star (Bullock, 1953; Feder, 1963; Phillips, 1976), *M. mollis* never moved rapidly away from *O. validus*, in most instances clamping down against the substrate and remaining immobile until the sea star had moved off its dorsal mantle. Such a behavioral response is consistent with a chemical means of defense.

The common antarctic sea anemone *Urticiniopsis antarcticus* is also a likely predator of mobile gastropods such as *M. mollis*. Individual anemones have been observed ingesting echinoderms and medusae that brush against the sticky tentacles (Dayton et al., 1974). Preliminary assays employing a single sea anemone suggest that when *M. mollis* is placed onto the tentacular crown of *U. antarcticus*, it elicits a rapid retraction of the tentacles (McClintock, personal observation). However, rather than the retracted tentacles engulfing the gastropod, as when fish pieces are presented, the tentacles push the gastropod upwards and eventually onto the tentacular crown. Although gastropods sometimes remain in this position for several minutes, ultimately the sea anemone leans strongly to one side and drops the gastropod to the substratum (McClintock, personal observation).

The present study expands the list of antarctic mollusks shown to possess chemical means of defense and is the first example in an antarctic lamellarian. The mantle tissues of the antarctic dorid nudibranch *Austrodoris kerguelensis* are rejected by several species of antarctic fish, while crude mantle homogenates cause tube-foot retractions in four antarctic sea stars (McClintock et al., 1992a). Davies-Coleman and Faulkner (1991) found *A. kerguelensis* contains a series of bioactive acid glycerides, which they suggest are produced de novo. Another dorid nudibranch, *Tritoniella belli*, has mantle tissues that cause feeding deterrence in antarctic fish and sea stars (McClintock et al., 1992a), and chimyl alcohol has been suggested to contribute to its defensive chemistry, perhaps derived from its soft coral diet (McClintock et al., in press). The pelagic antarctic pteropod *Clione antarctica* has also been shown to be chemically defended from fish predation (McClintock and Janssen, 1990). The compound specifically responsible for fish feeding deterrence in this pteropod (pteroenone) has recently been determined to be a hydroxyketone, which is likely produced de novo (Bryan et al., submitted; Yoshida, Bryan, Baker, and McClintock, in preparation).

Homarine induced feeding deterrence in the antarctic sea star *O. validus*. This water-soluble compound occurs in significant quantities in *M. mollis*. With the exception of a yellow pigment, extractions and subsequent chemical analyses did not indicate the presence of other secondary metabolites in *M. mollis*. It is possible that additional bioactive compounds exist, undetected due to their unstable nature or trace quantity. Homarine has been found in a wide diversity of marine invertebrates (Gasteiger et al., 1955; Baker and Murphy, 1976) and has been suggested to serve as an osmolyte (Gasteiger et al., 1955). Additionally,

homarine has been isolated from the gorgonians *Leptogorgia virgulata* and *L. setacea* and demonstrated to have chemical bioactivity, significantly inhibiting the growth of the potentially fouling benthic marine pennate diatom *Navicula salinicola* at naturally occurring concentrations (Targett et al., 1983). We found homarine-treated shrimp disks to cause significant feeding deterrence at concentrations of 0.4 and 4 mg homarine/50 mg wet shrimp disk (8 and 80 mg/g wet shrimp disk). These concentrations are similar to the range of natural concentrations of homarine isolated from body tissues (0.52 to 2.91 mg/g wet tissue weight). Sea star feeding-deterrent responses to homarine-treated disks are conservative, as some percentage of this water-soluble compound diffused off the shrimp disk once placed in sea water during each experimental feeding trial. Moreover, as many shell-less gastropods such as nudibranchs sequester their defensive chemistry in the epithelial tissues or in specialized glands or mucus (Cimino et al., 1982; Thompson et al., 1982; Karuso, 1987), it is possible that *M. mollis* similarly concentrates its defensive chemistry.

Homarine occurred in all the primary body tissues of *M. mollis* (mantle, foot, and viscera). Although pooling of body components prevented an analysis of individual variability, pooled levels of homarine, when compared on a per unit wet tissue basis, were approximately 4.5 times higher in the viscera and 5.5 times higher in the foot than in the mantle. This relationship changes considerably when homarine levels are calculated as a function of dry weight with levels in the gut (11.3 mg/g dry wt) only twice as high as in the mantle (6.3 mg/g dry wt), and only half as high in the foot (23.8 mg/g dry wt). In sea hares, there is evidence that dietary secondary metabolites occur only in the viscera, and therefore do not serve defensive roles (Pennings and Paul, 1994). In contrast, homarine occurs in all the primary body tissues of *M. mollis* and is therefore a likely defensive compound. The presence of homarine in the viscera of *M. mollis* may be indicative of its dietary derivation (Karuso, 1987). Homarine was present in the epizooites that foul the tunic of *Cnemidocarpa verrucosa*, the primary prey of *M. mollis* (based on ^{1}H NMR spectra of crude extracts of epizooites). The lack of homarine in the tunic itself suggests that other metabolites are responsible for its unpalatability to fish (McClintock et al., 1992a) and that *M. mollis* may be grazing on the epizooites rather than the tunic. Nonetheless, the presence of homarine in a variety of marine invertebrates (Targett et al., 1983) suggests that de novo synthesis of homarine by *M. mollis* cannot be ruled out. Further work is needed to determine whether this is indeed the case.

In a provocative review, Faulkner and Ghiselin (1983) evaluate the evolution of chemical defense and shell loss in gastropods. One evolutionary scheme they suggest is a postadaptation process, with chemical defense evolving after shell loss. A second hypothesis presented is that chemical defense is a preadaptation to shell loss, which permitted individuals to survive without a shell. Such

evolutionary questions are inherently difficult to assess without the benefits of hindsight. Nonetheless, *M. mollis* is clearly a gastropod "caught" in the evolutionary process of losing its shell (all that remains of the shell is a thin flexible calcium carbonate sheet buried below a thick exposed mantle). As an example of a mesogastropod that has yet to completely lose its shell, the presence of chemical defense in *M. mollis* lends support to the preadaptational hypothesis.

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INFLUENCE OF OLEORESIN CONSTITUENTS FROM *Pinus ponderosa* AND *Pinus jeffreyi* ON GROWTH OF MYCANGIAL FUNGI FROM *Dendroctonus ponderosae* AND *Dendroctonus jeffreyi*

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Abstract—*Dendroctonus jeffreyi* and *D. ponderosae* are morphologically similar sympatric species of pine bark beetles over portions of their geographic ranges; however, *D. jeffreyi* is monophagous on *P. jeffreyi* while *D. ponderosae* is highly polyphagous. Both species carry a species of mycangial fungi that are also very similar in appearance. Growth of the two mycangial fungi and of the fungus *Leptographium terebrantis* (associated with the polyphagous and non-tree-killing *Dendroctonus valens*) in the presence of oleoresin constituents of host and nonhost conifers was tested by placing individual chemicals on agar growth medium and by growing the cultures in saturated atmospheres of the chemicals. The fungus associated with *D. jeffreyi* showed greater tolerance for chemical constituents placed on the growth medium than the other two fungi, and growth after three days was enhanced by heptane, the dominant constituent of *P. jeffreyi* oleoresin. Growth of all three species of fungi was reduced by the resin constituents when the chemicals were presented as saturated atmospheres. The results suggest that the influence of the tree on growth of the symbiotic fungi of the bark beetles during the initial attack process may be different than after colonization by the beetles is complete. The difference in the responses of the apparently related species of mycangial fungi may provide some new insight into the evolutionary history of these beetle/mycangial fungus/host tree systems.

Key Words—*Dendroctonus*, *Pinus*, Coleoptera, Scolytidae, bark beetles, mycangial fungi, inhibition, host resistance, monoterpenes, Ponderosa pine, Jeffrey pine.

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INTRODUCTION

Dendroctonus ponderosae Hopkins is a highly polyphagous primary bark beetle, colonizing 12 species of pines, including *Pinus ponderosa* Laws., in North America (Wood, 1982). *Dendroctonus jeffreyi* Hopkins, a species sympatric with *D. ponderosae*, is monophagous on *P. jeffreyi* Grev. & Balf, a host-plant species not utilized by *D. ponderosae*. The two beetle species were synonomized by Wood (1963), but were separated by Lanier and Wood (1968) on the basis of host range and subtle morphological difference. In addition, Smith (1965) described physiological differences between the two bark beetle species.

Pinus jeffreyi is considered to be an older species than *P. ponderosa* (Fowells, 1965), although the two species were in synonomy at one time (Munz and Keck, 1968). The current distribution of *P. jeffreyi* includes southern Oregon, California, and northern Baja California, while *P. ponderosa* is broadly distributed throughout western North America (Fowells, 1965). The geographic distributions the two pine species overlap in California. However, *P. jeffreyi* is often associated with slightly drier and colder sites than *P. ponderosa* (Fowells, 1965). Although not common, these two pines will occasionally form natural hybrids (Mirov, 1929).

Both *D. ponderosae* and *D. jeffreyi* are closely associated with specific fungi that are carried between host trees in maxillary mycangia of both sexes (Whitney and Farris, 1970; T.D. Paine, unpublished data). *D. ponderosae* is associated with *Ophiostoma clavigerum* (Robins.-Jeff. & Davids.) Harrington. A mycangial fungus with characteristics that are very similar to characteristics of *O. clavigerum* has been isolated from *D. jeffreyi* (T.D. Paine, unpublished data) but has not been described as a separáte species or as a form of *O. clavigerum*. These fungi are inoculated into trees during colonization by the beetle. The pines have two principal defenses against invasion by insects or infection by microorganisms: the preformed resin contained within resin ducts immobilizes or intoxicates colonizers, and an induced reaction isolates invading organisms with resinous necrotic lesions. The primary resin of *P. ponderosa* is predominantly monoterpene and resin acids; the resin of *P. jeffreyi* is composed primarily of *n*-heptane (Mirov, 1929; Smith, 1967; Anderson et al., 1969; Smith, 1971). The primary resin of *P. ponderosa* × *P. jeffreyi* hybrids is intermediate in character between the two parent species (Smith, 1963, 1967, 1982) (Table 1).

Dendroctonus ponderosae and *D. jeffreyi* overlap in geographic distribution in California as do the distributions of *P. ponderosa* and *P. jeffreyi*. The beetles and their mycangial fungi appear to be closely related, but the host ranges of the insects do not overlap. Smith (1963, 1965) demonstrated that the resin volatiles from the proper host were nontoxic to adult beetles, but volatiles from nonhost pines caused mortality. However, it is not known how the resin con-

TABLE 1. COMPOSITION OF OLEORESIN (%) FROM THREE PINE SPECIES AND ONE HYBRID[a]

	Pinus jeffreyi	*Pinus ponderosa*	*Pinus jeffreyi* × *Pinus ponderosa*	*Pinus contorta*
Heptane	91.89	0.00	32.50	0.00
Nonane	0.94	0.00	1.00	0.00
α-Pinene	0.57	6.30	7.00	6.00
Camphene	0.18	0.00	0.00	0.50
β-Pinene	1.76	26.40	19.50	6.00
D-3-Carene	0.47	36.20	22.50	10.90
Myrcene	0.53	13.30	7.50	3.40
Limonene	0.30	14.50	5.50	2.20
α-Phellandrene	0.00	0.00	0.00	1.00
β-Phellandrene	3.18	1.18	1.00	68.30
Terpinolene	0.00	2.00	1.50	1.60
Undecane	0.00	0.00	2.00	0.00
p-Cymene	0.00	0.00	0.00	2.10
4-Allylanisol	trace	trace	trace	trace

[a] From Smith (1963, 1967, 1982).

stituents from hosts or nonhosts affect growth of the fungi associated with the beetles. An understanding of the interrelationship between beetle phytophagy, host-tree defensive chemistry, and growth of symbiotic fungi could provide insight into the evolutionary relationships among these organisms. The objective of the study described here was to determine how individual monoterpene, resin acid, or hydrocarbon constituents affect growth rates of fungi associated with *D. ponderosae* and *D. jeffreyi*. *Leptographium terebrantis*, a nonmycangial fungus frequently associated with the highly polyphagous *Dendroctonus valens*, was included as a comparison because this beetle–fungus complex rarely kills naturally colonized host trees (Owen et al., 1987).

METHODS AND MATERIALS

The fungus associated with *D. jeffreyi* was isolated from the mycangia of beetles emerging from *P. jeffreyi* cut from the San Bernadino mountains (San Bernardino County, California). *Ophiostoma clavigerum* was isolated from *D. ponderosae* emerging from *P. ponderosa* and *L. terebrantis* was isolated from *D. valens*, also emerging from *P. ponderosa* (D.L. Wood and J.R. Parmeter, Jr., personal communication). All stock cultures of fungi were maintained on 3% potato–dextrose agar. For both experiments, potato–dextrose agar (3%) was poured into culture plates (100 × 15-mm, plastic plates were used for the growth

medium test and glass plates were used for the saturated atmosphere test) and allowed to solidify. Reference cultures of the fungi are maintained on potato–dextrose agar slants held under oil.

Fungal inocula were prepared in two ways. To test the growth response of fungi to host compounds placed onto the surface of the growth medium, inoculum was grown for three weeks in shake culture following inoculation of 250-ml flasks containing 100 ml of sterile 3% potato–dextrose broth and 250 pieces ($10 \times 2 \times 2$ mm) of sterile wooden matchsticks (match heads removed). Fungal-colonized wood pieces provided a uniform initial dose of inoculum for the culture plates (Paine, 1984a). However, it appeared that the inoculation of the fungus associated with *D. jeffreyi* failed to grow in the shake-culture flasks. Inoculum of this fungus for this test and all fungi for the saturated atmosphere test was prepared in the same way as our stock cultures. A disk (4 mm diameter) was cut from the actively growing margin of the source culture of fungus and placed onto the center of each study plate.

The growth media study plates were prepared by pipetting 1 ml of test chemical onto the center of each plate before inoculation and gently swirling the material around the plate surface. Test chemicals were obtained from commercial sources [4-allylanisole, *n*-heptane, (+)-limonene, β-myrcene, *n*-nonane, *n*-undecane (Sigma Chemical, St. Louis, Missouri) (+)-camphene, δ-3-carene, *p*-cymene, (−)-limonene, (±)-α-pinene, β-pinene (Aldrich Chemical, Milwaukee, Wisconsin); dipentene, β-phellandrene (SCM/Glidco, Jacksonville, Florida); α-phellandrene, (−)-α-pinene, (+)-α-pinene, and terpinolene (TCI America, Portland, Oregon)]. Oleoresins from *P. jeffreyi* and *P. ponderosa* were collected from living trees by drilling into the wood and inserting a tight-fitting vial into the hole. The vials and the accumulated resin were removed after 24 hr, placed on Dry Ice, returned to the laboratory, and stored at −60°C until needed. The plates were inoculated with one of the fungi within 4 h after test chemical had been applied to the agar surface. Each chemical treatment was replicated six times. Colony diameter was measured at three and five days after inoculation.

The culture plates for the saturated atmosphere test were similarly prepared and inoculated without any chemical treatment. Test chambers consisted of a 3.79-liter paint can with a wire rack in the bottom that supported a stack of six plates (two plates of each fungus species). Two milliliters of one of the test chemicals was placed in an open glass dish beneath the wire rack, the six inoculated culture plates were stacked in a random sequence on top of the rack, and the lid was tightly sealed on the can. Three cans were prepared for each of the 19 treatments including a blank control. The diameter of each fungal colony was measured after five days.

Results from both tests were analyzed using one-way analysis of variance

(Superanova, Abacus Concepts, Inc.). Differences among means ($\alpha = 0.05$) were separated using the Student-Neuman-Kuels test.

RESULTS

Growth of the mycangial fungus from *D. jeffreyi* was enhanced at three days by incorporation of *n*-undecane and *n*-heptane into the growth media but was inhibited compared to the control by seven test materials: 4-allylanisole, (+)-camphene, *p*-cymene, α-phellandrene, (+)-α-pinene, terpinolene, and *P. jeffreyi* oleoresin (Table 2). After five days of growth, growth on many of the

TABLE 2. COLONY DIAMETER OF MYCANGIAL FUNGUS ISOLATED FROM *D. jeffreyi* AND GROWN ON 3% POTATO-DEXTROSE AGAR WITH 1 ML OF INDIVIDUAL RESIN CONSTITUENTS SPREAD ACROSS SURFACE OF CULTURE PLATES[a]

	Diameter (mm), $\bar{X}$ (SE)	
	Three days[b]	Five days[c]
4-Allylanisole	4.00 a (0.00)	4.00 a (0.00)
(+)-Camphene	8.17 ab (0.98)	16.17 a (2.44)
δ-3-Carene	28.33 def (2.91)	68.67 de (7.10)
p-Cymene	8.25 ab (1.60)	47.50 c (12.24)
Dipentene[d]	29.17 def (2.54)	83.33 e (1.05)
n-Heptane	67.50 h (2.11)	85.00 e (0.00)
(−)-Limonene	25.33 cde (1.50)	83.50 e (0.81)
(+)-Limonene	38.00 f (4.07)	84.17 e (0.83)
β-Myrcene	23.17 cde (2.80)	74.17 e (3.53)
n-Nonane	26.00 cde (4.24)	78.33 e (4.11)
α-Phellandrene	18.00 bcd (2.78)	57.83 cd (5.87)
(−)-α-Pinene	32.40 ef (2.23)	85.20 e (0.20)
(+)-α-Pinene	9.50 ab (1.41)	31.17 b (2.95)
(±)-α-Pinene	31.00 ef (1.03)	85.00 e (0.00)
(−)-β-Pinene	23.83 cde (2.47)	76.33 e (2.59)
P. jeffreyi resin	15.50 ab (1.41)	57.17 cd (8.55)
P. ponderosa resin	24.20 cde (3.77)	80.00 e (2.07)
Terpinolene	4.67 a (0.67)	4.67 a (0.67)
n-Undecane	51.17 g (4.93)	82.67 e (1.50)
Control	31.80 ef 1.56)	79.80 e (2.35)

[a] Fungal growth was measured after three and five days.
[b] $F = 35.96$, $df = 19, 95$, $P < 0.0001$. Means followed by different lowercase letters are significantly different ($P < 0.05$).
[c] $F = 51.39$, $df = 19, 95$, $P < 0.0001$. Means followed by different lowercase letters are significantly different ($P < 0.05$).
[d] Dipentene is a mixture of 51% limonene, 33.9% β-phellandrene, and 2.8% terpinolene.

treatments had reached the edge of the plate. However, the same seven chemicals continued to suppress growth (Table 2).

A different pattern emerged when *O. clavigerum* associated with *D. ponderosae* was grown on plates containing the chemical constituents. No compound enhanced growth at three days, but 13 test materials [4-allylanisole, (+)-camphene, δ-3-carene, *p*-cymene, dipentene, (−)-limonene, (+)-limonene, *n*-nonane, α-phellandrene, (+)-α-pinene, (−)-β-pinene, *P. jeffreyi* oleoresin, and *P. ponderosa* oleoresin] inhibited growth (Table 3). Three chemicals (4-allylanisol, terpinolene, and *p*-cymene) inhibited growth after five days; the racemic mixture of α-pinene significantly enhanced growth (Table 3).

TABLE 3. COLONY DIAMETER OF MYCANGIAL FUNGUS *Ophiostoma clavigerum* ISOLATED FROM *D. ponderosae* AND GROWN ON 3% POTATO-DEXTROSE AGAR WITH 1 ML OF INDIVIDUAL RESIN CONSTITUENTS SPREAD ACROSS SURFACE OF CULTURE PLATES[a]

	Diameter (mm), $\bar{X}$ (SE)	
	Three days[b]	Five days[c]
4-Allylanisole	2.50 a (0.00)	2.50 a (0.00)
(+)-Camphene	13.17 def (2.23)	43.83 bc (3.17)
δ-3-Carene	11.33 de (0.92)	32.33 bc (0.95)
p-Cymene	2.58 a (0.08)	10.33 a (0.76)
Dipentene[d]	10.33 cd (1.12)	32.00 b (1.83)
n-Heptane	21.00 i (1.41)	44.00 bc (1.67)
(−)-Limonene	13.17 def (0.87)	40.33 bc (1.82)
(+)-Limonene	12.67 def 0.67)	39.83 bc (2.12)
β-Myrcene	15.50 efg (1.34)	47.33 c (2.79)
n-Nonane	11.50 de (0.85)	44.00 bc (8.60)
α-Phellandrene	5.58 ab (1.21)	33.67 bc (6.08)
(−)-α-Pinene	16.67 fgh (0.61)	41.17 bc (2.15)
(+)-α-Pinene	4.60 ab (0.40)	31.67 b (3.68)
(±)-α-Pinene	20.00 hi (2.22)	69.67 d 2.76)
(−)-β-Pinene	13.50 def (0.56)	46.00 bc (0.73)
P. jeffreyi resin	5.17 ab (0.31)	33.00 bc (2.31)
P. ponderosa resin	7.83 bc (0.31)	46.33 bc (0.88)
Terpinolene	2.50 a (0.00)	2.50 a (0.00)
n-Undecane	18.17 ghi (0.31)	40.33 bc (2.56)
Control	19.17 ghi (0.17)	40.33 bc (3.14)

[a]Fungal growth was measured after three and five days.
[b]$F = 35.59$, $df = 19, 99$, $P < 0.0001$. Means followed by different lowercase letters are significantly different ($P < 0.05$).
[c]$F = 25.74$, $df = 19, 100$, $P < 0.0001$. Means followed by different lowercase letters are significantly different ($P < 0.05$).
[d]Dipentene is a mixture of 51% limonene, 33.9% β-phellandrene, and 2.8% terpinolene.

Leptographium terebrantis was inhibited by 13 of the resin constituents [4-allylanisole, (+)-camphene, δ-3-carene, *p*-cymene, dipentene, (+)-limonene, *n*-nonane, (−)-α-pinene, (+)-α-pinene, (−)-β-pinene, terpinolene, *P. jeffreyi* oleoresin, and *P. ponderosa* oleoresin] tested at three days (Table 4). Nine compounds [4-allylanisole, δ-3-carene, *p*-cymene, dipentene, *n*-nonane, (+)-α-pinene, (−)-β-pinene, terpinolene, and *P. jeffreyi* oleoresin] continued to inhibit growth after five days. Terpinolene, 4-allylanisol, and resin from *P. jeffreyi* were the most inhibitory at five days, but the affect of the resin on fungal growth was not different from (+)-α-pinene and *n*-nonane at three days.

Although *n*-heptane enhanced growth of the mycangial fungus isolated from *D. jeffreyi* when it was placed on the surface of the growth media, the same

TABLE 4. COLONY DIAMETER OF *Leptogrophium terebrantis* ISOLATED FROM *D. valens* AND GROWN ON 3% POTATO-DEXTROSE AGAR WITH 1 ML OF INDIVIDUAL RESIN CONSTITUENTS SPREAD ACROSS SURFACE OF CULTURE PLATES[a]

	Diameter (mm), $\bar{X}$ (SE)	
	Three days[b]	Five days[c]
4-Allylanisole	2.50 a (0.00)	2.50 a (0.00)
(+)-Camphene	17.17 cde (3.67)	64.83 def (6.67)
δ-3-Carene	17.33 cde (1.71)	48.17 c (1.30)
p-Cymene	21.33 def (3.56)	54.67 cd (6.83)
Dipentene[d]	16.50 cde (1.34)	49.83 c (3.18)
n-Heptane	37.33 i (0.88)	77.50 fgh (0.50)
(−)-Limonene	30.00 ghi (1.59)	75.67 fgh (1.58)
(+)-Limonene	26.17 fgh (0.70)	79.50 gh (1.93)
β-Myrcene	31.50 hi (1.65)	75.67 fgh (2.70)
n-Nonane	13.33 bcd (1.67)	48.33 c (1.93)
α-Phellandrene	31.67 hi (5.28)	85.00 h (0.00)
(−)-α-Pinene	25.33 fgh (1.05)	60.17 cde (2.07)
(+)-α-Pinene	11.83 bc (1.49)	47.67 c (5.33)
(±)-α-Pinene	32.50 hi (1.31)	83.50 h (0.76)
(−)-β-Pinene	22.17 efg (0.70)	58.83 cd (3.03)
P. jeffreyi resin	6.50 ab (0.29)	35.50 b (1.94)
P. ponderosa resin	14.50 cde (2.13)	76.33 fgh (2.01)
Terpinolene	2.50 a (0.00)	2.50 a (0.00)
n-Undecane	29.00 fghi (1.39)	66.83 defg (4.48)
Control	36.50 i (0.96)	71.67 efgh (1.23)

[a] Fungal growth was measured after three and five days.
[b] $F = 26.85$, $df = 19, 98$, $P < 0.0001$. Means followed by different lowercase letters are significantly different ($P < 0.05$).
[c] $F = 56.29$, $df = 19, 98$, $P < 0.0001$. Means followed by different lowercase letters are significantly different ($P < 0.05$).
[d] Dipentene is a mixture of 51% limonene, 33.9% β-phellandrene, and 2.8% terpinolene.

compound significantly inhibited growth when the atmosphere was saturated with volatiles (Table 5). In addition, 15 compounds were inhibitory, and there were no significant differences among the racemic blend or either individual enantiomer of α-pinene. *Ophiostoma clavigerum* and *L. terebrantis* were significantly inhibited by all volatile chemical treatments (Table 5). As was demonstrated for the fungus associated with *D. jeffreyi*, there were no differences among the three α-pinene treatments for either of the two other species of fungi tested.

DISCUSSION

The two bioassay techniques used in the study produced different responses of the fungi that were dependent on the method of presentation of the host resin constituents. Previous studies of the response of fungi associated with bark beetles have used either saturated atmospheres (Cobb et al., 1968; Bridges, 1987) or incorporation into the growth medium (Raffa et al., 1985), but not both. Because the culture plates used in the growth medium study were not sealed, some of the test chemicals may have volatilized and dissipated. In contrast, the concentration of chemicals in the saturated atmosphere test may have remained high throughout the duration of the experiment. However, there may also have been physiological differences in the response of the fungi to the different modes of presentation, as evidenced by the response of the two mycangial fungi to heptane and the enantiomers or racemic mixtures of α-pinene and limonene. For example, growth of the fungus associated with *D. jeffreyi* was enhanced by heptane on the medium after three days but was inhibited by the same compound under saturated atmosphere conditions. Although $(-)$-α-pinene and the racemic form did not effect growth of that fungus when presented on the medium, $(+)$-α-pinene was inhibitory. However, both of the α-pinene enantiomers were inhibitory when presented as saturated atmospheres, and growth in a saturated atmosphere of the racemic mixture was not different from growth on the untreated control. Neither enantiomer of limonene was inhibitory to the *D. jeffreyi* fungus when on the media, but saturated atmospheres of either compound inhibited growth. *Ophiostoma clavigerum*, associated with *D. ponderosae*, was not inhibited by any form of α-pinene in saturated atmospheres or by $(-)$-α-pinene provided on the growth medium, but like the mycangial fungus of *D. jeffreyi*, $(+)$-α-pinene on the medium inhibited growth after three days. The racemic mixture of α-pinene, however, enhanced growth of *O. clavigerum* after five days when the chemical was placed on the culture plates. Enantiomers of limonene were inhibitory irrespective of the method of presentation. In contrast, *O. clavigerum* was not inhibited by *n*-heptane on the medium, but growth was reduced under saturated atmospheric conditions. Bridges (1987) demon-

TABLE 5. COLONY DIAMETER OF THREE FUNGI ASSOCIATED WITH *Dendroctonus* SPECIES GROWN ON 3% POTATO-DEXTROSE AGAR FOR FIVE DAYS IN SATURATED ATMOSPHERES OF RESIN CONSTITUENTS

	Diameter (mm), $\overline{X}$ (SE)		
	D. jeffreyi fungus[a]	Ophiostoma clavigerum[b]	Leptographium terebrantis[c]
4-Allylanisole	5.33 a (1.33)	4.00 a (0.00)	4.00 a (0.00)
(+)-Camphene	43.67 c (2.65)	14.83 bc (2.57)	36.17 ef (1.14)
δ-3-Carene	60.83 d (1.40)	24.17 de (2.65)	37.50 ef (2.23)
p-Cymene	5.83 a (0.83)	4.00 a (0.00)	4.00 a (0.00)
Dipentene[d]	33.50 bc (6.44)	12.33 ab (2.33)	34.83 e (2.09)
n-Heptane	13.00 a (1.06)	4.00 a (0.00)	6.83 a (0.31)
(−)-Limonene	30.17 b (1.62)	10.17 ab (0.48)	21.00 bc (1.48)
(+)-Limonene	36.67 bc (0.61)	13.17 abc (2.27)	23.50 bc (2.43)
β-Myrcene	35.17 bc (0.75)	11.17 ab (2.07)	28.00 cd (1.46)
n-Nonane	34.50 bc (3.52)	10.00 ab (1.10)	16.17 b (0.31)
α-Phellandrene	54.50 d (3.64)	25.17 e (4.78)	48.17 gh (1.70)
β-Phellandrene	70.00 ef (2.41)	18.83 bcde (2.06)	48.67 gh (2.22)
(−)-α-Pinene	58.50 d (3.18)	23.67 de (1.93)	42.67 fg (1.38)
(+)-α-Pinene	58.83 d (1.05)	26.00 e (1.55)	47.50 gh (1.88)
(±)-α-Pinene	62.83 de (1.78)	21.67 cde (4.04)	47.84 gh (1.76)
β-Pinene	34.67 bc (1.28)	11.17 ab (2.07)	32.50 de (2.28)
Terpinolene	39.83 bc (0.83)	11.67 ab (1.20)	20.67 bc (0.80)
n-Undecane	74.67 f (2.70)	26.17 e (2.21)	53.50 h (3.61)
Control	69.83 ef (4.67)	34.00 f (2.18)	70.83 i (4.31)

[a] $F = 62.51$, df $= 18, 95$, $P < 0.0001$. Means followed by different lowercase letters are significantly different ($P < 0.05$).
[b] $F = 15.49$, $df = 18, 95$, $P < 0.0001$. Means followed by different lowercase letters are significantly different ($P < 0.005$).
[c] $F = 83.86$, $df = 18, 95 < 0.0001$. Means followed by different lowercase letters are significantly different ($P < 0.05$).
[d] Dipentene is a mixture of 51% limonene, 33.9% β-phellandrene, and 2.8% terpinolene.

strated that the enantiomers of host terpenes could affect mycangial fungi differently, but rather than being inhibitory as shown in the study presented here, (+)-α-pinene enhanced the growth of *Ceratocystiopsis ranaculosis* Bridges and Perry (*Sporothrix*) associated with *Dendroctonus frontalis* Zimmermann. He suggested that because (+)-α-pinene was the form normally encountered by the beetle-fungus complex in the host tree loblolly pine, the fungus was adapted to grow in the presence of this compound.

The growth of the fungus associated with *D. jeffreyi* was primarily inhibited in the growth medium study after three days by oleoresin constituents not normally found or found in only trace amounts in *P. jeffreyi* resin and was not

inhibited by major resin constituents normally associated with *P. ponderosa* and *P. contorta* (Table 1), host trees of *D. ponderosae*. The principal exception was strong inhibition by whole resin from *P. jeffreyi*. Saturated atmospheres of all the chemicals except racemic α-pinene, β-phellandrene, and undecane were inhibitory. However, the fungi would normally be in direct contact with the oleoresin constituents in the natural substrate for growth, the inner bark and wood of the host tree. Hyphae would contact oleoresin within the resin ducts of the host or in the induced reactions produced in the host in response to invasion (Gambliel et al., 1985; Paine et al., 1987). It is possible that the fungi could encounter saturated atmospheres of the resin within the confines of the bark beetle galleries; however, by the time the galleries are constructed, resin flow and resin pressure associated with tree resistance to beetle attack would be minimal. Growth stimulation of the mycangial fungus of *D. jeffreyi* by heptane suggests that the fungus may be adapted to utilize the dominant resin constituent of *P. jeffreyi* as a carbon source.

Ophiostoma clavigerum growth was unaffected by heptane on the growth medium, although it was highly inhibitory in saturated atmospheres. This compound would not be normally encountered by the *D. ponderosae–O. clavigerum* association, despite the wide host range of the beetle. However, despite the larger host range, this fungus was inhibited by twice the number of oleoresin constituents in the growth medium study than the fungus associated with the monophagous *D. jeffreyi*.

Leptographium terebrantis was very similar to *O. clavigerum* in the number of compounds that were inhibitory in both studies. Owen et al. (1987) suggested that *L. terebrantis* frequently associated with *D. valens* can be highly pathogenic, although the beetle is rarely associated with tree mortality. Because *D. valens* is associated with living trees (unlike the other two *Dendroctonus* species, which are tree-killing species), colonizing beetles and the progeny would be continually associated with fresh resin while in the tree. Therefore, the sensitivity of its associated fungus to resin constituents of the host trees is a surprising result.

Several hypotheses have been proposed to explain the intimate association of bark beetles and fungi. Many of the primary tree-killing beetles must overcome the defenses of their host tree to reproduce successfully (Berryman, 1972; Wood, 1972). Because trees killed by the beetles were observed to be infected by staining fungi that could be pathogenic and because these fungi could be isolated from the insects, it was proposed that tree mortality could be attributed to the associated fungi (Basham, 1970; Mathre, 1964; Horntvedt et al., 1983; Paine, 1984b). However, trees may be killed in the absence of staining fungi (Hetrich, 1949; Bridges et al., 1985). The staining fungi are not mycangial species but are found as external contaminants on the body surface; their ecological association with bark beetles is probably more related to proximity within

host trees than to symbiosis. The staining species are not within a protected environment, the mycangium, during the colonization process and, thus, would probably be in more direct contact with the primary resins of newly colonized trees.

In contrast, the relationship between the mycangial fungi and the beetles appears to be symbiotic (Barras, 1973; Goldhammer et al., 1990); the fungi may be a source of nutrition for the developing larvae (Whitney, 1982; Bridges, 1983, 1985), although the fungal-derived nutrition may be most important for late-instar larvae and teneral adults in chambers constructed for pupation (Whitney, 1971). In addition, there are indications from other bark beetle systems that the mycangial fungi may displace or outcompete the staining fungi within the inner bark. This may limit the potential detrimental impact of these contaminant fungi on the developing insect larvae (Barras, 1970; Bridges and Perry, 1985; Whitney and Cobb, 1972). The mycangial fungi are not only protected, but growth is supported within the mycangia (Whitney and Farris, 1970; Barras and Perry, 1972; Paine and Birch, 1983). Growth of the symbiotic fungi into host tissue would occur most commonly after the tree defenses had been overcome and contact with free-flowing host resin would be minimal. If growth of the mycangial fungi were not inhibited, the fitness of beetle progeny would be enhanced. However, if the colonization process were prolonged so that the trees were capable of producing the induced responses, then suppression of fungal growth by induced resins containing highly fungitoxic compounds [i.e., 4-allylanisol (Gambliel et al., 1985)] would result in significantly reduced fitness for the beetle larvae.

Dendroctonus ponderosae and *D. jeffreyi* appear to be closely related, but it is not clear if they are descended from a common ancestor or if one is the ancestor of the other. The two mycangial fungi associated with the two beetle species also appear to be closely related based on morphology and spore type (T.D. Paine, unpublished data). The host range of *D. jeffreyi* is limited to the older pine species with the more restricted current geographic distribution. However, the isolates from *D. jeffreyi* appear to be more tolerant of host oleoresin components than the isolates from *D. ponderosae*, which colonizes the broader host range. A host range shift associated with a speciation event would require changes in the physiological tolerances, host seeking behavior, and chemical communication system of the beetle, as well as changes in the physiological tolerances in the mycangial fungi. However, it does appear in this system that the fungal component of a monophagous beetle–fungal complex is not less tolerant of novel chemicals than the fungus associated with the polyphagous beetle species.

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WINTER HOST COMPONENT REDUCES COLONIZATION BY BIRD-CHERRY-OAT APHID, *Rhopalosiphum padi* (L.) (HOMOPTERA, APHIDIDAE), AND OTHER APHIDS IN CEREAL FIELDS

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Abstract—Methyl salicylate, a volatile component of *Prunus padus*, the winter host of *Rhopalosiphum padi*, was found to reduce colonization of the summer host by this aphid. The compound was identified by gas chromatographic analysis coupled with recordings from cells in the primary rhinarium on the sixth antennal segment of the aphid. Methyl salicylate eliminated the attractancy of oat leaves to spring migrants in olfactometer tests. In Sweden, this compound significantly decreased colonization of field grown cereals by *R. padi* and in the U.K., populations of *Sitobion avenae* and *Metopolophium dirhodum* were significantly lower on treated plots.

Key Words—Aphid, *Rhopalosiphum padi*, Homoptera, Aphididae, cereal, electrophysiology, methyl salicylate, behavior, field study.

INTRODUCTION

The bird-cherry-oat aphid, *Rhopalosiphum padi* (L.) (Homoptera, Aphididae), is an host alternating species: bird-cherry, *Prunus padus* L. (Rosaceae), is the

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winter or primary host and a wide range of cereals and grasses (Poaceae = Gramineae) are summer or secondary hosts. *R. padi* is an important pest that causes economic damage to cereals both as a virus vector and as a phloem feeder (MAFF, 1982).

The behavioral ecology of *R. padi* is strongly influenced by olfactory stimuli of various types (Pettersson, 1994). Interactions with the winter host have been studied, particularly with regard to the autumn migration and the factors affecting reproductive success during this process (Leather 1981, 1986; Pettersson, 1993, 1994). There are differences in attractiveness of *P. padus* for oviposition, and the immigrants select among individual plants, thus allowing the survival and reproduction of their offspring to be optimized. The effects on population size in the succeeding year have been analyzed using suction trap data and direct estimates of the feeding population (Wiktelius et al., 1990).

Spring migration of *R. padi* is not well understood. Aggregation behavior on cereal plants has been observed, and the existence of an aggregation pheromone, associated with migration onto the summer host, has been demonstrated (Pettersson, 1994). It was also shown that leaves of *P. padus* inhibited the activity of the aggregation pheromone. This effect is comparable with a plant resistance factor decreasing a settling response and could have value in control strategies for masking host attractancy or as a repellent, since even a 20% decrease in settling would have an economic impact on damage by this pest (Wiktelius and Pettersson, 1985).

In this study, volatiles from *P. padus* are investigated using high-resolution gas chromatography coupled with single cell recordings from *R. padi* olfactory receptors. One component is identified as having significant electrophysiological activity. Behavioral responses of *R. padi* spring migrants to this compound are examined in olfactometer assays and field trials, and effects on the other major cereal aphid pests, the grain aphid *Sitobion avenae* (Fab.) and the rose-grain aphid *Metopolophium dirhodum* (Walk.), are also described.

METHODS AND MATERIALS

Aphids. For olfactometer studies, spring morphs of *R. padi* were raised from eggs laid on twigs of *P. padus*, collected at the Swedish University of Agricultural Sciences, Uppsala, Sweden. The twigs were placed in wet sand in a greenhouse at a temperature of 18–22°C and a 12:12 hr light–dark regime. Leaves produced on the twigs supported aphids after egg hatch long enough for alate spring migrants to develop. Alate virginoparous cereal aphids for electrophysiological studies were reared on oats, *Avena sativa* L. (Poaceae), c.v. Commander, at 20°C and a 16:8 hr light–dark regime.

Entrainment of Volatiles. Volatiles were entrained from *P. padus* twigs

with foliage standing in water and from pots of oat seedlings (*ca.* 10 cm) with or without colonies of *R. padi*. Air, dried and purified by passage through an activated 5 Å molecular sieve and charcoal, was drawn at 1 liter/min through a glass culture vessel (Quickfit FV range, 1l) containing plant material. Volatiles were entrained onto Porapak Q, which had been purified by washing with ether (5 ml) and heating for 12 hr at 150°C under a stream of nitrogen. Collected volatiles were desorbed from the Porapak by elution with freshly distilled diethyl ether. The resulting extract was concentrated under a stream of nitrogen and stored in sealed glass ampoules at −20°C.

Gas Chromatography (GC). Air entrainment volatiles were separated on an AI 93 gas chromatograph equipped with a cold on-column injector, a flame ionization detector (FID), and a 50 m × 0.32 mm id HP-1 bonded phase fused silica capillary column. The oven temperature was maintained at 40°C for 1 min and then programmed at 5°/min to 100°C and then at 10°/min to 250°C. The carrier gas was hydrogen.

Coupled Gas Chromatography-Mass Spectrometry (GC-MS). A capillary GC column (50 m × 0.32 mm id HP-1) was directly coupled to the MS and integrated data system (70–250 VG Analytical). Ionization was by electron impact at 70 eV, 230°C. The GC was maintained at 30°C for 5 min and then programmed at 5°/min to 180°C. Tentative identifications by GC-MS were confirmed by comparison with authentic samples and then by peak enhancement on GC (Pickett, 1990). Methyl salicylate was obtained from Aldrich and diluted in hexane for electrophysiological and behavioral assays.

Single Cell Recording (SCR). Electrophysiological recordings from olfactory cells associated with the distal primary rhinaria of cereal aphid virginoparae were obtained using tungsten microelectrodes. The indifferent electrode was positioned in the first antennal segment, and the recording electrode was then brought into contact with the rhinarium until impulses were recorded. Permanent copies of the action potentials generated by the olfactory cells were obtained by standard methods (Wadhams et al., 1982). The stimulus was delivered into a purified airstream (1 liter/min) flowing continuously over the preparation. The delivery system, employing a filter paper in a disposable Pasteur pipet cartridge, has been described previously (Wadhams et al., 1982). The impulse frequency was determined as the number of impulses elicited during the first 1 sec after stimulus application.

Coupled Gas Chromatography-Single Cell Recording (GC-SCR). The GC-SCR system, in which the antennal preparation is directly coupled to the capillary column gas chromatograph, has been described previously (Wadhams, 1990). Simultaneous records of the FID response and of the action potential frequencies were obtained by detecting with a level discriminator and plotting by means of a voltage/frequency convertor.

Olfactometry. Behavioral assays on *R. padi* spring migrants were carried

out in a Perspex olfactometer (100 mm diam.) as described by Pettersson (1970), with a weak airstream directed towards the center from each of four side arms. The test stimulus was placed at the end of one of the arms, with oat leaves in the other three arms as a control. Treatments comprised (1) oat leaves + 5 spring migrants; (2) as (1) + 2 μl eluate from *P. padus* entrainment; and (3) as (1) + methyl salicylate applied in a 10-μl capillary sealed at one end. Ten aphids were introduced into the center of the chamber and their positions were noted every 2 min for 20 min. Each experiment was replicated five or six times, and results were analyzed by paired t test; the number of visits into the treatment arm were compared with the mean of the visits into the three control arms. If aphids in the arena showed little activity, the experiment was terminated and the aphids changed.

Field Experiment: Sweden 1992. A commercial field of barley, *Hordeum sativum* Pers. (Poaceae), c.v. Pernilla, was sown to give five replicates of five treatments in a randomized block arrangement: plot size was 2 × 5 m, with 1 m between plots. Methyl salicylate was applied in two ways. A: an emulsion (1% methyl salicylate, 2% xylene, 0.3% Ethylan B.V. wetter, 96.7% water) was sprayed directly onto the plants using an electrostatic rotary atomizer (Arnold and Pye, 1981); the plots were treated every day at noon during the experimental period, and aphid counting was started on the day after the first application. B: six slow-release polyethylene vials (WP/5, Fisons), having four top holes (1 mm diam.) and containing 50 mg methyl salicylate, were positioned on 40-cm-high sticks in three pairs evenly distributed in each plot. Three control treatments were also included. These were—C: emulsion as described for A, omitting methyl salicylate; D: empty vials distributed as in B; and E: no treatment. The experiment was situated 200 m from a 12-m suction trap and was started when *R. padi* were found regularly in the trap. Total numbers of alate *R. padi* on the plots were determined by inspecting each plant for three consecutive days. On the fourth day, alatae were counted on a randomly selected 1-m^2 area in each plot. Analysis of variance (ANOVA) was done on the raw and log-transformed data for each day. The log transformation used was $x = \log_{10(y+1)}$, where x and y represent the transformed and untransformed data, respectively. The least significant difference test (LSD) was applied to the results at $P = 0.05$.

Field Experiment: U.K. 1993. Treatments were set out in a 5 × 5 quasi complete Latin square in spring-sown wheat, *Triticum aestivum* L. (Poaceae), c.v. Canon. Each plot was 3 × 5 m with 3 m between plots, the guard strips comprising 0.5 m fallow, 2 m wheat, and 0.5 m fallow. Methyl salicylate was released from three point sources provided by polyethylene bags suspended 5–10 cm above the crop and spaced 1 m apart in the center of each plot. Four different release rates were tested, calculated by weighing the sources every 10–11 days to be 1.0, 4.9, 46.5 and 125.4 mg/plot/day. Total numbers of aphids

along a 1-m line in the center of each plot were recorded twice a week over the period May 11 to July 26 and analyzed by ANOVA.

RESULTS

Coupled GC-SCR analysis of volatiles from undamaged *P. padus*, using recordings from the distal primary rhinaria of alate *R. padi* virginoparae, showed a peak with high electrophysiological activity (Figure 1). The active compound was tentatively identified from GC-MS as methyl salicylate, and this was confirmed by peak enhancement on GC with authentic compound. GC-MS of volatiles from oats, either with or without colonies of *R. padi*, by mass fragmentography of the diagnostic ions at *m/z* 92, 120, and 152, showed no trace of methyl salicylate at the appropriate retention time. The dose-response

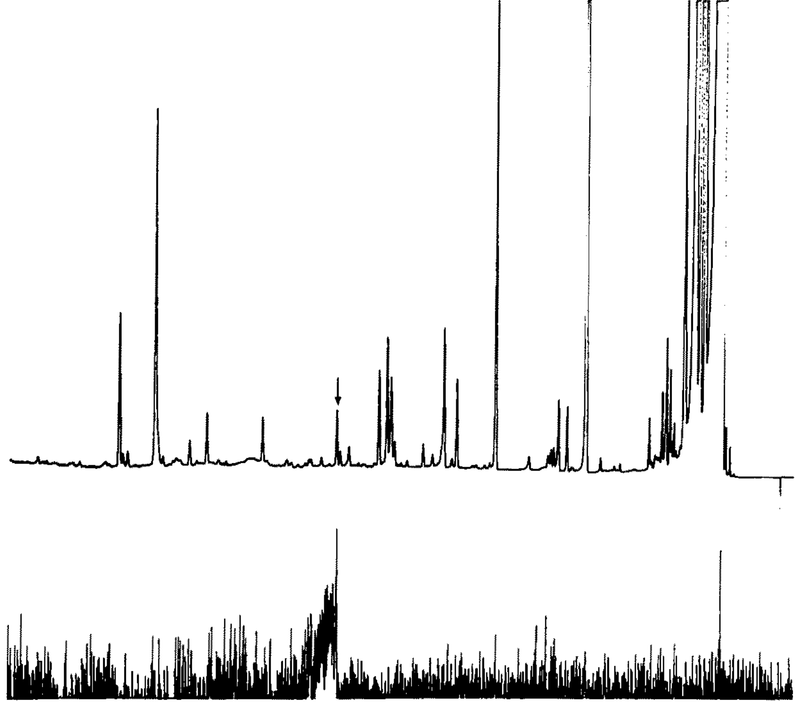

FIG. 1. GC-SCR of volatiles from *Prunus padus*. Upper trace: FID response; lower trace: action potential summation from cells in the distal primary rhinarium of *Rhopalosiphum padi*. Arrow: peak from methyl salicylate.

curve for the methyl salicylate cell (Figure 2A) showed a high level of sensitivity, with a threshold concentration of 10^{-9}g. Similar methyl salicylate-specific cells were found in the distal primary rhinaria of *S. avenae* (Figure 2B) and *M. dirhodum* (Figure 3). For *R. padi*, olfactometer tests (Table 1) showed that spring migrants feeding on oats released volatiles that attracted other spring migrants. This activity was inhibited by volatiles from *P. padus* leaves, and a similar effect was observed with methyl salicylate.

In the Swedish field experiment on barley in 1992, methyl salicylate applied either as an emulsion or from slow-release vials significantly reduced settling by *R. padi* spring migrants (Table 2). ANOVA of the log-transformed data showed no differences between the three control treatments. Therefore, the data for these treatments were combined and reanalyzed. Back-transformed means are included for clarity. In the U.K. field trial on wheat in 1993, total numbers of aphids on each treatment were recorded over a two-month period (Figure 4). Although numbers of *R. padi* were too low to analyze statistically, there was a significant reduction in populations of *S. avenae* and *M. dirhodum* associated

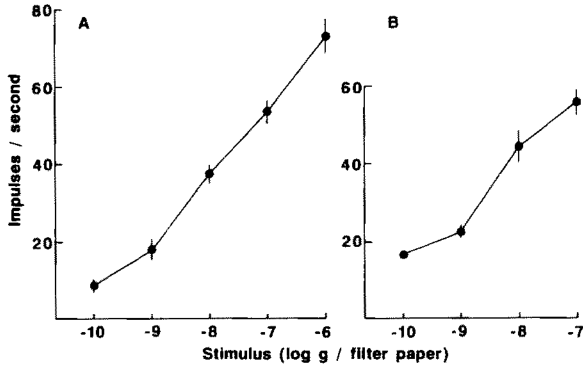

FIG. 2. Dose-response curves of olfactory cells in the distal primary rhinaria to methyl salicylate (each point is the mean of two stimulations): (A) *Rhopalosiphum padi*, (B) *Sitobion avenae*.

FIG. 3 Olfactory cell in the distal primary rhinarium of *Metopolophium dirhodum*: response to methyl salicylate at 10^{-7} g (bar = 1-sec stimulation).

TABLE 1. RESPONSES OF *Rhopalosiphum padi* SPRING MIGRANTS TO DIFFERENT STIMULI IN OLFACTOMETER[a]

Stimulus	Mean number aphids		P	Replicates
	Treated arm	Each control arm[b]		(N)
Oats + 5 spring migrants	27.2	16.3	<0.001	5
Oats + 5 spring migrants + *Prunus padus* volatiles	22.2	23.2	NS	6
Oats + 5 spring migrants + methyl salicylate	21.5	23.1	NS	6

[a]Cumulative counts over 20 min. NS = not significantly different at $P = <0.05$, paired t test.
[b]Control = oat leaves alone.

TABLE 2. FIELD TRIAL, SWEDEN 1992: EFFECT OF METHYL SALICYLATE ON COLONIZATION OF BARLEY BY *Rhopalosiphum padi*

Treatment	Log transformed mean no. spring migrants per plot[a]			
	Day 1	Day 2	Day 3	Day 4
Methyl salicylate (emulsion)	0.407ab (1.55)	0.156a (0.43)	0.739a (4.48)	0.709a (4.12)
Methyl salicylate (vials)	0.216a (0.64)	0.120a (0.32)	0.658a (3.55)	0.756a (4.70)
Combined controls[b]	0.576b (2.77)	0.426b (1.67)	1.025b (9.59)	1.028b (9.67)
SE[c]	0.101	0.107	0.097	0.057

[a]Values in the same column followed by different letters differ significantly (LSD test, $P < 0.05$). Values in parentheses are back-transformed means.
[b]The control treatments (blank emulsion, blank vials, no treatment) were not significantly different from each other, so are combined here for brevity.
[c]SE = standard error of difference between means.

with the methyl salicylate treatments. Overall reductions of alatae and apterae for both species were of the order of 40–50% ($P < 0.001$). No significant differences were observed between the four release rates.

DISCUSSION

Olfactory cells in the distal primary rhinarium responding specifically to methyl salicylate have now been found in many aphid species and morphs (Pickett et al., in preparation). The ubiquity of such receptors suggests that this

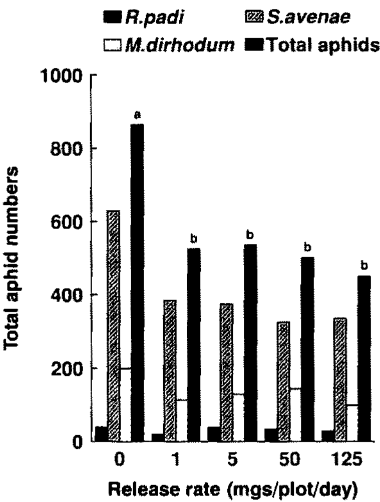

FIG. 4. Field trial, U.K., May 11–July 26, 1993: total aphid numbers along a 1-m line in the center of each plot (difference *a* from *b*, $P < 0.001$).

compound may be involved in several signaling processes in aphid chemical ecology. For *R. padi*, the absence of methyl salicylate from summer host volatiles, in addition to its abundance in the winter host *P. padus*, would point strongly to a role in host alternation.

The general importance of volatile semiochemicals in aphid chemical ecology is now being recognized, and evidence is accumulating for the role of nonhost plant volatiles as repellents and agents capable of masking host kairomones (Pickett et al., 1992). Thus, the black bean aphid, *Aphis fabae* Scop., and the damson-hop aphid, *Phorodon humuli* (Schrank), are repelled by organic isothiocyanates released by members of the Brassicaceae (= Cruciferae), upon which these insects do not feed (Nottingham et al, 1991; Pickett et al., 1992; Isaacs et al., 1993). This is analogous to the role of methyl salicylate for *R. padi*, as a component of the winter host acting as a repellent for the spring morphs. Methyl salicylate may fulfil a similar function for *M. dirhodum* in the migration from the rosaceous winter host. However, its role in the life-cycle of *S. avenae* is not clear, as this species remains throughout the year on cereals and grasses. The response to this compound could relate to an earlier host alternation that no longer exists. Alternatively, the methyl salicylate may be acting as a volatile signal related to host plant defense since it is the methylated metabolite of salicylic acid, a systemic plant component inducing a range of defense mechanisms. Although such a role for salicylic acid is not well characterized in the Poaceae, it is biosynthesized as part of the inducible phenylal-

anine ammonia-lyase (PAL) system that is responsible for plant phenolic-based defense (Ward et al., 1991), known to be active against some aphids (Grayer et al., 1992). Indeed, the predatory mite *Phytoseiulus persimilis* Athias-Henriot (Acarina: Phytoseiidae) is reported to employ methyl salicylate, released from Lima bean, *Phaseolus lunatus* L. (Fabaceae = Leguminosae) on feeding by the phytophagous mite *Tetranychus urticae* Koch (Acari: Tetranychidae), as a foraging stimulus (Dicke and Sabelis, 1988; Takabayashi et al., 1991; Bruin et al., 1992). Furthermore, methyl salicylate eliminates attraction of *A. fabae* to its host *Vicia faba* L. (Hardie et al., 1994), which is also in the Fabaceae, a family with a well-established inducible chemical defense based on the PAL system (Dixon and Paiva, 1993). In addition, methyl salicylate is present in hops, *Humulus lupulus* (L.) (Cannabaceae), at higher levels when colonized by *P. humuli*, and in the olfactometer this compound eliminated the attractancy of other host-derived compounds (Campbell et al., 1993).

The significant activity of methyl salicylate against cereal aphids in the field trials indicates its potential in the development of novel control strategies. The overall reductions observed in these trials would be sufficient to allow the population to be controlled by natural enemies (Wiktelius and Pettersson, 1985). Methyl salicylate is a common constituent of foods, naturally and as a flavoring agent, which should facilitate registration if it is to be developed for agricultural use.

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DIFFERENTIAL INDUCTION OF TOMATO FOLIAR PROTEINS BY ARTHROPOD HERBIVORES

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Abstract—The effects of mechanical and chemical damage and three types of biotic damage on the activities of four foliar proteins of the tomato plant (*Lycopersicon esculentum* Mill var. Castlemart) were assayed. Proteinase inhibitor, polyphenol oxidase, peroxidase, and lipoxygenase activities were assayed in damaged leaflets and compared with activities in undamaged leaflets. These proteins are putative plant defenses in tomato. Differential induction of these proteins by the various damage-treatments was demonstrated, such that different subsets of the four proteins were induced by different types of damage. This study clearly demonstrates the ability of plants to respond differentially to different types of damage. Possible mechanisms for this differential induction and the implications of differential induction for plant defense are discussed.

Key Words—Plant-insect interactions, induced defense, proteinase inhibitors, polyphenol oxidase, peroxidase, lipoxygenase, *Lycopersicon esculentum*, *Helicoverpa zea*, *Liriomyza trifolii*, *Aculops lycopersici*, phenolics, allelochemicals, insect nutrition.

INTRODUCTION

Rapid phytochemical responses to attack by insects and pathogens include enzyme activation, enzyme induction, increased production of small organic molecules and proteins (secondary metabolites), and synthesis of structural polymers at the site of injury (Bostock and Stermer, 1989; Duffey and Felton, 1989; Bowles, 1990). The importance of this dynamism to plant–microbe interactions has been recognized for several decades (Kuć, 1972), and recent evi-

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dence strongly supports the contention that a causal relationship exists between the induction of phytoalexins and plant resistance to pathogens (Hain et al., 1993).

Intensive study of rapid biochemical responses to insect herbivory is more recent than is the study of biochemical responses to pathogens; hence, the former field is less developed. Many researchers have, perhaps prematurely, hypothesized that plant responses to insects represent active defenses specifically against insects and that the initiation of these defenses only after attack by insects significantly reduces the cost of defense to the plant. Although evidence for this hypothesis remains equivocal (Hunter and Schultz, 1993; Tallamy and Raupp, 1991; Fowler and Lawton, 1985), alternative hypotheses (e.g., plant responses as simply wound-healing responses or as defenses against pathogen ingress at the wound site) are less often considered (Baldwin, 1994).

Elucidation of the ecological role(s) of induced responses is not currently possible, in part because most plant responses to insects remain poorly characterized. The biochemistry and molecular biology of signal transduction and phytochemical response have been extensively studied in only one system, the proteinase inhibitors of tomato (Ryan et al., 1985). The interaction of phytochemical induction with ontogenetic patterns of phytochemical expression has likewise received little attention (Wolfson, 1991). In addition, very few chemical responses have been spatially and temporally mapped (i.e., the time course and spatial extent of induction characterized (Karban and Myers, 1987; Baldwin, 1993)), despite the fact that the rapidity, duration, and spatial extent of induction of a given phytochemical will determine what organisms are affected by that chemical. Another little-studied aspect of phytochemical induction, one of particular relevance to our study, is the control, coordination, and potential interactions of multiple inducible phytochemicals in a plant. Most plant responses involve changes in the levels of multiple chemical factors (Baldwin, 1994); thus, induced resistance must emerge from a complex interplay of enzyme and end-product induction and suppression. Nonetheless, studies of induced responses are usually restricted to a single chemical or single class of chemical factors. Furthermore, those chemicals that have been investigated have primarily been small organic compounds; relatively little is known about the induction of structural polymers or enzymes by insects.

The tomato, *Lycopersicon esculentum*, provides a useful model for investigating the aspects of plant responses discussed above. Tomato foliage contains at least five insect-inducible proteins: two major proteinase inhibitors (PIs) and three oxidative enzymes, polyphenol oxidase (PPO), peroxidase (POD), and lipoxygenase (LOX). The induction of the serine proteinase inhibitors of tomato foliage has, as previously noted, been extensively characterized (Ryan et al., 1985), and several studies have implicated these proteins in plant defense against insects (Broadway et al., 1986; Ryan et al., 1985; Johnson et al., 1989). How-

ever, Wolfson (1991) has recently shown that reduced performance of insects on tomato foliage is not always correlated with increased levels of PIs; thus, the reduced growth of insects on wounded tomato foliage cannot be attributed solely to PIs.

We have been investigating the role of the oxidative enzymes PPO, POD, and LOX in plant defense against insects in tomato foliage. Both PPO and POD catalyze the oxidation of phenolics (in tomato foliage, primarily chlorogenic acid and rutin) to quinones; LOX catalyzes the formation of hydroperoxides from certain unsaturated fatty acids, notably the essential nutrients linoleic and linolenic acid. Both quinones and hydroperoxides are directly toxic to microorganisms and insects, and POD and LOX are associated with the formation of active oxygen species and free radicals, which also exhibit antibiotic properties (Elstner, 1980). In addition, quinones and hydroperoxides act as strong electrophiles, thereby alkylating amino acids. The alkylation of (often essential) amino acids results in a reduction in the digestibility, utilizability, and, ultimately, the nutritional value of plant protein (Felton et al., 1989a, 1992; Duffey and Felton, 1991). POD is also involved in lignin formation, which plays a role in woundhealing and which may be involved in defense against pathogens (Bostock and Stermer, 1989), and LOX is essential to the production of signal molecules, such as jasmonic acid, which may activate defense pathways (Farmer and Ryan, 1990). Increases in the activities of all three of these enzymes are correlated with resistance to pathogens and/or insects, and all three enzymes have been shown to reduce the growth of lepidopteran larvae in artificial diets (Shukle and Murdock, 1983; Gentile et al., 1988; Felton et al., 1989a, 1992, 1994; Duffey and Felton, 1991; Siedow, 1991; Stout, Workman, and Duffey, unpublished data).

In this study, we report the multiple and differential induction of PIs, PPO, POD, and LOX by five diverse types of damage and discuss possible mechanisms by which these responses are controlled and coordinated. This study serves as a prelude to a more detailed characterization of the induction of these proteins and its role in effecting resistance in tomato foliage.

METHODS AND MATERIALS

Plants and Insects. Tomato plants (*L. esculentum* cv. Castlemart) were grown in sterilized soil 4-in. pots in a greenhouse for 30–40 days (five to six fully expanded leaves). Plants were watered daily and fertilized weekly with a 15-30-15 P-N-K fertilizer. In winter, natural light was supplemented by fluorescent light (12:12 hr light–dark cycle).

Leafminers (*Liriomyza trifolii* Burgess) were from a laboratory colony maintained on chrysanthemum. *Helicoverpa zea* Boddie (corn earworm, tomato

fruitworm) larvae were hatched from eggs obtained from the USDA Southern Field Crop Insect Management Laboratory (Stoneville, Mississippi) and reared on an artificial diet until the second or third instar. Russet mites (*Aculops lycopersici* Massee) were originally collected from greenhouse infestations and were maintained on tomato plants in laboratory colonies under constant light and temperature.

Damage protocols. In a typical experiment, groups of plants were subjected to various levels of one of five types of damage and were subsequently maintained in a growth chamber (L16 : 8D cycle; 25°C) for one to five days depending on damage type. Control plants (undamaged plants of equivalent age) were maintained in the same growth chambers as damaged plants. Plants were then assayed for PPO, POD, LOX, and PI activities, and the activities of these proteins in damaged and control plants were compared. For all experiments, the terminal leaflet and the leaflet pair immediately adjacent to the terminal leaflet (hereafter, called the three terminal leaflets) of the fourth leaf (from the cotyledons) of five to six leaf stage plants were damaged and assayed. Only damaged leaflets were assayed for chemical changes; systemic induction of these enzymes (i.e., induction in undamaged leaves of damaged plants) was not considered. The importance of LOX as a component of signaling pathways was also not addressed by this study, as any changes in plant chemistry involved in signal transduction are likely to be more rapid than the changes assayed in these experiments.

Damage types were designed to simulate biotic (caterpillar, mite, or leafminer feeding), mechanical (forcep crushing), or chemical (immersion in soap solution) forces, as described next.

Chewing Damage. Zero (control), one, two, or three second- or third-instar larvae of *H. zea* were confined to the three terminal leaflets of the fourth leaves using a cloth bag and allowed to feed for 8 h. After the feeding period, caterpillars were removed and the plants were categorized according to the level of damage received: control (no damage), low ($\leq 2\%$ of leaf area eaten), medium (2–10% of leaf area eaten), or high (10–20% of leaf area eaten). Because preliminary studies had indicated that induction was maximal at 48 h, plants were assayed for chemical changes 40 h after removal of caterpillars.

Mining. Three to four adult female leafminers (*Liriomyza trifolii*) were confined to the three terminal leaflets of the fourth leaves using a cloth bag and allowed to oviposit for 1–5 h. This produced a range of mine densities. Five days after oviposition, plants were categorized according to average mine density per leaflet: control (0 mines/leaflet), low (1–2 mines/leaflet), medium (3–4 mines/leaflet), or high (4+ mines/leaflet). The plants were then assayed for chemical changes.

Mite Feeding. Zero or ~500 russet mites (*Aculops lycopersici*) were transferred to each of the terminal three leaflets of the fourth leaves from heavily

infested colony plants on excised leaf pieces and confined to the leaflets by applying Stikem pest glue (Seabright Enterprises) around the petiole of the leaflet. These mites are extremely small (0.2 mm), and densities as high as those used in this study can be observed in the field (Anonymous, 1990). Only one density of mites was used in these experiments because our source colony did not produce enough mites to allow the use of multiple densities. Mites were allowed to feed for three days, after which time assays for chemical changes were performed.

Forcep Crushing (Mechanical Damage). Each of the three terminal leaflets of the fourth leaf received zero (control), one, two, or three injuries, all perpendicular to the midvein. The plants were wounded by crushing the leaflet tissue between a pair of forceps; the size of each wound was approximately 2 cm long and 0.2 cm wide, and wounding did not sever the leaflet. Plants were assayed 48 h after wounding.

Soap Dipping. The three terminal leaflets of the fourth leaf were immersed a total of zero, one, two, or three times for approximately 5 sec in a label-strength (4%) solution of Safer insecticidal soap (Safer, Inc.). Leaflets dipped three times in soap were immersed once per day beginning three days before assays were performed, those dipped twice were dipped daily beginning two days before assays, etc. Control leaflets were dipped daily in distilled water for three days. Assays were performed 24 h after the last soap or water immersion.

Protein/Enzyme Assays. In order to determine enzyme activities, the terminal (damaged) leaflet of the fourth leaf was excised with a razor blade, weighed, and used immediately for assays. Enzymes were extracted from individual leaflets by macerating them separately, using a tissue grinder (Tekmar Company), in ice-cold pH 7 phosphate buffer with 7% polyvinylpolypyrrolidine according to Felton et al. (1989b). The macerated tissue was centrifuged at 11,000*g* for 10 min and the supernatant (enzyme extract) used directly for enzyme assays. PPO and POD activities were measured spectrophotometrically as the increase in OD_{470}/min/g, following the method of Ryan et al. (1982). PPO and POD assays used caffeic acid and guaiacol/H_2O_2, respectively, as substrates (these and all other chemicals were purchased from Sigma, St. Louis, Missouri). The LOX assay used linoleic acid as a substrate and measured the appearance of conjugated dienes at 234 nm (Hildebrand and Hymowitz, 1981). The reaction mixture consisted of 16 μl enzyme extract mixed with 2.9 ml of a 0.4 mM solution of linoleic acid dispersed in 0.1 M phosphate buffer (pH 7.0) containing Tween-20 (0.1% by volume). Change in absorbance was monitored for approximately 2 min. Enzyme activities are reported as ΔOD/per minute per gram of leaf tissue. For LOX, an increase of 1 OD unit corresponds to approximately 40 μmol of conjugated diene formed, assuming a molar extinction coefficient of 2.5×10^4 M^{-1} cm^{-1} (Koch et al., 1992). The PI assay used one leaflet of the damaged leaflet pair of the fourth leaf and was based on the ability of plant

extracts to inhibit chymotryptic activity. A leaflet from the damaged leaflet pair was used instead of the terminal leaflet because it was not possible to assay oxidative enzymes and PIs from the same leaflet. Leaf extracts were prepared by grinding leaflets in a 50 mM Tris HCl (pH 7.8) buffer (3 ml/g leaf tissue) containing 7% polyvinylpolypyrrolidine, 1.67 mM phenylthiourea, 0.3 M KCl, and 0.4 mM ascorbic acid. This extract was immediately frozen for later use. For PI assays, the frozen leaf extract was thawed and centrifuged at 13,000*g*, and 25 μl of the resulting supernatant was added to 25 μl of a 0.001 N solution of HCl containing 0.001 mg of chymotrypsin. This mixture was then allowed to incubate for 10 min. Following incubation, the chymotrypsin/PI extract mixture was added to 2.9 ml of 0.5 mM benzoyl tyrosine ethyl ester (BTEE, a chymotrypsin substrate) in a methanol/phosphate buffer (0.1M, pH 8.0) mixture (12:13 ratio) and the increase in absorbance at 256 mm recorded for approximately 3 min. A 25-μl aliquot of chymotrypsin without plant extract was used to determine uninhibited chymotrypsin activity. Proteinase inhibitor activities are reported as percent chymotrypsin activity relative to this control run with BTEE and chymotrypsin only.

Statistics. For all experiments except those involving leafminers, at least five plants were used at each damage level. The number of plants at each damage level was variable in leafminer experiments. Three replicates of the general experiment were performed for each damage type, although proteinase inhibitors were not assayed in one of the replicates of the mite experiment. Data for enzyme activities were subjected to a square root transformation to correct for heterogeneity of variance (variance usually increased with the mean; see below) and transformed data were analyzed using two-way ANOVA, with damage treatment and replicate as the main effects. Proteinase inhibitor data were analyzed by two-way ANOVA after arcsin transformation. The use of two-way ANOVA to analyze these data allowed the identification of damage types for which the slopes of the damage-response curves differed between replicates [manifested in the statistical analysis as a significant ($P < 0.05$) treatment-replicate interaction]. In such cases, each replicate was reanalyzed separately as transformed data using a one-way ANOVA (with treatment as the main effect). Although analyses were performed on transformed data, untransformed values are presented in graphs and figures to facilitate biological interpretation.

RESULTS

Elucidation of the major result of this paper—the dual influence of damage type and environmental factors on induced responses—first requires a consideration of two methodological issues. The first issue, common to nearly all studies of induced responses, is the appropriateness of the control treatment.

Because the chemical assays used in this study are destructive, chemical activities could not be assayed from the same plant both before and after the damage event or monitored by an internal control. This necessitated the use of separate control plants with which the damaged group was compared; a statistically significant difference between the two groups was interpreted as induction. This approach is valid as long as no unanticipated fluctuations in chemical levels occur in the control plants. The experiments reported here are susceptible to such problems because of the small sample sizes ($N \approx 6$) necessitated by the labor-intensive nature of the assays. Although care was taken to treat control and damaged plants identically, save for the damage treatment itself, it is, of course, impossible to absolutely ensure that none of the control plants were inadvertantly induced.

The second issue relates to the comparison of chemical activities in plants exposed to different damage types. The use of several densities of herbivores or intensities of artificial damage produced a range of intensities for each damage type; for four of the five damage types, four categories of damage, reflecting increasing intensities of damage, were differentiated. Because levels of damage were categorical and because no common estimate of amount of tissue damaged was possible, levels of damage cannot be directly compared between damage types (e.g., a high level of chewing damage is not necessarily equivalent to a high level of leaf-mining damage). Thus, changes in the levels of plant chemicals caused by different damage treatments can be compared only at a qualitative level (i.e., the presence/absence of induction of a given protein). Only within each damage treatment can quantitative statements be made.

These considerations aside, our results show that both damage type and environmental conditions affect the nature of a plant's response. At a qualitative level, and without regard to between-replicate differences in protein induction (see below), all treatments induced one or more of the foliar proteins; however, different subsets of the four proteins were induced by different types of damage (Table 1). Of the four types of damage, chewing damage (*H. zea* feeding) induced the greatest number of the four proteins: PPO, PIs, and LOX were induced by this type of damage. Leafminer feeding, in contrast, induced only POD. Mite feeding had a significant effect on POD and LOX activities, as did soap immersion. Mechanical damage induced PPO and PIs.

In Figures 1–5, data from all three replicates are combined for each damage type and activities at each damage level are expressed relative to control activities. Although there were qualitative and quantitative differences in protein responses between replicates (see below), combining the replicates in this manner conveys the general impact of the five damage types on the activities of the four proteins. The mean magnitude of induction at the highest level of damage for all enzymes ranged from approximately 1.5- to 3-fold above constitutive levels (Figures 1–5). The increases in PPO and PI activities seen at the higher

TABLE 1. EFFECTS OF FIVE DAMAGE TYPES ON FOLIAR ACTIVITIES OF POLYPHENOLOXIDASE (PPO), PEROXIDASE (POD), LIPOXYGENASE (LOX), AND PROTEINASE INHIBITORS (PI)

	Protein: Presence/absence of induction			
Damage type	PPO	POD	LOX	PI
Caterpillar feeding	+	−	+	+[c]
Leafminer feeding	−	+[b]	−	−
Mite feeding	−	+	+	−
Soap dipping	−	+	+	−
Mechanical damage	+	−	−	+

[a] Presence/absence of induction derived from data in Table 2. A + indicates a statistically significant ($P < 0.05$) treatment effect and a − indicates no significant effect in all three replicates unless otherwise indicated.
[b] In one of three replicates of this experiment, leafminer feeding had a significant effect on POD activity.
[c] Statistically significant induction in two of three replicates.

levels of leafminer damage (Figure 5) result from high constitutive activities in the first replicate, which had the largest sample sizes at the higher levels of damage. If the individual replicates are examined (Table 2), it is evident that the apparent response to damage is actually due to a bias introduced by the first replicate.

The data in Table 2, in addition to showing the differential effect of damage type on chemical levels, show that chemical response often varied among replicates of a given experiment. Because these experiments were performed throughout the year, this between-replicate variation most probably reflects the influence of as-yet unidentified environmental factors (see Discussion). Significant variation between replicates in responsiveness to a given damage type was manifested in the statistical analysis as a significant treatment–replicate interaction. Such treatment–replicate interactions were not found for all damage types, or for all proteins within a damage type. When present, however, the results from each replicate are presented separately in Table 2; when not present (i.e., response to damage essentially similar between replicates), results from the three replicates are combined in Table 2. An example of significant variation between replicates in the responsiveness of plants to a damage treatment is the response of POD activity to soap immersion. Soap immersion had a significant effect on POD levels in all three replicates of the experiment, as signified by the P values in the last column of Table 2. However, the magnitude of response with respect to level of damage varied greatly between replicates; in the first and third replicates of the soap immersion experiment, induction of POD in

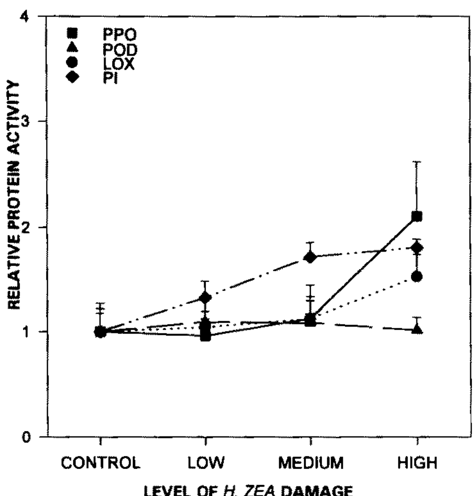

FIG. 1. Relative mean activities (+S.E.) of the foliar proteins polyphenol oxidase (PPO), peroxidase (POD), lipoxygenase (LOX), and proteinase inhibitors (PI) in leaflets subject to three levels of damage caused by feeding of *Helicoverpa zea* larvae. Leaflets were damaged by enclosing zero (control), one (low), two (medium), or three (high) larvae on the three terminal leaflets of the fourth leaf of six-leaf tomato plants and allowing the larvae to feed for 8 hr. Chemical activities were assayed 48 hr after initiation of damage. Each point represents the mean of 14–15 plants from three replicates of the experiment. Activities are expressed relative to the mean activity of control plants; mean activities at each damage level were divided by the mean activity of the controls to yield relative activities. Values presented are untransformed data.

damaged plants was much less marked (approximately 1.5- to 2-fold induction at the highest damage level) than in the second replicate of this experiment (approximately 4-fold induction). As a result of this variability, the slopes of the lines obtained by plotting the various levels of the damage treatment against POD activities at these levels differed significantly, and it is this difference in slope that is responsible for the statistically significant interaction. Furthermore, for some damage types, the effect of damage differed qualitatively between replicates. Leafminer feeding, for example, induced POD activity in the first but not the second or third replicates. The most illustrative example of this, however, was the first replicate of the *H. zea* feeding experiments, in which damage failed to induce PIs. This was surprising, since the phenomenon of PI induction by caterpillar feeding is well established in the literature.

In addition to the between-replicate variability noted above, the chemical responsiveness of individual plants exposed to the same level of the same type

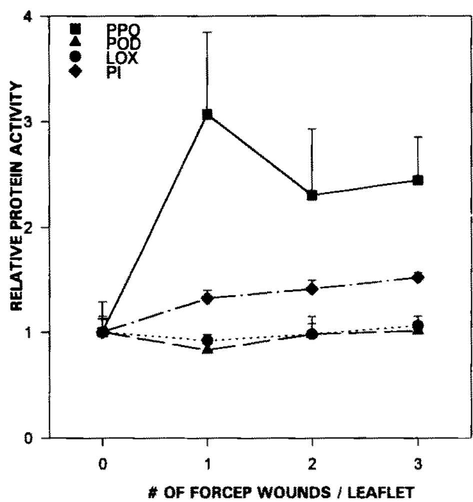

FIG. 2. Relative mean activities (+S.E.) of the foliar proteins polyphenol oxidase (PPO), peroxidase (POD), lipoxygenase (LOX), and proteinase inhibitors (PI) in response to damage inflicted by crushing leaflet tissue with forceps. The three terminal leaflets of the fourth leaf of six-leaf tomato plants received zero (control), one, two, or three wounds per leaflet, all perpendicular to the midvein. Wounds did not sever the leaflet and measured approximately 2 × 0.2 cm. Chemical activities were measured in damaged leaflets 48 hr after damage. Each point represents the mean of 14–18 plants from three replicates of the experiment. Activities are expressed relative to the mean activity of control plants; mean activities at each damage level were divided by the mean activity of the controls to yield relative activities. Values presented are untransformed data.

of damage were usually not uniform, a fact evidenced by high levels of variability within a treatment level. Even in cases of relatively clear induction, plants experiencing the same level of damage of a given damage type varied considerably in their responsiveness to damage. For example, PPO activities in individual plants exposed to the highest level of *H. zea* damage varied between 0.6 and 8 times the average levels found in control plants (data not shown). Similarly, POD activities in plants exposed to the highest level of soap damage varied between 1.3 and 7.1 times the average levels found in control plants (data not shown). There was a highly significant positive relationship between the means of the activities of the three oxidative enzymes at the various damage levels and the corresponding standard deviations ($r^2 = 0.77$; $P < 0.001$); thus, variability in the levels of these chemicals generally increased as the mean foliar level of an enzyme increased.

We occasionally observed a stimulation of chymotrypsin activities by

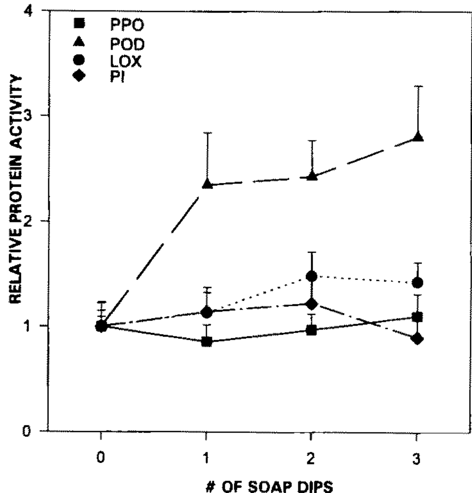

FIG. 3. Relative mean activities (+S.E.) of the foliar proteins polyphenol oxidase (PPO), perioxidase (POD), lipoxygenase (LOX), and proteinase inhibitors (PI) in response to damage inflicted by dipping tomato leaflets into a solution of insecticidal soap. The three terminal leaflets of the fourth leaf of five-six leaf tomato plants were dipped in a 4% solution of Safer insecticidal soap. Leaflets were dipped a total zero (control), one, two, or three times, once per day, over the course of three days. Chemical activities in damaged leaflets were determined 24 hr after the final dip. Each point represents the mean of 15 plants from three replicates of the experiment. Activities are expressed relative to the mean activity of control plants; mean activities at each damage level were divided by the mean activity of the controls to yield relative activities. Values presented are untransformed data.

extracts of control plants (most evident in the second replicate of the *H. zea* experiments). We have at present no explanation for this phenomenon, but, since it was relatively uncommon and its magnitude relatively small, we do not believe that it is indicative of any serious problem with the procedure used to assay chymotrypsin inhibitor. The plant extracts used were crude, and some component(s) of the extracts evidently interacted in some way with the chymotrypsin and/or its substrate to increase the apparent chymotryptic activities of the mixtures.

DISCUSSION

The results of this study clearly demonstrate that plants can respond differentially to different herbivores and types of damage. This conclusion is made on the basis of changes in protein activities in plants subject to five types of

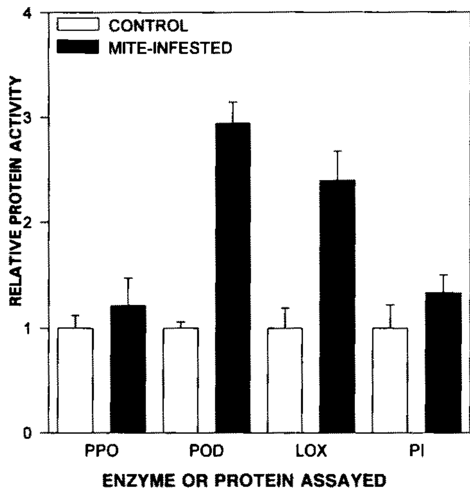

FIG. 4. Relative mean activities (+S.E.) of the foliar proteins polyphenol oxidase (PPO), peroxidase (POD), lipoxygenase (LOX), and proteinase inhibitors (PI) in response to damage resulting from russet mite feeding. Leaflets were infested by transferring approximately 500 mites from a laboratory-maintained mite colony to each of the three terminal leaflets of the fourth leaves. Mites were confined to these leaflets by an application of pest glue around the petiole. Pest glue, but no mites, was applied to the petioles of control plants. Protein activities in damaged leaflets were determined three days after infestation. Each point represents the mean of 12–19 plants from three replicates of the experiment. Activities are expressed relative to the mean activity of control plants; mean activities at each damage level were divided by the mean activity of the controls to yield relative activities. Values presented are untransformed data.

damage. Multiple isoforms of each of the proteins assayed in this study probably exist in tomato foliage (Mohan and Kolattukudy, 1990; Droillard et al., 1993), and more than one isoform of each protein may be responsible for the observed changes in activity. We will refer to each of these proteins in the singular for simplicity. Chewing damage induces PPO, PIs, and LOX, while crushing leaf tissue with forceps induces on PPO and PIs. Both surface feeding (mites) and soap dipping induce LOX and POD, and leaf mining induces only POD. The induction of PPO and PIs in tomato by chewing insects and mechanical damage has been previously reported, as has the induction of LOX and POD by mites (Green and Ryan, 1972; Duffey and Felton, 1991). This is the first report of the induction of POD and/or LOX in tomato by soap treatment or leaf mining, and of the induction of LOX by chewing damage.

Differential induction of phytochemicals by insects has also been shown to occur, albeit less markedly, in at least three other systems. Hartley and Lawton

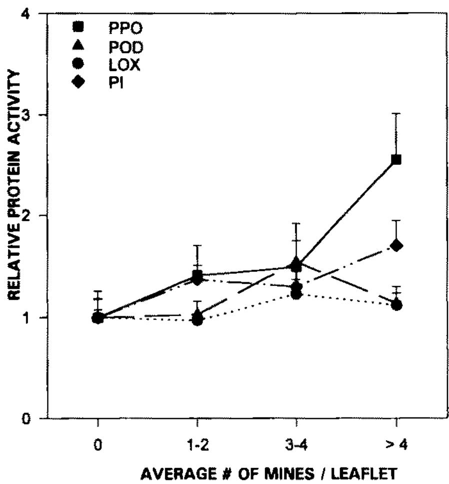

FIG. 5. Relative mean activities (+S.E.) of the foliar proteins polyphenol oxidase (PPO), perioxidase (POD), lipoxygenase (LOX), and proteinase inhibitors (PI) in response to three levels of damage caused by leafminer feeding. Leaflets were infested with leafminers by enclosing three to four adult female leafminers (or no leafminers as a control) on the three terminal leaflets of the fourth leaf and allowing the leafminers to oviposit for 1-5 hr. This resulted in a range of miner densities. Five days later, plants were categorized as to average mine density/leaflet and chemical assays were performed on damaged leaflets. Each point represents the mean of 7-23 plants from three replicates of the experiment. Activities are expressed relative to the mean activity of control plants; mean activities at each damage level were divided by the mean activity of the controls to yield relative activities. Values presented are untransformed data.

(1987, 1991) demonstrated that chewing and mining induced different amounts and possibly different types of phenolics and induced phenylalanine ammonia lyase (a key enzyme in the phenylpropanoid pathway) to different degrees. Similarly, Olson and Roseland (1991) showed that feeding by the sunflower beetle induced the coumarins scopoletin and ayapin to higher levels than did thrips feeding, although induction after thrips feeding was more rapid. More recently, Felton et al., (1994) observed differential induction of soybean foliar proteins by a chewing insect and a phloem-feeding insect. In this latter study, feeding by a phloem-feeding insect was found to induce lipoxygenase, peroxidase, polyphenol oxidase, and ascorbate oxidase activities, while feeding by a leaf-chewing insect was found to induce only lipoxygenase and ascorbate oxidase. Thus, the ability of plants to generate distinct responses to different types of herbivory may be a general and heretofore under-appreciated aspect of plant–insect interactions. In addition, these results confirm and extend the findings of

TABLE 2. EFFECT OF FIVE DAMAGE TYPES ON ACTIVITIES (±SE) OF FOUR PROTEINS IN TOMATO FOLIAGE[a]

Protein	Damage level				
	Control[b]	Low	Medium	High	P^c
Helicoverpa zea feeding					
PPO	8.11 ± 2.16 (15)	7.80 ± 1.86 (15)	9.11 ± 2.62 (15)	17.01 ± 4.11 (14)	0.014
POD	39.01 ± 8.64 (15)	42.38 ± 9.21 (15)	41.94 ± 9.59 (15)	39.31 ± 4.50 (14)	0.77
LOX	3.29 ± 0.55 (15)	3.41 ± 0.27 (15)	3.67 ± 0.56 (15)	5.01 ± 0.69 (14)	0.004
PI					
Replicate 1	23.36 ± 3.55 (15)	37.08 ± 6.55 (5)	30.40 ± 1.79 (5)	28.02 ± 2.47 (5)	0.16
Replicate 2	119.94 ± 4.13 (15)	52.63 ± 20.67 (4)	32.67 ± 14.12 (6)	24.4 ± 8.25 (5)	0.0005
Replicate 3	46.62 ± 8.71 (5)	40.73 ± 11.10 (6)	10.48 ± 1.61 (4)	11.03 ± 3.24 (4)	0.027
Forcep crushing					
PPO	6.75 ± 1.95 (15)	20.73 ± 5.25 (16)	15.52 ± 4.27 (16)	16.49 ± 2.77 (16)	0.0005
POD	22.02 ± 2.85 (16)	18.25 ± 1.84 (18)	21.53 ± 3.80 (4)	22.22 ± 2.04 (16)	0.36
LOX	3.13 ± 0.47 (16)	2.87 ± 0.18 (16)	3.06 ± 0.32 (16)	3.32 ± 0.30 (16)	0.61
PI	54.42 ± 6.12 (16)	37.03 ± 3.80 (16)	32.77 ± 3.95 (16)	27.43 ± 2.23 (16)	0.0004
Soap damage					
PPO					
Replicate 1	5.14 ± 1.61 (5)	3.26 ± 0.44 (5)	4.63 ± 1.49 (5)	2.19 ± 0.19 (5)	0.21
Replicate 2	3.92 ± 1.39 (5)	6.94 ± 1.47(5)	8.9 ± 2.01 (5)	9.42 ± 1.94 (5)	0.086
Replicate 3	13.79 ± 3.46 (5)	9.43 ± 2.93 (5)	8.56 ± 2.27 (5)	13.63 ± 2.35 (5)	0.34
POD					
Replicate 1	17.18 ± 2.68 (5)	24.39 ± 2.72 (5)	39.73 ± 8.22 (5)	26.32 ± 2.08 (5)	0.014
Replicate 2	36.14 ± 11.01 (5)	138.03 ± 31.54 (5)	109.96 ± 24.07 (5)	151.67 ± 27.03 (5)	0.006
Replicate 3	45.93 ± 4.12 (5)	70.85 ± 11.25 (5)	91.20 ± 5.47 (5)	100.30 ± 7.15 (5)	0.0004
LOX	3.72 ± 0.33 (15)	4.22 ± 0.70 (15)	5.50 ± 0.85 (15)	5.27 ± 0.70 (15)	0.045
PI					
Replicate 1	68.36 ± 8.9 (5)	56.34 ± 11.69 (5)	60.0 ± 11.3 (5)	89.14 ± 6.75 (5)	0.089
Replicate 2	90.16 ± 9.49 (5)	94.68 ± 5.32 (5)	97.1 ± 1.94 (5)	85.32 ± 4.38 (5)	0.40
Replicate 3	48.64 ± 9.59 (5)	43.04 ± 5.27 (5)	36.22 ± 3.71 (5)	42.22 ± 1.17 (5)	0.51

Leafminer feeding					
PPO					
Replicate 1	31.85 ± 8.76 (4)	39.18 ± 7.10 (9)	40.26 ± 10.51 (6)	42.65 ± 4.33 (6)	0.83
Replicate 2	7.24 ± 1.58 (6)	8.86 ± 1.87 (9)	11.43 ± 1.90 (3)	5.01 ± 0.0 (1)	0.56
Replicate 3	7.85 ± 3.37 (5)	7.49 ± 1.57 (5)	5.63 ± 1.51 (5)		0.76
POD					
Replicate 1	23.58 ± 1.05 (4)	34.19 ± 3.92 (9)	59.86 ± 11.75 (6)	48.06 ± 4.0 (6)	0.0027
Replicate 2	27.14 ± 6.63 (6)	32.26 ± 7.76 (9)	32.52 ± 7.71 (3)	20.03 ± 0.00 (1)	0.87
Replicate 3	70.11 ± 10.32 (5)	73.22 ± 10.32 (5)	83.18 ± 14.03 (5)		0.77
LOX					
Replicate 1	3.61 ± 0.63 (4)	2.91 ± 0.18 (9)	3.25 ± 0.57 (6)	3.69 ± 0.60 (6)	0.60
Replicate 2	3.27 ± 0.37 (6)	3.0 ± 0.16 (9)	4.3 ± 0.71 (3)	2.23 ± 0.00 (1)	0.075
Replicate 3	2.49 ± 0.35 (5)	3.18 ± 0.27 (5)	4.17 ± 0.96 (5)		0.18
PI					
Replicate 1	32.32 ± 4.92 (4)	25.56 ± 4.29 (9)	26.77 ± 2.78 (9)	28.58 ± 4.7 (6)	0.74
Replicate 2	67.91 ± 9.79 (6)	55.50 ± 7.07 (9)	35.23 ± 17.06 (3)	88.92 ± 0.0 (1)	0.17
Replicate 3	76.06 ± 12.81 (5)	67.51 ± 13.36 (5)	82.81 ± 9.25 (5)		0.74
Mite feeding					
PPO	5.70 ± 0.67 (19)			6.88 ± 1.50 (18)	0.26
POD	36.40 ± 2.29 (19)			107.08 ± 7.30 (18)	0.0001
LOX	2.17 ± 0.41 (18)			5.22 ± 0.61 (18)	0.0001
PI	65.06 ± 7.62 (14)			53.38 ± 5.88 (12)	0.16

[a] Values listed are mean activities (±SE, with *n* values in parentheses) of the proteins at various damage levels. Units of enzyme activity are ΔOD_{470}/min/g leaf tissue (wet weight) for PPO and POD and ΔOD_{234}/min/g leaf tissue for lipoxygenase. PI activities are expressed as percent activity relative to a control run without leaf extract; lower chymotrypsin activities represent higher proteinase inhibitor activities (see text). Although untransformed data are presented in the table, statistical analyses used square root (enzyme activities) or arcsin (proteinase inhibitor activities) transformed data. For proteins in which the slopes of the damage–response curves varied significantly between replicates, the results of each replicate are listed separately.

[b] Four damage levels were used for experiments involving *H. zea* and leafminer feeding, soap dipping, and leaf crushing; fewer damage levels were used for some leafminer and mite experiments.

[c] *P* values denote the significance level of the effect of treatment.

several other researchers that alterations in secondary metabolism that follow mechanical wounding may not accurately simulate alterations in metabolism that follow biological damage (insect feeding) (Baldwin, 1990; Lin et al., 1990). Crushing of leaf tissue, a form of damage that has been used to simulate insect damage (e.g., Green and Ryan, 1972) did not mimic any of the other damage types with reference to the four protein activities assayed. Interestingly, however, soap dipping induced the same subset of proteins as did mite feeding.

Presumably, differences in response to different damage types are due to differences in signals generated at the wound site. These signals may be of plant (e.g., cell wall fragments) (Ryan et al., 1985) or insect (e.g., salivary factors) origin. Microorganisms associated with insects or unsterilized forceps may also be responsible for some of the responses observed (Grisham et al., 1987). Salivary factors, such as hydrolytic enzymes or plant growth regulators (Miles, 1969; Hori, 1976), have been shown to be involved in PAL induction in birch (Hartley and Lawton, 1991) and in the induction of resistance in soybean (Lin et al., 1990). Lin et al. (1990) concluded that the strength of an induced response may depend on the number of wounded cells in contact with healthy cells. Other factors possibly responsible for the differential response (differential release of signals) include the duration (persistence) and/or magnitude of a single damage event, the cell or tissue type(s) affected by damage, and the types of forces brought to bear on the leaf (feeding mechanism) (Baldwin, 1990). In regard to the latter factor, even insect species with ostensibly similar modes of feeding can differ markedly in the ways in which they remove leaf material or cell contents from a plant (Bernays and Janzen, 1988; Parrella et al., 1985).

It is not possible to discern from this study what factors are important in producing differential responses in tomato foliage. The fact that feeding by *L. trifolii*, an insect which mines the palisade mesophyll (Parrella et al., 1985), had the least marked effect on protein activities in this study suggests that the cell or tissue type damaged partly determines the response engendered. This is supported by the fact that mite feeding and immersion in soap solution, both of which probably have their greatest impact on epidermal cells, induce the same subset of proteins (Table 1). The generally positive relationship between damage intensity and magnitude of response (Figures 1–5) suggests that the number of cells damaged influences the response. The role of salivary factors in the induction of tomato foliar proteins warrants further investigation.

The induction of POD and LOX appear to be controlled independently (i.e., are induced via separate signal transduction pathways) in tomato foliage. POD but not LOX was induced by leafminer feeding, and LOX but not POD was induced by *H. zea* feeding. In contrast, PPO and PIs may be under coordinate control, as both proteins are only induced by mechanical damage and *H. zea* feeding. The induction of both POD and LOX by mite feeding and PPO/PIs and LOX by *H. zea* feeding is evidence that multiple signals can be released,

and multiple signal transduction pathways activated, by insect feeding. Most likely, more than one of the factors discussed in the previous paragraph operate during a single damage event to create an "array of inductive signals" (Grisham et al., 1987).

Chemical responses of plants subject to similar types and intensities of damage are often found to vary in an unpredictable fashion between experiments or replicates of an experiment (Coleman and Jones, 1991; Haukioja and Hanhimaki, 1985). This variability is usually attributed to the influence of plant age, history, and environmental factors (Karban and Myers, 1989). In these experiments, we found the chemical responses of tomato foliage to the same type of damage to vary in groups of plants grown at different times of the year. This variability may be due, in part, to differences in the average intensities of damage inflicted on groups of plants in the different replicates. In addition, since these experiments were performed throughout the year, day length and light intensity may be responsible for some of the observed variability between experiments (see Karban, 1987). We have also demonstrated that leaf age has both a quantitative and qualitative effect on inducibility of these proteins (Stout, Workman, and Duffey, unpublished); thus, small differences in leaf age between replicates may have also contributed to the variability in our results. A complete and systematic investigation of the effects of environmental conditions on inducibility is an important direction for future research.

We also found that individual plants subject to approximately the same level of damage were not uniform in their responses, a fact reflected in the large standard errors associated with the means of the chemical activities (Table 2, Figures 1–5). Again, this variability may be due to small differences in the intensities of damage suffered by different plants in a treatment group, or it may be due to small differences in leaf age, etc. Importantly, however, it may also reflect an inherent stochasticity in the responses of tomato foliage to damage.

Our results are consistent with, but do not constitute strong evidence for, the putative role of these proteins in induced defense against insects, since increases in the activities of these proteins following damage are expected to have a negative impact on the nutritional value of foliage for insects (see *Introduction*). Furthermore, this study raises the interesting possibility of differential resistance; that is, because the induced state varies qualitatively with the inducing factor, the effectiveness of induced resistance against a given type of herbivore may also depend on the inducing factor. However, the actual biological effect of the induction of these proteins depends on several additional factors. A number of metabolites in addition to PIs and oxidative enzymes are induced by insect feeding (Stout and Duffey, unpublished data), and interactions with these induced chemicals or with constitutive plant chemicals may decrease the efficacy of PIs or oxidative enzymes as defenses (i.e., biological effect depends on chemical context). PPO, for example, has been shown to be capable of

inactivating PIs in the presence of catecholic phenolics (Felton et al., 1989b; Workman, Felton, and Duffey, unpublished). In addition, the activity of these proteins may be altered in the insect gut by interactions with gut pH, surfactants, proteases, etc. (Felton et al., 1989a). The efficacy of these proteins as defenses against the damaging insect or subsequent insects will also depend on the rapidity, duration, and spatial extent of induction (Karban and Myers, 1989). Thus, the relative role of a single induced chemical in effecting resistance depends on a complex interplay of factors. Given this complexity, and the multiple biological activities expressed by these proteins (e.g., involvement in wound healing or lignin formation; see *Introduction*), it is clear that mere inducibility by insect feeding does not constitute evidence for a defensive role against insects.

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MALE LURES FOR MEDITERRANEAN FRUITFLY (*Ceratitis capitata* WIED.): STRUCTURAL ANALOGS OF α-COPAENE

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Abstract—Nine sesquiterpenes structurally related to the potent male Mediterranean fruit fly lure (+)-α-copaene were tested in a series of field bioassays to determine their male medfly attractiveness relative to one another and to (+)-α-copaene itself. This study was carried out to determine the relative importance of the various substructure components of the (+)-α-copaene molecule in eliciting an attractive response in the male fly. Tests indicated that any deviation from the three-dimensional structure of (+)-α-copaene leads to major losses in male fly attractancy. The tested analogs fell into two groups, based on their levels of attraction: (+)-α-ylangene, (+)-β-copaene, (+)-β-ylangene, and (−)-α-copaene were found to be somewhat attractive, although much less so than (+)-α-copaene, while (+)-cyclosativene, (+)-cyclocopacamphene, (+)-longicyclene, (+)-longipinene, and (−)-*trans*-α-bergamotene were not attractive.

Key Words—Attractants, medfly, *Ceratitis capitata*, Diptera, Tephritidae, male lures, α-copaene, β-copaene, α-ylangene, β-ylangene, cyclosativene, cyclocopacamphene, longicyclene, longipinene, *trans*-α-bergamotene, Angelica seed oil, *Angelica archangelica*, enantiomers.

INTRODUCTION

α-Copaene, a tricyclic sesquiterpene, has been shown to be the major factor responsible for the attractiveness of angelica seed oil to the male Mediterranean fruit fly (Fornasiero et al., 1969; Guiotto et al., 1972). The angelica seed oil

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component is dextrorotatory (Jacobson et al., 1987) and was subsequently shown to be present in angelica seed oil from one source as a mixture of enantiomers: 98.6% (+) and 1.4% (−) (Flath et al., 1994). Direct field bioassay comparisons of the (+) and (−) enantiomers demonstrated that (+)-α-copaene is much more attractive to the medfly male than is the (−) isomer. In a previous study, one of the present authors (R.T.C.) and coworkers found angelica α-copaene to be two to five times more attractive to male flies than trimedlure in field tests (Cunningham, 1989). Because (+)-α-copaene is so active, there is much interest in its potential use as a male lure, or parapheromone, in survey trap arrays for early detection of medfly introductions to the mainland United States. The major limitation to its use is its scarcity; although α-copaene (either enantiomer) is widely distributed in the plant kingdom, its concentration is typically very low, so there is no convenient and inexpensive essential oil or plant extract available as a practical source. Published synthetic approaches to the compound are not economically practical for its production, and the product is racemic (Heathcock, 1966; Heathcock et al., 1967; Corey and Watt, 1973). Even if a synthetic sequence is nonstereoselective, it could prove useful if it provided a low-cost route to the compound, for there is no evidence at present that the (−) enantiomer interferes with detection of and response to the (+) enantiomer by the male medfly. The major problem in designing a synthesis of α-copaene is the complexity of the 15-carbon structure. If only a portion of the α-copaene structure were needed to induce a response in the male fly, then the synthetic complexity might be reduced considerably.

One approach to an assessment of substructure importance is to compare the attractancies of (+)- and (−)-α-copaene with those of structurally related sesquiterpenes. There are numerous naturally occurring compounds potentially available for this purpose (Figure 1). The most similar compound structurally is the double-bond isomer, β-copaene. The isopropyl configurational isomer is α-ylangene, which also has a double-bond isomer, β-ylangene. Ring enlargement, converting the isopropyl group to a *gem*-dimethyl, leads to α-longipinene and its double-bond isomer, β-longipinene. If the isopropyl-bearing ring of α-copaene is opened, leaving a 4-methylpent-3-enyl substituent on the methyl-substituted tertiary carbon, *trans*-α-bergamotene is obtained. Again, *trans*-β-bergamotene is the double-bond isomer. If several bonds in α-copaene are shifted, enlarging the cyclobutane ring to cyclopentane and forming a cyclopropane ring at the former double-bond position, the saturated tetracyclic compound cyclocopacamphene is produced (the corresponding monoterpene compound pair are α-pinene and tricyclene). The isopropyl configurational isomer of cyclocopacamphene is cyclosativene. Finally, if the isopropyl-bearing ring of either of these two latter compounds is enlarged, converting the isopropyl group to a *gem*-dimethyl, longicyclene is obtained. The $C_{15}H_{24}$ structures in Figure 1 depict those sesquiterpenes with structural features most similar to those of α-copaene.

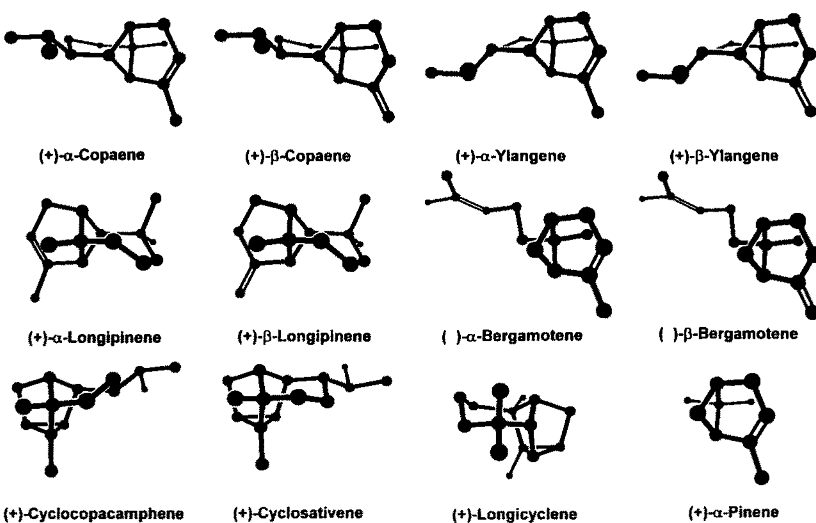

FIG. 1. Absolute stereochemistry of (+)-α-copaene and closely-related $C_{15}H_{24}$ structural analogs; (+)-α-pinene is included for reference. The α- and β-bergamotene absolute stereostructures could not be found in the literature, so the configurations shown are only relative. Structures were drawn using Alchemy III (TRIPOS Associates), employing the minimization routine to determine the minimum energy conformation for each molecule, then "ball and stick" and "perspective" display options to generate a pseudo-three-dimensional depiction of each molecule.

Since several of these compounds have been isolated by us in related studies, while some of the others can be found in various essential oils or are commercially available, we decided to purify those readily obtainable and submit them for field bioassays, comparing their attractancies to that of dextrorotatory α-copaene, which we had previously isolated from angelica seed oil [(+)/(−) = 98.6/1.4; Flath et al., 1994]. If a given structural change is found to have little effect upon the attractiveness of the resulting compound, then that portion of the α-copaene molecule may not be critical to the male medfly's detection of and response to α-copaene.

In this paper, the terms "dextrorotatory" and "levorotatory" are used when referring to bulk isolates having positive or negative specific rotations. The prefixes "(+)" and "(−)" are used when referring to specific enantiomer spatial structures.

Each of the compounds mentioned above can exist in two enantiomeric forms, those shown in Figure 1 and their mirror images [we could locate no literature references unambiguously depicting the absolute stereochemistry of

(+)-*trans*-α-bergamotene; the configuration shown in Figure 1 is a relative one only]. Figure 1 therefore actually provides the structures of 21 analogs of (+)-α-copaene, including (−)-α-copaene. The $C_{15}H_{24}$ structures in Figure 1 contain from three (bergamotenes) to seven (cyclocopacamphene and cyclosativene) asymmetric carbons. Fortunately the rigid structures tie the centers of asymmetry together, so they cannot be inverted individually, leading to even more possible structural isomers. Experimentally, it was impossible to fully address the topic of enantiomers in this study by obtaining samples of each of the 21 possible structures for bioassay comparisons. Information about the enantiomeric makeup of naturally occurring sequiterpenes identified in the literature is minimal at best. Most literature identifications are based upon GC-MS data, but even when components are isolated for characterization, only their specific rotations are noted (if optical activity is even addressed). There is therefore no way to select suitable sources for the desired enantiomers. Nine of the 11 structural analogs (or 21 possible enantiomeric structures) were purified and compared with (+)-α-copaene in field bioassays.

METHODS AND MATERIALS

Compound Sources. The samples tested are listed in Table 1, along with their sources, purities, enantiomeric distributions and specific rotations.

Sample Isolation and Purification. The trimedlure was purchased from UOP. Commercial trimedlure is a mixture of eight structural isomers, each of which consists of a racemic mixture of two enantiomers (Warthen et al., 1993). α-Longipinene, cyclosativene, and longicyclene were purchased from Fluka Chemical Corp., who isolated them from *Pinus longifolia*. They were used without further treatment. The other components were isolated from angelica seed oil, cubeb oil, lemon oil, "orange juice carbonyls" (OJC; a commercially available fraction of orange essence oil), or the dried fruits of *Schisandra chinensis* ("Wu wei zi" in Chinese herbal medicine). The oils were fractionated by a combination of preparative silica gel chromatography and vacuum distillation. Short-path distillation was employed to remove monoterpenes, and Teflon spinning band fractional distillation was used to provide purified samples of each of the two α-copaene enantiomers. In several instances final isolations were achieved with silver nitrate-impregnated silica gel chromatography (15% w/w), or by preparative GC on methylsilicone- and on Carbowax 20 M-coated substrates. Typical procedures have been outlined in a previous publication (Flath et al., 1994). Isolation of α-ylangene from *S. chinensis* fruit included simultaneous steam distillation–ether extraction (Flath and Forrey, 1977) of the finely ground dried berries, followed by vacuum distillation of the resulting concentrate, then silica gel fractionation. GC analysis of α-ylangene-containing

TABLE 1. ISOLATED ANALOGS OF (+)-α-COPAENE

Compound	Reference	Source	GC Purity		Enantiomer Distribution[a]		Specific rotation[b]
			DB-1	DB-Wax	Plus	Minus	
trans-α-Bergamotene[c]							
dextro[d]	T45-137-4	Angelica	98.5	97.8	95.7	4.3	+49.57 (c 1.70)
levo	MFA1-186-4C	Lemon	99.1	98.3	0.0	100.0	−53.21 (c 1.70)
α-Copaene							
dextro	MFA1-54-4	Angelica	95.3	96.1	98.6	1.4	+5.92 (c 2.13)
levo	MFA1-46-12	Cubeb	98.7	98.2	0.3	99.7	−5.39 (c 2.06)
β-Copaene[e]							
dextro	MFA1-158-1	Angelica	96.0	94.4	61.4	38.6	+0.10 (c 1.56)
	MFA1-158-3	OJC	97.8	99.0	95.4	4.6	+9.97 (c 0.74)
Cyclocopacamphene[e] dextro	MFA1-158-4	OJC	99.4	99.4	no separation		+41.46 (c 2.40)
Cyclosativene[e] dextro	MFA1-158-5	*P. longifolia*	99.5	99.3	no separation		+77.81 (c 3.20)
Longicyclene[e] dextro	MFA1-160-1	*P. longifolia*	96.8	95.7	no separation		+30.35 (c 1.88)
α-Longipinene[e,f] dextro	MFA1-160-3	*P. longifolia*	97.4	97.6	no separation		+30.12 (c 1.82)
α-Ylangene[e] dextro	FFA2-84-2	*S. chinensis*	96.2	96.5	100.0	0.0	+55.62 (c 2.19)
β-Ylangene[e] dextro	MFA1-158-2	Angelica	97.3	96.2	91.9	8.1	+46.47 (c 1.01)

[a] Determined with Cyclodex B capillary column.
[b] Calculated Specific Rotations not corrected for impurities.
[c] Neither enantiomer of trans-β-bergamotene was available for testing.
[d] Dextrorotatory trans-α-bergamotene was not isolated in sufficient quantity for field bioassay (Flath et al., 1994).
[e] Levorotatory samples were not available for testing.
[f] Neither enantiomer of β-longipinene was available for testing.

fractions was complicated by the compound's thermal instability. In a hot injector (210°C), a portion of the injected sample rearranged into a number of unidentified products. This problem was circumvented by switching to a cool on-column injector, and injecting samples as dilute hexane solutions.

Sample Analysis, Identification, and Quantitation. Identifications were based upon GC-FID and GC-MS examinations of fractions and purified components, comparing experimental retention index and mass spectral data with those of authentic samples [See Flath et al. (1994) for instrumentation specifics and Table 1 enantiomer ratio determinations, using a Cyclodex B capillary column (J & W Scientific)]. Thin-film infrared spectra were compared with literature reference spectra in order to further validate identifications. Both β-copaene and β-ylangene from angelica seed oil were converted to the α-isomers to further verify their identities and to permit determination of their enantiomeric ratios (Flath et al., 1994). The β-isomers could not be resolved into separate enantiomer peaks with the cyclodextrin-based capillary column.

Bioassay Design. The field bioassay design and test insect status are described in detail in our previous publication (Flath et al., 1994). Briefly, test samples (five replications of each sample) were applied to dental wicks suspended in Jackson traps. Sample loadings of 0.010 ml/wick were used in all of the field tests except the one summarized in Table 2, where 0.020 ml/wick loadings were used. Sterile flies (5–10 days old; male–female ratio approx. 1 : 1) obtained from the USDA-ARS mass rearing facility in Hawaii were released uniformly (30–60 thousand flies/day) in a randomized field plot design trap array hung in a macadamia nut orchard near Hilo, Hawaii. The sticky insert cards from the Jackson traps were removed each day, and replaced with another insert before the next fly release.

RESULTS AND DISCUSSION

Results from three of the field bioassay tests are summarized in Tables 2–4. Table 4 of our previous publication (Flath et al., 1994) presented data from a field test that included the remaining Table 1 test samples: two β-copaene isolates, β-ylangene, cyclocopacamphene, and cyclosativene. Three samples (trimedlure, angelica α-copaene, and cubeb α-copaene) were included in all four of these bioassay experiments, both to provide points of reference when comparing bioassay results from different tests and to further clarify the relative attractiveness of the three samples under the test conditions employed. Mean first-day catches with trimedlure were typically lower than fly catches on subsequent days. This may be due to overly high release rates of vaporized trimedlure from the freshly loaded traps, inducing some repulsive response in the test flies. There is little indication of a similar effect with the angelica α-copaene

TABLE 2. MALE FLY CATCH–REFERENCE GROUP PLUS α-YLANGENE (5 REPLICATIONS)

Treatment (0.020 ml/wick)	Purity (%)[a]	Enantiomer ratio (+)	Enantiomer ratio (−)	Post treatment day (mean + SE)[b] Day 0	Day 1	Day 2	Day 3	Day 4	Day 7	Day 8
Trimedlure				137.60 ± 29.93bc	174.20 ± 25.06b	303.00 ± 16.57a	246.20 ± 20.70a	138.00 ± 39.11b	3.20 ± 0.49b	1.60 ± 0.68b
Angelica α-copaene—										
MFA1-54-4	95.3	98.6	1.4	287.20 ± 17.57a	314.40 ± 14.58a	318.80 ± 24.89a	189.00 ± 30.60b	212.60 ± 29.17a	247.40 ± 47.87a	174.80 ± 38.85a
Starke Angelica seed oil										
(neat)				115.80 ± 24.72bc	16.60 ± 1.63d	3.60 ± 0.75b	3.40 ± 0.60c	0.80 ± 0.58c	0.60 ± 0.40b	1.80 ± 1.20b
Cubeb α-copaene—										
MFA1-46-12	98.7	0.3	99.7	89.80 ± 20.86c	64.20 ± 17.90cd	2.80 ± 0.86b	1.40 ± 0.24c	1.20 ± 0.73c	1.40 ± 0.75b	0.20 ± 0.20b
S. chinensis α-ylangene—										
FFA2-84-2	96.2	100.0	0.0	166.20 ± 28.01b	129.00 ± 45.27bc	29.20 ± 8.91b	24.20 ± 12.08c	24.60 ± 10.25c	5.80 ± 3.85b	0.60 ± 0.24b
Starke Angelica seed oil cut—										
MFA1-82-2				23.80 ± 7.34d	21.20 ± 7.69d	38.40 ± 17.96b	16.80 ± 13.12c	1.40 ± 0.51c	0.60 ± 0.40b	3.80 ± 3.56b
F (*df*)				19.494 (5, 20)	21.352 (5, 20)	108.034 (5, 20)	36.987 (5, 20)	17.058 (5, 20)	25.432 (5, 20)	22.185 (5, 20)
EMS				1995.700	3015.047	1065.417	1585.857	2401.300	1968.937	1127.190
kLSD				54.374	66.633	38.629	47.688	59.939	53.577	40.693

[a] Determined with DB-1 column.

[b] Analysis of variance. Mean separation by Waller-Duncan (1969) kLSD test. Mean catches within a column followed by the same letter are not significantly different ($P < 0.05$).

TABLE 3. MALE FLY CATCH–REFERENCE GROUP PLUS COPAENE ANALOGS (5 REPLICATIONS)

Treatment (0.010 ml/wick)	Purity (%)[a]	Enantiomer ratio (+)	Enantiomer ratio (−)	Posttreatment day (mean ± SE)[b] Day 0	Day 1	Day 2	Day 3	Day 4
Trimedlure				105.00 ± 20.28c	280.80 ± 19.66a	56.40 ± 18.91b	0.40 ± 0.40b	0.80 ± 0.37b
Angelica α-copaene— MFA1-54-4	95.3	98.6	1.4	230.40 ± 6.12a	186.20 ± 40.61b	88.40 ± 14.78a	60.40 ± 23.07a	36.40 ± 9.78a
Cubeb α-copaene— MFA1-46-12	98.7	0.3	99.7	79.00 ± 11.51c	3.40 ± 2.66c	1.20 ± 0.97c	0 ± 0b	0.40 ± 0.24b
Angelica β-copaene— MFA1-158-1	96.0	61.4	38.6	191.00 ± 22.88b	305.20 ± 33.59a	55.20 ± 11.70b	19.80 ± 11.60b	6.20 ± 2.75b
P. longifolia longicyclene— MFA1-160-1	96.8	not resolved		4.20 ± 2.62d	0 ± 0c	0.40 ± 0.40c	0.20 ± 0.20b	0.20 ± 0.20b
P. longifolia longifolene— MFA1-160-2	100.0	not resolved		2.00± 2.00d	0 ± 0c	0 ± 0c	0± 0b	0.80 ± 0.58b
P. longifolia longipinene— MFA1-160-3	97.4	not resolved		12.40 ± 5.64d	0.80 ± 0.20c	0 ± 0c	0 ± 0b	0.80 ± 0.20b
F (*df*)				69.564 (6, 24)	51.202 (6, 24)	15.084 (6, 24)	5,451 (6, 24)	11.607 (6, 24)
EMS				615.514	1962.236	455.126	475.126	76.712
kLSD				29.092	52.087	25.826	28.738	10.751

[a] Determined with DB-1 column.
[b] Analysis of variance. Mean separation by Waller-Duncan (1969) kLSD test. Mean catches within a column followed by the same letter are not significantly different ($P < 0.05$).

TABLE 4. MALE FLY CATCH—REFERENCE GROUP PLUS COPAENE ANALOGS (5 REPLICATIONS)

Treatment (0.01 ml/wick)	Purity (%)[a]	Enantiomer ratio		Posttreatment day (mean ± SE)[b]				
		(+)	(−)	Day 0	Day 1	Day 2	Day 3	Day 4
Trimedlure				159.80 ± 20.74b	287.80 ± 60.81a	181.00 ± 26.29a	72.00 ± 39.45b	10.40 ± 3.14b
Angelica α-copaene—								
MFA1-54-4	95.3	98.6	1.4	396.20 ± 33.29a	181.00 ± 42.45b	133.60 ± 6.39b	160.6 ± 12.03a	127.2 ± 16.80a
Cubeb α-copaene—								
MFA1-46-12	98.7	0.3	99.7	129.20 ± 38.67b	0.20 ± 0.20c	0 ± 0c	0 ± 0c	0 ± 0b
Lemon *trans*-α-bergamotene—								
MFA1-186-4C	99.1	0.0	100.0	104.00± 16.10bc	0± 0c	0 ± 0c	0 ± 0c	0 ± 0b
Starke Angelica seed oil cut—								
MFA1-82-2				58.80± 19.83c	63.00 ± 14.66c	15.20 ± 3.23c	1.00 ± 0.77c	0 ± 0b
F (*df*)				26.772 (4, 16)	16.526 (4, 16)	47.810 (4, 16)	16.246 (4, 16)	56.017 (4, 16)
EMS				3252.300	4761.850	760.490	1543.685	278.960
kLSD				70.210	86.405	33.405	49.234	20.301

[a] Determined with DB-1 column.

[b] Analysis of variance. Mean separation by Waller-Duncan (1969) kLSD test. Mean catches with a column followed by the same letter are not significantly different ($P < 0.05$).

[predominantly the (+) enantiomer: +/− = 98.6/1.4], or with the cubeb α-copaene [predominantly the (−) enantiomer: +/− = 0.3/99.7]; in most field trials fly catches were highest on the first day. The only exception appears in Table 2, where the mean catch with angelica α-copaene on the first day was slightly smaller than that on the next day. There are major differences in the relative effectiveness of the angelica and cubeb α-copaenes, however. Not only did traps baited with angelica material collect three to six times as many flies on the first day as did the traps containing cubeb α-copaene, but on following days the angelica material continued to demonstrate attractiveness, while the cubeb α-copaene traps caught few if any flies after the second day. Clearly, the male medflies used in the field bioassays were discriminating between the two possible enantiomers of α-copaene, responding almost exclusively to the (+) enantiomer.

In general, when attempting to assess the relative attractiveness of sesquiterpene samples from the bioassay results, it appears to be more useful to compare fly catches after the first day. During the first day, all sample traps caught some flies, no matter what the sample. This suggests that there may be some nondiscriminating response induced in the flies when exposed to high air concentrations of such compounds. As the release rate of the samples from the individual traps decreases with time, the flies appear to be more selective in their attractive responses to the individual sample vapor gradients. All of the sesquiterpenes tested have similar volatilities, so differences in fly catches should be indicators of relative attractiveness among the samples tested, and should not be appreciably influenced by differences in sample release rates.

The structural analog most closely related to α-copaene is β-copaene, with an exocyclic methylene rather than a methyl-substituted endocyclic double bond. Two dextrorotatory β-copaene samples were available for testing (see Table 4 of Flath et al., 1994). The angelica-derived sample was a mixture of both enantiomers, 61.4% (+) and 38.6% (−). The other, isolated from orange essence oil material (OJC), was 95.4% (+). An insufficient quantity of the latter sample was available to fully load all of the replicate traps; one of the five replications was underdosed, so male fly catch totals for this sample were probably slightly depressed. Both samples trapped more flies on the second day than on the first, but the counts then dropped off on following days. Differences in enantiomeric purity [% (+)] did not cause significant differences in fly catch; if anything, the less-pure angelica sample caught higher numbers of males. Overall, the results in Table 4 of the previous publication indicated that (+)-β-copaene was less attractive than (+)-α-copaene and that this difference was more pronounced as time passed. By the end of day 3, the β-copaene catches had dropped to background, while the (+)-α-copaene isomer retained considerable attractiveness.

α-Ylangene, the isopropyl configurational isomer of α-copaene, was also

reported to be an attractive component of angelica seed oil. Fornasiero et al. (1969) and Guiotto et al. (1972) reported that they found both compounds equally attractive in their laboratory bioassays. The enantiomeric composition of the α-ylangene in the angelica oil used in our present study was estimated to be approximately 85% (+), 15% (−) (Flath et al., 1994). Unfortunately, this compound is present at too low a concentration (0.03%) in angelica seed oil to make practical its isolation from the oil in sufficient quantity for field bioassay. Several grams of dextrorotatory α-ylangene [all (+); no (−) enantiomer detected] were isolated instead from the dried fruits of *Schisandra chinensis*, the source used by Motl et al. (1965) and Ohta and Hirose (1969) in their original determinations of the structure and stereochemistry of α-ylangene. When *S. chinensis*-derived (+)-α-ylangene was evaluated along with trimedlure, the (+)- and (−)-α-copaenes, and neat angelica seed oil in a field bioassay, it was found to be appreciably attractive to male medflies, although not to the same degree as (+)-α-copaene (Table 2). The listed results partly corroborate the findings of Fornasiero et al. (1969) and Guiotto et al. (1972), who reported both α-ylangene and α-copaene from Angelica seed to be attractive and to be equally effective. (The angelica seed oil cut MFA1-82-2 was included in several of the bioassays, but is not germane to this discussion. It is predominantly humulene, but includes a number of other sesquiterpenes, including several percent of β-copaene.)

We were unable to isolate and compare angelica α-ylangene directly with its double bond isomer, β-ylangene, which we did isolate from the oil. The (+) enantiomer predominates in this latter sample [91.9% (+)-β-]. Bioassay data in Table 4 of Flath et al. (1994) indicated that (+)-β-ylangene was initially attractive, although less so than (+)-β-copaene. After the third day, attractiveness was no longer apparent.

In cyclocopacamphene and cyclosativene, the α-pinene-related portion of the α-copaene and α-ylangene structures is converted to a tricyclene-related segment; the ring bearing the isopropyl group is unchanged. This modification appears to completely disrupt the attractiveness of the two molecules, since neither cyclo compound was found to produce any significant male fly catches [Table 4 of Flath et al. (1994)]. Enantiomeric differences cannot be completely addressed, because neither of the two compounds could be resolved into enantiomeric pairs on the cyclodextrin column. However, a −42° specific rotation has been reported for the product of a stereoselective synthesis of (−)-cyclocopacamphene (Piers et al., 1975), and the sample used by us has a specific rotation of +41.46°. This would indicate that the OJC cyclocopacamphene is largely, if not totally the (+) enantiomer. Piers and coworkers also prepared (+)-cyclosativene and reported a specific rotation of +84°. The cyclosativene obtained from Fluka and used in the bioassay has a specific rotation of +77.81° and is thus predominantly the (+) enantiomer.

The α-pinene portion of α-longipinene's structure is identical to that of

α-copaene and α-ylangene. However, the isopropyl-bearing ring of the latter two compounds is enlarged in α-longipinene to include the tertiary carbon of the isopropyl grouping, forming a four-carbon bridge with *gem*-dimethyl substitution (Figure 1). Again, no enantiomer separation could be achieved with the cyclodextrin column; however, the specific rotation of the α-longipinene sample was +30.12°, and a literature value of +36.9° has been reported for a sample isolated from *Pinus sylvestris* (Westfelt, 1967). Bioassay data in Table 3 indicate that this skeletal modification eliminates the bioactivity of (+)-α-copaene [and presumably, of (+)-α-ylangene]. After a small initial fly catch, the totals for following days dropped to background levels. Longicyclene is related to cyclocopacamphene and cyclosativene as α-longipinene is related to α-copaene and α-ylangene; it has a four-carbon bridge bearing a *gem*-dimethyl group, rather than a three-carbon bridge with an isopropyl group. Since neither of the cyclo compounds displayed any appreciable bioactivity, it is not surprising that longicyclene is inactive as well (Table 3). A specific rotation of +33.6° has been reported (Nayak and Dev, 1968) for longicyclene. A value of +30.35° was determined for the sample used in the field test. Longifolene was included in the field tests largely because it was available and because it has some structural features in common with longipinene. There are, however, major differences in structure between longifolene and α-copaene.

If the isopropyl-substituted ring of α-copaene or α-ylangene is opened, the *trans*-α-bergamotene structure is obtained. While the α-pinene substructure retains its rigidity, the remaining portion of the molecule is now free to rotate in space, constrained only by the planarity of the trisubstituted double bond. The *trans*-α-bergamotene enantiomer having the same absolute configuration for its α-pinene substructure as that found in (+)-α-copaene would be the preferred one for testing. We have been unable to find any literature source fully defining the absolute configuration of either *trans*-α-bergamotene enantiomer, so we cannot say with certainty that the α-pinene portion of (+)-*trans*-α-bergamotene has the same absolute stereochemistry as the α-pinene portion of (+)-α-copaene. A small quantity of *trans*-α-bergamotene isolated from angelica seed oil in our previous study (Flath, et al., 1994) had a specific rotation of +49.57° and an enantiomer ratio of 95.7% (+), 4.3% (−). Unfortunately, insufficient material for field bioassay tests could be isolated from the seed oil. The *trans*-α-bergamotene used in the current field bioassay (Table 4) was instead isolated from lemon oil, one of the few readily available sources containing appreciable concentrations of *trans*-α-bergamotene. However, this material is the opposite enantiomer to that predominating in angelica oil. The lemon oil-derived sample's specific rotation was −53.21°, and cyclodextrin capillary GC indicated that it is solely the (−)-enantiomer. Male fly response to this compound was similar to that seen with (−)-α-copaene: an appreciable first day catch, followed by no fly catches after the first day.

In summary, the results above indicate that any deviation from the absolute stereochemistry of (+)-α-copaene leads to a $C_{15}H_{24}$ product that is likely to be much less attractive to the male medfly. This study does not directly address the possibility that simpler analogs having fewer carbon atoms might still display useful activity, since we limited our test samples (except for trimedlure) to naturally occurring $C_{15}H_{24}$ analogs. As such, whenever a partial structure is changed, a new three-dimensional substructure is unavoidably introduced. The bioassay results are really a reflection of the effects of this total change on the attractiveness demonstrated by (+)-α-copaene itself; (−)-α-copaene being a special situation in which only the absolute stereochemistry of the intact "parent" molecule is changed. However, there is no evidence to indicate that such new three-dimensional substructures are in any way repellent to the male medfly, so the reduced attractiveness of the various tested analogs likely reflects the effects of disrupting the (+)-α-copaene stereostructure.

Based upon the data in Tables 2–4 (this publication) and Table 4 of Flath et al. (1994), it is possible to group the 10 tested samples from Table 1 according to their observed attractancy to the male medfly. The dextrorotatory angelica α-copaene [(+)/(−) = 98.6/1.4] stands alone as the most potent attractant and is the most long-lived in the traps. The three structurally related dextrorotatory samples, *S. chinensis* α-ylangene [(+)/(−) = 100.0/0.0], OJC β-copaene [(+)/(−) = 95.4/4.6], and angelica β-ylangene [(+)/(−) = 91.9/8.1], are of roughly equivalent attractancy but are considerably less effective than dextrorotatory α-copaene in attracting male flies to traps. In addition, their observed attractancy is relatively short-lived. Levorotatory cubeb α-copaene [(+)/(−) = 0.3/99.7] falls at the lower end of this second group. It is basically the mirror-image enantiomer of the (+)-α-copaene predominating in the most potent sample. The field tests demonstrated some low level of activity, but only for the first day or two. The remaining five test samples were essentially nonattractive to the male medfly, especially if first-day mean catches are considered to be the result of nondiscriminating fly responses to uncommon volatiles. These five include the three dextrorotatory *P. longifolia*-derived samples cyclosativene, longicyclene, and longipinene; the dextrorotatory OJC cyclocopacamphene; and the levorotatory lemon *trans*-α-bergamotene [(+)/(−) = 0.0/100.0].

When comparing the mean male fly catches for angelica α-copaene with those for the other test samples, not only do the data indicate that it is more potent than the other samples, but also that it exhibits this activity for a significantly greater time interval when tested in the trap design employed in this study. This longevity is most pronounced in the Table 2 data, where the trap loading was 0.020 ml/wick, rather than 0.010 ml/wick, as in the other field tests. Because of the numerous physiological and environmental factors that may influence fly behavior, it is hazardous to compare data obtained from different field bioassays. However, it appears that the higher sample loading (and

presumably higher initial release rates) did not induce higher mean male fly catches during the first days of the bioassay, when compared with those recorded in the other three field bioassays, where sample amounts were one half as large. Instead, the 0.020-ml loading ensured trap effectiveness over a greater total number of days. Therefore, under the conditions determined by the bioassay design, site location, and choice of trap, the dextrorotatory angelica α-copaene could probably be used more efficiently in combination with a fixative or retardant to moderate the initial sample release rate and extend its trapping effectiveness lifetime over a still longer interval.

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REPEATABILITY OF FEMALE RESPONSE TO IPSDIENOL ENANTIOMERIC MIXTURES BY PINE ENGRAVER, *Ips pini* (COLEOPTERA: SCOLYTIDAE)

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Abstract—Repeatability, a measure of the proportion of variance in a character that occurs among rather than within individuals, is assessed for the phenotypic trait of female response preference for enantiomeric blends of ipsdienol in *Ips pini* at two pheromone concentrations—1 μg and 5 μg of ipsdienol. Average female response shows greater repeatability at the higher pheromone dosage when females are tested in two successive sets of five assays than when assayed in three successive sets or at the lower dosage. Repeatability within each set of five assays is highest for the first set and decreases thereafter. Thus the response phenotype of females for enantiomeric blends of ipsdienol in this experiment is context dependent; female choice of an enantiomeric blend differed between dosages and among sets of assays.

Key Words—*Ips pini*, Coleoptera, Scolytidae, repeatability, female choice, response phenotype, bark beetle, pheromone, communication systems, multiple measurements, individual variation, ipsdienol, enantiomers.

INTRODUCTION

Repeatability, a measure of the proportion of variance in a character that occurs among rather than within individuals, is an important statistic for population and ecological geneticists. Not only can repeatability be used to assess the reliability of multiple measurements on an individual, but it also provides an upper limit to the value of heritability, i.e., the degree to which phenotypes are determined by the genes transmitted from the parents (Falconer, 1981). The

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study of communication systems, whether the system is of a visual, auditory or chemical nature depends upon the ability of the investigator to accurately measure the values of a trait and the response to (or preference/nonpreference for) those values (Mayr, 1972). Likewise, the evolution of a communication system depends upon the phenotypic variation among individuals and the degree to which natural selection can act on this variation. By partitioning the phenotypic variation observed in a population into variation due to differences among individuals and that found within individuals, a greater understanding of how the system is evolving can be attained.

The repeatability of a signal (trait) produced by an individual has been examined directly or indirectly for a wide variety of animals in auditory communication systems (Gerhardt, 1991; De Winter, 1992; Gratson, 1993; Tuckerman et al., 1993) and in the pheromonal communication systems of moths (Barrer et al., 1987; Du et al., 1987; Witzgall and Frerot, 1989) and bark beetles (Teale et al., 1994). Fewer studies have examined the repeatability of individual response (preference); of these, repeatability was examined in an indirect manner through mark–recapture (Cardé et al., 1976), heritability studies (Roelofs et al., 1986; Collins and Cardé, 1990), or in studies of learning ability (Kyriacou et al., 1992; De Winter and Rollenhagen, 1993) (but see Tuckerman et al., 1993). Only Boake (1989) directly measured the repeatability of responses of *Tribolium castaneum* to conspecific pheromones.

The male of *Ips pini*, upon locating a suitable host and beginning excavation of a nuptial chamber, produces an aggregation pheromone consisting predominantly of ipsdienol (2-methyl-6-methylene-2,7-octadien-4-ol) (Stewart, 1975) and lanierone (4,4,6-trimethyl-2-hydroxy-2,5-cyclohexadiene-1-one) (Teale et al., 1991) that is attractive to conspecifics of both sexes. Ipsdienol is a chiral compound and the ratio of the (*S*)-(+) and (*R*)-(−) enantiomers produced by individual males varies among and within populations (Lanier et al., 1980; Miller et al., 1989). Female response to the enantiomeric blends varies accordingly (Lanier et al., 1972; Birch et al., 1980; Mustaparta et al., 1980, 1985; Teale and Lanier, 1991). Within a population, there is a strong correlation between mates for the enantiomeric blend produced by the male and the female's average response (preference) in a bioassay (Teale et al., 1994).

Herein we investigate the repeatability of a female's response to different enantiomeric blends in an olfactometer that provides the female with eight choices of blends ranging from 2.0 to 97.5% (*R*)-(−)-ipsdienol. Additionally, two dosages are used to ascertain the effect of dosage differences on the estimate of a female's preference.

METHODS AND MATERIALS

Bioassay Apparatus. The response preferences of females to varying compositions of the enantiomers were ascertained by using an olfactometer that presents eight different pheromone blends representing a range of ipsdienol enan-

tiomers: 2%, 16.5%, 30%, 43.5%, 57%, 70.5%, 84%, 97.5% (*R*)-(−)-ipsdienol in a 1 μg/μl solution with ethanol. Lanierone, a minor component of the aggregation pheromone of *Ips pini*, was added to the ipsdienol in a ratio of 1 : 100 (lanierone–ipsdienol). These blends were created at the beginning of the study in sufficient quantity to be used throughout the repeatability assays; concentration ratios were checked with the gas chromatography–mass spectrometer.

The olfactometer was constructed of seven 3-mm-ID Nalgene Y tubes in a sequentially bifurcating pattern with interconnecting pieces of glass tubing of the same inner diameter (Figure 1). The eight pheromone blends were arrayed at the end of each of the four distal Y tubes. Between the last Y tube and the glass tube with the pheromone bait was a pitfall trap constructed of a 3-mm-ID Nalgene T tube. Two pieces of 64-mm-thick plate glass supported the apparatus and kept it level to prevent geotaxis. The apparatus was raised 2.5 cm above the table by three thumb screws to facilitate leveling and to accommodate the T tube pitfall traps. To avoid phototaxis, a dim red light was centered above the olfactometer in an otherwise dark room.

For each assay (six to eight individuals run once through the olfactometer), the placement of the baits was constant. All eight baits (filter paper strips) were impregnated nearly simultaneously with pheromone and attached to the olfactometer while the air flow was blocked. Charcoal-filtered air flowed through the apparatus at a rate of 80 ml/min and, upon exiting, was removed by vacuum. After the initiation of air flow, a 5-min interlude was designated to allow the airflows to equalize in all arms of the tubing and the solvent to evaporate. The

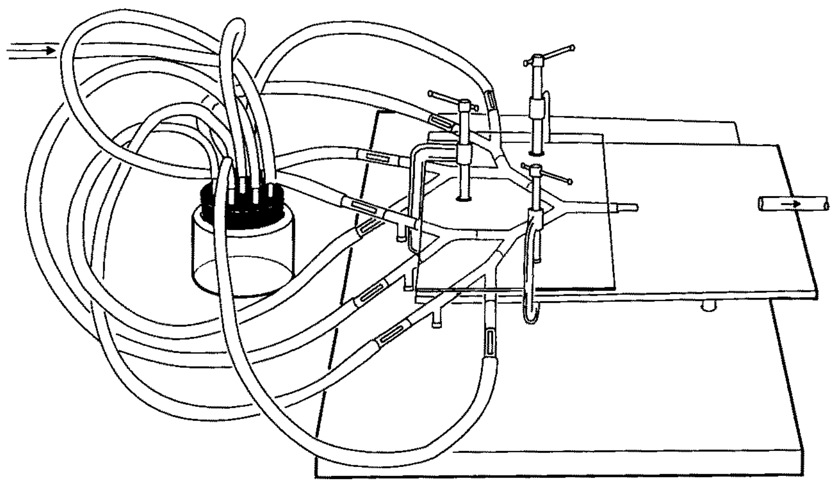

FIG. 1. The olfactometer. All Y tubes and T tubes were made of Nalgene plastic.

used baits were discarded after all beetles (six to eight) were assayed once. Then the olfactometer was reloaded and the enantiomeric blends were rearranged by twisting the tube junctions into a different spatial configuration. Our purpose for rearranging the baits was twofold: (1) at no time was any particular spatial arrangement of pheromone blends repeated during a day's set of assays [a total of five assays of each beetle (= set) was conducted daily] and (2) any particular tube and section of tubes always had the same pheromone running through it to eliminate the possibility of lingering pheromone contamination by a different blend. The tubing was cleaned and baked at 75°C overnight.

Experimental Treatment. At the beginning of the experiment, unrelated females were selected from newly emerged, laboratory-reared progeny of randomly mated pairs of field-collected *Ips pini* from the same population used for phenotypic and genetic correlation studies (Teale et al., 1994; Hager and Teale, unpublished manuscript). Once removed from the emergence cans, the beetles were put on moist filter paper in a Petri dish for 24 hr preceding the commencement of the first set of assays. The females were tested three times (three sets of five assays) over six days. Thus approximately 42 hr passed between the sets of assays for an individual. Between sets, the beetles again were placed individually in Petri dishes lined with damp filter paper but given a 10 × 20-mm white pine phloem disk to feed on for the first 18 hr. In order to keep preassay conditions the same, after 18 hr the food was removed and the beetles were left on moist filter paper for the 24 hr prior to the next set of assays.

A female was placed in the airflow beyond the end of the first Y tube where she could experience the mixture of all blends. As she proceeded up the tubing, her choices became more specific. When the female chose one of the eight blends, she entered the T tube associated with that blend, fell into the pitfall trap, was removed immediately from the apparatus, and placed in a holding dish. A 1-min interval was taken to allow the airflow to reequilibrate before the next female was tested. Approximately 45 min to an hour passed between an individual's successive assays.

Each beetle was assayed five times in a day (= 1 set of assays), and the average of her responses taken as her response phenotype, that is, her preference for a particular enantiomeric blend. The rationale for assaying the female several times and obtaining an average response phenotype is as follows. The percentage of (*R*)-(−)-ipsdienol differed by 13.5% due to the desire to expose the females to the widest range of enantiomeric blends. The necessarily categorical nature of the bioassay is not reflective of the nearly continuous nature of ipsdienol blends a female would be exposed to in the field. For instance, a female's preference may lie somewhere between any two of the eight choices at the end of the apparatus. Her choice between these two in any given assay more than likely would be random. Thus, the mean of multiple tests can more accurately

estimate an individual female's preference as compared to the mode that would reflect the categorical nature of the enantiomeric blends used.

Two dosages were used in the assays: 1 μl (= 1 μg) and 5 μl (= 5 μg) of ipsdienol enantiomeric blends. Females were only tested under one or the other dosage—no female was subjected to both dosage treatments. Although initially more than 20 beetles were assayed at least through the first set of assays, due to death, escape, or nonresponse of a beetle (eight, one, and five beetles, respectively), only 20 females for each dosage were used in most of the analyses as that was the number of beetles that completed all three sets of assays.

Data Analysis. A repeated measures analysis of variance was performed on the data for each dosage treatment and for either two or three measurements (sets of assays). Repeatability (R) was determined using the formula given by Becker (1984) where

$$R = \frac{\sigma_w^2}{\sigma_{w'}^2 + \sigma_e^2}$$

and σ_w^2 is the mean square error within treatments (MS_w) minus the mean square error among measurements (MS_e) for the individuals (σ_e^2) divided by the number of measurements per individual. Thus σ_e^2 represents the differences among measurements within the individuals and σ_w^2 represents the differences among individuals. In this case, a high value of repeatability indicates that most of the differences in response are attributable to the differences among individuals rather than differences between the successive measurements done on each of the individuals. Standard errors of this measurement of repeatability were also calculated using the formula presented by Becker (1984).

Repeatability was examined at two levels for each dosage. The first was repeatability of a beetle's response within a set of five assays. This gives an estimate of how repeatable an individual's response is in a single day. Recall that if a female's true phenotypic response preference is between two of the presented ratios, then she may go to either one in any given assay. Thus, due to the constraints of using wide differences in enantiomeric blends in the assays, repeatability is expected always to be lower than the ideal value of 1 within a set of assays. The second level of repeatability is that of mean response of an individual across each set of assays; it indicates how closely the choices of beetles repeat themselves in subsequent sets of assays.

RESULTS

Both the number of measurements (two versus three sets of assays) and the ipsdienol dosage affect the estimate of repeatability (Table 1). Repeatability estimates are higher when the mean response of the females is compared for the

TABLE 1. ANALYSIS OF VARIANCE TABLE AND REPEATABILITY MEASUREMENTS OF FEMALE RESPONSE AT DIFFERENT PHEROMONE DOSAGES FOR TWO AND THREE SETS OF ASSAYS

Trials (N)	Ipsdienol dosage (μl)	Source of variation	df	Sums of squares	Mean squares	F ratio and P value	Repeability estimate ($R \pm$ SE)
2	1	among individuals	19	3864.98	203.42	$F = 2.29$	0.40 ± 0.19
		within individuals	20	1779.00	88.95	$P < 0.072$	
	5	among individuals	19	12334.30	649.17	$F = 13.31$	0.86 ± 0.06
		within individuals	20	975.89	48.79	$P < 0.0001$	
3	1	among individuals	19	4988.46	262.55	$F = 1.89$	0.23 ± 0.08
		within individuals	40	5553.03	138.83	$P < 0.10$	
	5	among individuals	19	19508.13	1026.74	$F = 1.90$	0.23 ± 0.15
		within individuals	40	21648.84	541.22	$P < 0.10$	

first two sets; repeatability is also greater in the 5-μl treatment than the 1-μl treatment. Repeatability estimates decrease when mean response is compared for all three sets at either dosage.

Although the same females were used in the three sets of assays for a single dosage treatment, the overall mean response across the three sets differed for the 1-μl dosage but not for the 5-μl concentration (Table 2). The lack of change in the estimate of repeatability of mean response between two and three sets at the 1-μl dosage may be confounded by the apparent response to the ipsdienol blends composed of a higher percentage of the negative enantiomer. Despite the differences in the mean percentage of (*R*)-(−)-ipsdienol that females responded to in the 1-μl treatment, for both dosages there was a consistent decrease in repeatability of the mean response of females between sets as successive sets were conducted (Table 3). In each dosage treatment, the change in repeatability of response is approximately halved between the first and third sets of assays. An unknown change in the percentage of (*R*)-(−)-ipsdienol in the pheromone

TABLE 2. AVERAGE FEMALE RESPONSE VALUES EXPRESSED IN PERCENTAGE OF (*R*)-(−)-IPSDIENOL FOR FEMALES IN EACH SET OF FIVE ASSAYS AT TWO DIFFERENT DOSAGE TREATMENTS[a]

Pheromone dosage (μg)	Average response [% (*R*)-(−)-ipsdienol]			ANOVA results, repeated measures
	Trial 1	Trial 2	Trial 3	
1	39.6 ± 13.5a 22.4	47.8 ± 13.7ab 26.2	51.0 ± 11.4b 22.4	$F = 5.08$; 2, 38 *df*, $P < 0.011$
5	50.3 ± 19.2a 38.6	51.7 ± 18.2a 37.7	53.2 ± 14.4a 30.8	$F = 0.569$; 2, 38 *df* $P < 0.57$

[a] Means with different letters are significantly different. Values underneath each mean are the coefficients of variation.

TABLE 3. REPEATABILITY MEASURES FOR MEAN RESPONSE OF FEMALES BETWEEN ANY TWO GIVEN TRIALS AT TWO DOSAGE TREATMENTS

Trials	Repeatability (±SE)	
	1 μg ipsdienol	5 μg ipsdienol
1 vs. 2	0.40 ± 0.19	0.86 ± 0.06
2 vs. 3	0.20 ± 0.22	0.62 ± 0.14
1 vs. 3	0.16 ± 0.22	0.47 ± 0.18

baits cannot account for the observed differences because the same pheromone mixtures were used throughout the experiment and we alternated the dosage treatment on a weekly basis with the different groups of test females.

The repeatability of a female's response within sets (the five assays) decreases across sets (Table 4). It appears that females become less choosy after successive exposures to the pheromone; repeatability within the third set is significantly lower than in the first set. Only the 5-μl dosage is shown for this analysis for two reasons. First, the overall mean percentage of (*R*)-(−)-ipsdienol to which females responded did not differ among the three sets (Table 2). Second, repeatability within a set of assays at the 1-μl ipsdienol treatment did not differ from zero in any of the three sets.

Lastly, an examination of the frequency distribution of individual mean responses for the first two sets (average of the 10 assays) in the two dosage treatments reveals an interesting pattern. The distribution of the mean response of females from the 10 assays moves away from a bell-shaped curve at the 1-μl dosage towards a bimodal one under the high ipsdienol treatment (Figure 2; contingency analysis; $\chi^2 = 15.39$, 4 *df*, $P < 0.005$; with response below 40% (*R*)-(−) ipsdienol pooled together and those above 60% (*R*)-(−)-ipsdienol pooled together).

DISCUSSION

There are three major findings of this study. First, the amount of ipsdienol used in the bioassay affects the measure of repeatability of response to varying enantiomers. Second, the estimate of repeatability is affected by the number of

TABLE 4. REPEATABILITY MEASURES OF INDIVIDUAL RESPONSE WITHIN A SET OF ASSAYS COMPARED ACROSS 3 SETS AT 5 μg DOSAGE[a]

Trial	Repeatability (±SE)	MS_e	MS_w	Lower confidence limit	Upper confidence limit
First	0.20 ± 0.08 (n = 31)	839.53	1902.6	0.064	0.393
Second	0.109 ± 0.079 (n = 27)	919.35	1478.6	−0.018	0.300
Third	−0.035 ± 0.061 (n = 22)	1063.7	884.35	−0.123	0.135

[a] Sample sizes are given in parentheses. Repeatability measures within a trial for the 1-μg dosage treatment are not reported here because in all three sets of assays they did not differ significantly from zero.

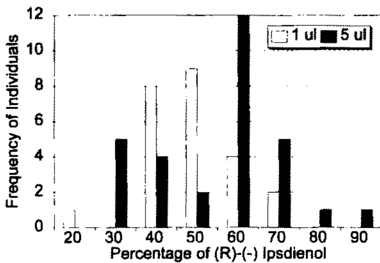

FIG. 2. Frequency distribution of the mean response of individual females to the ipsdienol enantiomeric blends [expressed in % (*R*)-(−)-ipsdienol] for the two different pheromone concentrations. The data for the mean response of females are taken from the average response of each female from the first two sets of assays (= 10 responses/female).

measurements (sets of assays) used to determine a female's response phenotype. Third, an assayed female shows a range of acceptable pheromone blends rather than a single preferred blend when run repeatedly.

Interpretation of studies of dosage effects in several species of moths (Roelofs, 1978; Linn and Roelofs, 1981, 1985; Cardé and Charlton, 1984) and a bark beetle (Stock and Borden, 1983) has led to the general consensus that higher dosages stimulate the receiver to respond to a greater range of pheromone blends (enantiomeric or otherwise) than would "normally" occur at lower concentrations. In these studies the responses of the signal receivers were reported for the study population as a whole. It is unknown whether the observed variation in the response window is a reflection of individual differences or a wide window of response for each individual (Boake, 1989). However, Cardé et al. (1976) and Collins and Cardé (1989) have found that some males are more responsive than others over a wide range of component blends in the oriental fruit moth, *Grapholita molesta*, and the pink bollworm, *Pectinophora gossypiella*.

Our results indicate that, for this population of *Ips pini*, the variation observed in the response window is a reflection of individual differences expressed to a greater extent at the higher dosage than the lower ipsdienol dosage. The high repeatability values, together with the high coefficients of variation in the 5-μl treatment, mean that there is a refinement in the specificity of response by the individual females rather than a mere broadening of response by all females. Indeed, the repeatability statistic provides information on how much variation is due to inter- versus intraindividual variation. Females at the 1-μl dosage are actually less consistent in response; the repeatability estimate within a set of assays does not differ from zero in any of the sets, and the mean response of individuals for 10 assays (the first two sets of assays) cluster around 40–50% (*R*)-(−)-ipsdienol (Figure 2). It is difficult to translate these laboratory

results to a field situation, but if low ipsdienol concentrations equate with small population sizes and if females are under a time constraint to locate a host and mate, then under these conditions females may not be able to afford to be choosy and will respond to, and mate with, a male regardless of her phenotypic "preference" for a particular enantiomeric blend.

There are two non-mutually exclusive explanations for the decrease in repeatability observed in successive sets of assays. First, physiological factors affecting the females may change with the successive sets. Beetle response to pheromones (usually measured as the number of beetles responding to a given treatment) has been shown to vary according to age, desiccation, lipid content of the fat bodies, and flight activity (Atkins, 1966; Bennet and Borden, 1971; Hagen and Atkins, 1975; Gast et al., 1993). Our females were sexually mature before the first set of assays and aged on average six days during the testing period. Although the females were fed and watered between the assay sets, their lipid reserves could have become depleted over time. Additionally, the airflow during the assays could have resulted in desiccation. An equal proportion of beetles (four each) died between the first and second sets as died between the second and third sets, and these deaths may be attributed to severe desiccation.

Nevertheless, these physiological factors cannot be teased apart from the possibility that the decrease in repeatability both between and within sets of assays is due to learning. A female may become less choosy after selecting one to five different blends in a set of assays and not obtaining a mate (Domjan, 1992). Prior experience can influence an individual's response to a pheromone or a particular blend of the pheromone. Collins and Cardé (1989) showed that preexposure to pheromone had no influence on male response of *P. gossypiella* when tested after 24 hr but did affect response when done an hour beforehand. Cardé and Charlton (1984) stated that the threshold of response in moths is raised by repeated exposures to the pheromone. Stock and Borden (1983) found that for the bark beetle *Dryocoetes confusus* prior testing to different pheromone doses did not affect the response in further tests. In nonpheromone signal–response systems, exposure to the signal also affects response [e.g., katydids (Tuckerman et al., 1993), fruit flies (Kyriacou et al., 1992)]. Thus it remains possible that females may be learning and changing their response based on lack of mating success at the various enantiomeric blends chosen earlier.

In conclusion, the estimate of repeatability of response to enantiomeric blends of ipsdienol by *Ips pini* females is a complex function of the number of measurements (sets of assays) and the dosage during the assays. Both male pheromone production (Teale et al., 1994) and female response to specific enantiomeric blends has significant repeatability while still showing some variation. Variability in the character(s) used in mate choice and in the preference for the trait will have major effects on the way in which the system can evolve (Boake, 1989). Therefore, if mate choice models are to be incorporated into biological

control plans, it is imperative that we examine individual variability in pheromone production and response.

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CRAYFISH FEEDING RESPONSES TO ZEBRA MUSSELS DEPEND ON MICROORGANISMS AND LEARNING

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Abstract—Three species of crayfish (*Orconectes virilis*, *O. rusticus*, and *Cambarus robustus*) were tested for feeding responses to potential food odors from mollusks (either zebra mussels, *Dreissena polymorpha*, or native gastropods). In all three crayfish species, feeding responses to odor cues were shown only by individuals experienced with feeding on a prey type. Individuals exposed to just the smell of prey organisms did not show feeding responses, indicating the role of associative learning in diet breadth. Establishment of a learned association took more than one feeding experience but once established lasted more than three weeks. When microbial enzymatic degradation of food protein was eliminated, either by UV radiation or microfiltration, feeding responses were eliminated even for crayfish experienced with a prey type.

Key Words—Feeding, learning, microorganisms, crayfish, *Orconectes virilis*, *Dreissena polymorpha*.

INTRODUCTION

It is widely acknowledged that animals can learn characteristics of items in their diet and that diet breadth is often experience-dependent (Croll and Chase, 1980; Connaughton and Epifanio, 1993; Derby and Atema, 1981; Waldbauer and Friedman, 1968; Wood, 1968). However, the role of learning in the recognition of food by invertebrates is less frequently acknowledged. In addition, although the role of bacteria in ecosystems is well known, the role of bacteria or other agents of degradation in producing food-related cues has been studied in only a few cases (Ikeshoji, 1984; Vissen, 1986). Furthermore, no study has shown that learned responses to food odors are dependent on microorganisms.

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Crayfish are the dominant invertebrates in many freshwater ecosystems (Flint and Goldman, 1975; Momot et al., 1978). They are polytrophic (Hobbs, 1993), feeding predominantly on algal and detrital material scraped from surfaces, but show strong feeding responses to amino acids emanating from animal protein (Tierney and Atema, 1988). It has been suggested that the feeding activity of crayfish can have very strong effects on both whole communities (Chambers et al., 1990) as well as on particular species, such as zebra mussels, *Dreissena polymorpha* (Ludyanskiy et al., 1993).

The native crayfish *Orconectes virilis* has been reported to feed upon *D. polymorpha* in the laboratory (Love and Savino, 1993). Thus initial observations of the lack of response to either intact zebra mussels or the odor of crushed zebra mussels by *O. virilis* that had no experience with that species of mollusk suggested that learning could influence feeding behavior in crayfish. The following experiments were designed to test the role of past experience with food types in the responses of individuals of three species of crayfish (*Orconectes virilis*, *O. rusticus*, and *Cambarus robustus*) to food odor cues. During the course of these experiments, the response patterns of crayfish suggested investigation of the possible role of microbial degradation of food proteins in the generation of food cues.

METHODS AND MATERIALS

The experiments were done in the laboratory in Ann Arbor, Michigan, and at the University of Michigan Biological Station in Pellston, Michigan. The individuals of *Orconectes virilis* (Hagen, 1870) were collected from either the Maple River in northern Michigan or Fleming Creek near Ann Arbor, Michigan. The individuals of *Cambarus robustus* (Girard, 1852) utilized were collected from Paint Creek near Ypsilanti, Michigan. None of these waters supported any zebra mussels *Dreissena polymorpha* (Pallas, 1773) at the time the crayfish were collected; thus the crayfish were naive with regards to that species. The *Orconectes rusticus* (Girard, 1852) used were obtained from a commercial supplier in Wisconsin and had been maintained in the laboratory for two months prior to the start of treatments for these experiments. Zebra mussels were collected from Lake Erie, Monroe Beach, in Monroe, Michigan. The native gastropods *Stegnicola elodes* (Say, 1821) and *Aplexa elongata* (Say, 1821) were utilized in experiments with *O. rusticus* and were collected from a drainage ditch near the Huron River in Ann Arbor.

All the crayfish used in these experiments were adults and were maintained and fed in 30-gal aquaria with constant aeration and filtration. Crayfish were fed daily with either canned tuna (the control *O. rusticus*), commercial shrimp pellets (controls for *O. virilis* and *C. robustus*), or freshly crushed mollusks.

After being fed a prey type for the designated time period (see below), crayfish were placed individually in visually isolated 10-gal aquaria with a clay burrow and 12.5 liters of water, which was continually aerated. After a 24-hr adjustment period, test solutions were introduced to individual crayfish and each was observed for 5 min during the late morning or early afternoon. The latency to first feeding responses and the duration of feeding responses were recorded. Feeding was recognized either by the combination of locomotion, elevated body and raised chelae (while this can be the initial response to some other stimuli, it is the initial response shown to the introduction of food), or by elevated body and rapid scraping movements of the chelated ambulatory legs (Ameyaw-Akumfi, 1977). Ten replicates were run for each treatment.

Statistical analyses were by one-way ANOVA with individual contrasts. All differences reported were associated with $P < 0.05$ and most were associated with $P < 0.01$. Differences in latency and duration among treatments or tests were (almost) always similar, i.e., both were significantly different or both were not significant; thus only duration measures are reported in most situations.

Experience Tests. To test the role of past experience with a food type, individuals were fed for two weeks with freshly crushed mollusks or not fed mollusks. Following this, test solutions were prepared by placing 5 g of prey flesh in 200 ml of distilled water, mixing, and filtering through Fisher P8 coarse filter paper. Twenty milliliters of solution was introduced via syringe into a corner of an individual crayfish's aquarium and data recording started.

There are several mechanisms through which past experience can affect behavior. Associative learning involves the formation of an association or linkage between two features, while sensory sensitization involves changes in how an animal utilizes a single sensory input (Dickinson, 1980). To rule out sensitization as the mechanism for differences in feeding responses with experience, individuals of *O. virilis* that had no experience feeding upon zebra mussels were exposed daily for two weeks to a freshly prepared filtrate of zebra mussel but fed shrimp pellets at different times of the day (to eliminate the formation of an association between zebra mussel smell and food). These animals, which experienced only odor of zebra mussels, were tested with a standard zebra mussel filtrate.

There are two possible mechanisms of the formation of an association by crayfish. This is because individuals of *O. virilis* respond to some prey solutions with a combination of feeding and alarm responses (Hazlett, 1994). Thus, experienced animals could be learning either to associate prey smell with prey taste or could be learning to not respond to alarm substances from the prey and just respond to the food odors. Freeze–thawing apparently destroys the alarm substance (Hazlett, 1994), thus a freeze–thawed filtrate should contain only potential food cues, not alarm chemicals. Animals experienced with odor only were

tested with the freeze–thawed filtrate to distinguish between the two types of associative learning.

The temporal pattern of learning was studied in two ways. Individuals of *O. virilis* that had no experience feeding on zebra mussels were given one feeding experience (one crushed zebra mussel per crayfish overnight) and then placed in an observation aquarium and tested with zebra mussel filtrate. Extinction of responses was tested with crayfish that had two weeks of experience feeding on freshly crushed zebra mussels but then were given no further experience with that species and fed commercial shrimp pellets for 20 days. These individuals were tested with the standard filtrate and then tested a second time 20 days later (40 days after their last experience feeding on mussels).

Microbial Degradation. During a set of pilot experiments, a test solution was stored in a refrigerator for several hours prior to use. The solution produced no feeding responses with experienced crayfish during the standard 5-min period, but about 20 min later full feeding responses were shown by the crayfish. This suggested that cooling had some effect upon the test solutions, and one possibility was that microbial degradation had been slowed down. To test this possibility, two experiments were done. Crayfish experienced with a food type were used in both experiments. Individuals of *O. virilis* were given either standard filtrates of freshly crushed zebra mussels that had been at room temperature for 1 hr or filtrates that had been exposed for 1 hr to short wavelength UV radiation (254 nm) in shallow dishes. To control for the possibility that the radiation altered chemical cues (in addition to killing microorganisms), a UV-treated solution had 1 mg of trypsin (Nasco trypsin) added per 100 ml just prior to introduction of the solutions to the crayfish. This tested for the presence of suitable substrate in the UV-treated filtrate.

In the case of *O. rusticus*, filtrates of freshly crushed gastropods were prepared and then microfiltered through an Acrodisc filter (Gelman Sciences), which has a pore size of 0.2 μm. Bacteria cannot pass through this filter. This microfiltrate was introduced to experienced crayfish. To test for the presence of appropriate substrate in the microfiltrate, solutions were treated with trypsin and then introduced to crayfish. A control of trypsin in distilled water (with no substrate present) was also introduced to experienced crayfish.

RESULTS

When crushed mollusks were introduced to naive crayfish, no feeding responses were seen until contact was made with the mollusk flesh by setae on the crayfish's walking legs or maxillipeds. This was true for all three species of crayfish and both categories of mollusks. Once physical contact was made, the crayfish fed vigorously on crushed mollusks. However, even experienced

individuals of *O. virilis* and *Cambarus robustus* did not feed upon larger (20- to 25-mm-long) zebra mussels living with them. Very large individuals of *C. robustus* (carapace length 55–60 mm) were often observed scraping around the valves of mussels with their mouthparts but did not crush or break the valves during many weeks of postexperimental cohabitation.

Individuals of *O. virilis* that had experience with zebra mussels responded to zebra mussel odors with feeding responses, while crayfish without experience did not (Table 1, experiment 1 vs. 2). This experience involved associative learning rather than some form of sensitization because individual *O. virilis* that were exposed daily for two weeks to just the smell (but not the taste) of crushed zebra mussels behaved similarly to inexperienced crayfish (experiments 2 and 3). Because *O. virilis* shows an alarm response to some freshly crushed prey species (Hazlett, 1994), the role of experience could be to overrule a possible alarm response rather than to associate food odors (detected by the antennules)

TABLE 1. RESPONSES OF CRAYFISH FOLLOWING PRESENTATION OF TEST SOLUTIONS[a]

Experiment number and animal's experience	Test solution	Feeding response duration, sec (±SE)	
)rconectes virilis			
1 Fed ZM	Standard ZM	133	(35)
2 No ZM experience	Standard ZM	9	(8)
3 ZM odor experience	Standard ZM	11	(11)
4 ZM odor experience	Freeze-thaw ZM	27	(11)
5 24 Hours ZM exp.	Standard ZM	43	(15)
6 Fed ZM, 20 days w/o	Standard ZM	145	(34)
7 Fed ZM, 40 days w/o	Standard ZM	26	(9)
8 Fed ZM	Standard ZM	164	(24)
9 Fed ZM	ZM solution UV treated	79	(37)
10 Fed ZM	ZM solution UV treated + trypsin	161	(31)
)rconectes rusticus			
11 Fed snails	Standard snail	131	(31)
12 No snail experience	Standard snail	37	(15)
13 Fed snails	Tuna	212	(8)
14 Fed tuna	Tuna	241	(18)
15 Fed snails	Snail solution microfiltered	37	(11)
16 Fed snails	Snail solution microfiltered + trypsin	169	(39)
17 Fed snails	Trypsin alone	21	(15)
:ambarus robustus			
18 Fed ZM	Standard ZM	150	(35)
19 No ZM experience	Standard ZM	69	(20)

Ten crayfish per experiment. ZM = zebra mussel. *Dreissena polymorpha*.

and food tastes (detected by contact chemoreceptors on other appendages). To test this idea, freeze–thawed preparations of zebra mussel flesh were introduced, but the responses of crayfish that had been exposed only to zebra mussel odor were the same for the freeze–thawed solution and the standard solution (Table 1, experiments 3 and 4). Thus, the mechanism of learning appears to be the formation of an association between food odors and food taste.

Individuals of *O. virilis* given just one feeding with crushed zebra mussels did not show a feeding response to odor when tested 24 hr later (experiment 5), suggesting that repeated experiences are required for establishment of an association. Experienced individuals that had not been fed zebra mussels for three weeks still showed a full-strength response to food odors (experiment 6), but by six weeks without experience, their average response level had declined to control levels (experiment 7).

Individuals of *Orconectes rusticus* that had been fed a mixture of crushed gastropods showed a significantly stronger feeding response to crushed gastropods than those that had no experience with gastropods (Table 1, experiment 11 vs. 12). Individuals of *Cambarus robustus* also showed a significantly stronger feeding response to zebra mussel odor if they had prior experience with zebra mussel flesh in the previous 14 days (Table 1, experiment 18 vs. 19). Thus, experience plays a significant role both for different crayfish species and for different food types.

Individual *O. virilis* that were experienced with feeding on zebra mussels were tested with solutions of crushed zebra mussels treated with UV radiation to kill all microorganisms. Crayfish showed a nearly significant decrease in feeding duration ($P = 0.064$) and a significant increase in latency ($P = 0.017$) (experiment 8 vs. 9). Addition of trypsin to the UV-treated solutions fully restored the stimulating power of the solutions (experiment 10), indicating that the chemical substrate for odor production was not eliminated by the UV treatment.

In the case of *O. rusticus*, crushed snail solutions were coarse filtered and then microfiltered to remove all microorganisms. These solutions were introduced to individual *O. rusticus* experienced with feeding on those snails, but the crayfish showed no feeding response (experiment 11 vs. 15). When such microfiltered solutions were treated with the enzyme trypsin, a full feeding response was shown (experiment 16), indicating that appropriate substrate was present in the microfiltrate. No feeding was shown when the trypsin control was introduced (experiment 17).

DISCUSSION

These results indicate that crayfish do not respond to food odors from animal protein sources unless they have had experience with that protein source. Without an association between taste and odor gained while feeding on damaged

prey, odors apparently do not signal food to adult animals. Presumably under field conditions, contact with damaged prey could occur with some regularity. Although it appeared to take more than one feeding bout to form the association between taste and odor, once formed, this association persisted for at least three weeks. Although the same individuals were tested twice (at 20 and 40 days after the last experience with feeding on mussels), the results clearly indicate a decline in responsiveness somewhere between three and six weeks.

Perhaps the more important result from these experiments is that with the elimination of enzymatic activity of microorganisms (probably bacteria), which degrades protein sources, food odors are not produced in sufficient quantities to stimulate feeding by experienced animals. The small molecules that constitute food signals (amino acids and small polypeptides) for many aquatic organisms apparently do not appear in the water immediately after damage without degradation by microbe-sized particles (the microfiltration experiment). The strong responses shown to a food source such as canned tuna may relate to the degradation of proteins that has occurred during the canning process. The process of freezing and thawing also releases many autochthonous enzymes that can degrade proteins (Rittschof, 1980) and release the small molecules used as cues for the presence of larger, less volatile molecules. Rittschof (1993) reviews a number of cases where enzymatic activity is necessary for the generation of odor cues.

Thus, with experienced crayfish, elicitation of feeding appears to be microbe-dependent. I predict that we will find that most other organisms are dependent upon microbial degradation for food odor cues in nature.

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SYNTHESIS AND ANTIFUNGAL ACTIVITY OF ANALOGUES OF NATURALLY OCCURRING BOTRYDIAL PRECURSORS

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Abstract—Analog compounds of the proposed intermediates of the biogenetic pathway to botrydial have been synthesized. These compounds were tested for their potential antifungal activity against the phytopathogen *Botrytis cinerea*. Our results showed a fungistatic effect of some compounds on mycelium growth. The most significant effect was exerted by 2-α-hydroxy-2,3-dihydro-1-epiprobotrydial, which inhibited growth of *B. cinerea*. Some aspects of structure-activity relationships are discussed.

Key Words—Synthesis, antifungal, *Botrytis cinerea*, bioassay, structure-activity.

INTRODUCTION

Botrytis species are serious pathogens of a number of commercial plants (Whealer, 1969; Coley-Smith et al., 1980). *Botrytis cinerea* attacks a wide range of plants, causing several leaf-spot diseases and grey powdery mildews on lettuces and tomatoes, and rotting of strawberries and raspberries. *Botrytis alli*, *Botrytis byssoidea*, and *Botrytis squamosa* cause neck rots of onions, and *Botrytis tulipae* and *Botrytis fabae* produce a leaf-spot disease on beans, which can result in substantial crop losses (Coley-Smith et al., 1980). The rapid development of tolerance of *Botrytis* spp. to the commercial fungicides has led to an

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increase in the amount of these compounds used, with the subsequent additional problems of persistence in the ecosphere and its incorporation to the food chain. Since 1968, knowledge of *Botrytis cinerea* metabolism has increased quite rapidly. Several botrydial derivatives (Fehlhaber et al., 1974; Lindner and Gross, 1974; Cuevas and Hanson, 1977; Bradshaw and Hanson, 1980; Kimura et al., 1986; Kimata et al. 1985) and some nonsesquiterpenoids metabolites have also been detected (Overeem and Van Dijkman, 1968; Arpin et al., 1977; Welmar et al., 1979; Suga et al., 1984; Cutler et al., 1988).

Botrydial and structurally related compounds are characteristic metabolites of *Botrytis* species. Botrydial (Figure 1, **1**) is a bicyclic nonisoprenoid sesquiterpene, which was isolated from culture of the fungus *Botrytis cinerea* (Fehlhaber et al., 1974). The role of these molecules in fungus physiology is unknown. We have undertaken the synthesis of analogs of botrydial precursors and the study of their fungicidal activity in order to enhance our knowledge of these sesquiterpenes biosynthesized by *B. cinerea*.

METHODS AND MATERIALS

General Methods. Melting points were measured with a Kofler block Reichert-Jung apparatus and are uncorrected. ^{13}C and ^{1}H NMR spectra were recorded with a Varian Gemini 200 and Unity 400, IR spectra with a Perkin Elmer 881 instrument, and mass spectra were recorded on a VG12-250 spectrometer at 70 eV. Optical rotations were determined with a Perkin Elmer 241 polarimeter. HPLC was performed with a Hitachi L-6270 apparatus equipped with a UV-VIS detector (L 4250) and a differential refractometer detector (RI-71). TLC was performed on MN Alugran SIL G/UV 254 plates, 0.25 mm thick. Silica gel (Merck) was used for column chromatography.

Microorganism and Antifungal Assay. The culture of *Botrytis cinerea* employed in this work, *Botrytis cinerea* (UCA 992), was obtained from grapes

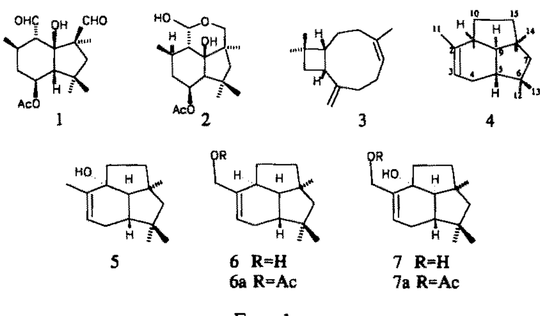

FIG. 1.

of the Domecq vineyard, Jerez de la Frontera, Cádiz, Spain. The culture of *Botrytis cinerea* is deposited in the Universidad de Cádiz, Facultad de Ciencias Mycological Herbarium Collection (UCA). The synthesized compounds were tested on *Botrytis cinerea* by the "poisoned food technique." Chemicals were solubilized in ethanol and incorporated into the culture medium (glucose, malt, peptone, agar) at a concentration of 10–300 ppm. The final alcohol concentration was identical in controls and treated culture. A 5-mm-diameter mycelial disk of an actively growing culture of *Botrytis cinerea* was placed in the center of a 6-cm-diameter agar plate. The diameter of the fungal colony was measured daily for seven days. Percent growth inhibition over control was calculated by following the method previously described (Patil et al., 1986).

Epoxidation of 1-Epiprobotrydial ***(4)***. See Gollnick and Shade (1970) and Collado et al. (1994). A solution of **4** (351 mg) in 2 ml of Et_2O was treated with 592 mg of MCPBA in 10 ml of Et_2O. The reaction mixture was stirred at 0°C for 7 hr. Then the solvent was removed and the crude product purified by column chromatography (CC) and HPLC (hexane–AcOEt 99 : 1) yielding 19 mg of unreacted starting material **(4)**, 132 mg of $2\beta,3\beta$-epoxy-1-epiprobotrydial (Figure 2, **17**, 35%) and 212 mg of $2\alpha,3\alpha$-epoxy-1-epiprobotrydial (**16**, 56%). $2\beta,3\beta$-epoxy-1-epiprobotrydial **(17)**, colorless oil; ν^{film} 2972, 2884, 1437, 1363, 1298, 1256, 1193, 1081, 832, 711 cm^{-1}. ^1H NMR: 0.82 (s, 3H, H-13), 0.95 (s, 3H, H-12), 1.03 (s, 3H, H-14), 1.33 (s, 3H, H-11), 1.38 (m, 1H, H-5), 1.40–1.56 (m, 4H, H-10, H-15, H-7, and H-7'), 1.62 (m, 1H, H-4), 1.80–2.00 (m, 3H, H-4', H-10', H-15'), 2.25 (ddd, 1H, J = 6.8, 8.2 and 9.2 Hz, H-1), 3.11 (br d, 1H, J = 2.1 Hz, H-3). ^{13}C NMR: 23.09 (q, C-13), 25.16 (q, C-11), 26.02 (t, C-15), 26.26 (t, C-4), 28.92 (q, C-12), 30.49 (q, C-14), 40.48 (s, *C-6), 41.26 (d, C-1), 42.36 (d, C-5), 42.97 (t, C-10), 46.30 (s,

4 — 16α epox. 17β epox. — 18α OH 19β OH — 20 — 21

I = MCPBA; II = $LiAlH_4$; III = $HFSO_3/ClFSO_2$; IV = H_2SO_4/Et_2O

FIG. 2.

C-8), 57.13 (d, C-9), 59.02 (t, C-7), 59.71 (s, C-2), 63.44 (d, C-3) (assignments may be interchanged). Mass spectrum: *m/z* 220 (12, M^+), 205 (16), 202 (2) [M^+-H_2O], 187 (21), 161 (55), 123 (52), 121 (42), 109 (81), 107 (57), 93 (52), 91 (67), 79 (68), 77 (64), 67 (40), 55 (55), 43 (100), 41 (98). 2α,3α-epoxy-1-epiprobotrydial **(16),** mp 81–82°C; ν^{KBr} 3396, 2958, 2883, 1441, 1360, 1214, 1118, 1060, 945, 862, 812, 753 cm^{-1}. ^1H NMR: 0.75 (s, 3H, H-13), 0.94 (s, 3H, H-12), 1.03 (s, 3H, H-14), 1.18 (ddd, 1H, $J_{5\text{-}9}$ = 12.8 Hz, $J_{5\text{-}4'}$ = 4.8 Hz and $J_{5\text{-}4}$ = 12.8 Hz, H-5), 1.28 (s, 3H, H-11), 1.62–1.78 (m, 3H, H-10, H-10′, H-9), 1.87 (ddd, 1H, $J_{4\text{-}4'}$ = 14.0 Hz, $J_{5\text{-}4'}$ = 4.8 Hz, $J_{3\text{-}4'}$ = 5.8 Hz, H-4′), 2.49 (m, 1H, H-1), 3.08 (d, 1H, $J_{3\text{-}4'}$ = 5.8 Hz, H-3). ^{13}C NMR: 22.16 (q, C-11), 22.66 (q, C-13), 25.73 (t, C-4), 27.97 (t, C-15), 28.94 (q, C-12), 31.00 (q, C-14), 40.45 (s, *C-6), 43.74 (t, C-10), 44.24 (d, C-1), 45.84 (s, *C-8), 47.63 (d, C-5), 55.10 (d, C-9), 59.28 (t, C-7), 62.08 (s, C-2), 62.46 (d, C-3) (* assignments may be interchanged). Mass spectrum: *m/z* 220 (11, M^+), 205 (21), 202 (2, M^+-H_2O), 187 (27), 176 (20), 161 (100), 151 (37), 135 (19), 123 (27), 109 (48).

2α-Hydroxy-2,3-dihydro-1-epiprobotrydial ***(18).*** Compound **16** (22 mg), dissolved in 5 ml of Et_2O, was treated for 16 hr with 0.3 ml of $LiAlH_4$ in Et_2O (1 M). Then, H_2O (10 ml) was slowly added and the reaction mixture extracted with Et_2O. The organic layer was washed with brine and dried over anhydrous Na_2SO_4. Evaporation of the solvent afforded a crude reaction product that was purified by CC (hexane–AcOEt, 99:1) yielding **18** (19 mg, 86%) as a colorless oil, $[\alpha]_D^{25}$ −36° ($CHCl_3$, c = 2.25 10^{-3}); ν^{film} 3373, 2947, 2862, 1453, 1370, 1298, 1186, 1133, 1053, 1023, 986, 940, 918, 898, 746 cm^{-1}. ^1H NMR: 0.81 (s, 3H, H-13), 0.97 (s, 3H, H-12), 1.02 (s, 3H, H-14), 1.09 (ddd, 1H, J = 12.4, 12.2, and 2.5 Hz, H-5), 1.16 (s, 3H, H-11), 1.23 (dddd, 1H, J = 12.2, 12.4, 12.3, and 3.5 Hz, H-4), 1.36–1.44 (m, 3H, H-15, H-15′, and H-4′ overlapped), 1.56–1.70 (m, 2H, H-10′, and H-3′), 1.84 (dd, 1H, J = 5.5 and 12.4 Hz, H-9), 2.09 (m, 1H, H-1). ^{13}C NMR: 21.56 (t, C-4), 23.37 (q, C-13), 27.52 (t, C-15), 29.46 (q, C-12), 29.62 (q, C-11), 30.32 (q, C-14), 37.78 (t, C-10), 40.72 (s, *C-6), 43.00 (t, C-3), 46.24 (s, *C-8), 50.78 (d, C-5), 50.86 (d, C-1), 56.43 (d, C-9), 59.11 (t, C-7), 72.66 (s, C-2) (*assignments may be interchanged). Mass spectrum: *m/z* 222 (31, M^+), 207 (25), 204 (10, M^+-H_2O), 189 (33), 177 (35), 165 (42), 149 (100), 137 (39), 121 (30), 109 (70), 95 (77), 81 (52), 71 (30), 55 (17), 43 (43), 42 (6).

2β-Hydroxy-1-epiprobotrydial ***(19).*** Compound **19** was prepared following the procedure described for **18.** Thus, 78 mg of **17** afforded 41 mg (52%) of **19** and 20 mg of starting material. 2β-Hydroxy-1-epiprobotrydial **(19),** mp 90–91°C, $[\alpha]_D^{25}$ −0.7° ($CHCl_3$, c = 6.05 × 10^{-3}); ν^{KBr} 3336, 2932, 2866, 1454, 1367, 1148, 1012, 908, 762, 652. ^1H NMR: 0.78 (s, 3H, H-12), 0.97 (s, 3H, H-13), 1.01 (s, 3H, H-14), 1.00 (ddd, 1H, J = 12.2, 3.9, and 11.9 Hz, H-4), 1.24 (ddd, 1H, J = 12.4, 12.5, and 2.8 Hz, H-5), 1.30 (s, 3H, H-11), 1.44–

1.52 (m, 2H, H-3 and H-4′), 1.52–1.70 (m, H-9, H-7, and H-7′), 1.81 (m, 1H, H-10′), 2.19 (m, 1H, H-1). ^{13}C NMR: 23.24 (t, C-4), 23.29 (q, C-13), 25.93 (t, C-15), 29.52 (q, C-12), 30.25 (q, C-14), 30.67 (q, C-11), 38.46 (t, C-10), 40.31 (s, *C-6), 42.63 (t, C-3), 46.32 (s, *C-8), 50.14 (d, C-1), 50.81 (d, C-5), 58.37 (d, C-9), 59.07 (t, C-7), 73.32 (s, C-2) (* assignments may be interchanged). Mass spectrum: *m/z* 222 (12, M^+), 207 (12), 204 (5, M^+-H_2O), 189 (23), 177 (18), 165 (23), 149 (55), 137 (36), 121 (28), 109 (77), 95 (100), 81 (64), 71 (65).

Treatment of 2α-Hydroxy-1-epiprobotrydial ***(18)*** *with $FSO_3H/ClFSO_2$.* To a cold flask (−120°C), under N_2 atmosphere, 0.15 ml of FSO_3H, 0.7 ml of $ClFSO_2$ and 46 mg of **18** were added. The reaction mixture was stirred for 45 min and then 5 ml of acetone–water (5:1) was slowly added. The mixture obtained was subjected to column chromatography, yielding 16 mg of unreacted starting material **(18),** 8 mg of 2α,3-dihydro-1α-hydroxy-1-epiprobotrydial (**20,** 17%) (Khomenko et al., 1985), and 1 mg of 10α-chloro-2α,3-dihydro-1α-hydroxy-1-epiprobotrydial **(21)**; mp 78–82°C, $[\alpha]_D^{25}$ −16.3° ($CHCl_3$, c = 1.6 10^{-3}), ν^{KBr} 3592, 2969, 2950, 2882, 1437, 1353, 1298, 1249, 1130, 1102, 1029, 972, 948, 883, 856, 810, 729, 685. ^{1}H NMR: 0.79 (s, 3H, H-12), 0.94 (s, 3H, H-13), 1.02 (d, 3H, $J_{11\text{-}2}$ = 6.7 Hz, H-11), 0.95–1.15 (m, 3H, H-5, H-4, and H-3), 1.31 (s, 3H, H-14), 1.49 (s, 2H, H-7 and H-7′), 1.55 (m, 1H, H-4′), 1.75–1.85 (m, 3H, H-9, H-2, and H-3′), 2.01 (dd, 1H, $J_{10\text{-}15}$ = 8.8 Hz, $J_{15\text{-}15'}$ = 14.2 Hz, H-15), 2.36 (dd, 1H, $J_{10\text{-}15'}$ = 9.0 Hz, $J_{15\text{-}15'}$ = 14.2 Hz, H-15′), 4.59 (dd, 1H, $J_{10\text{-}15}$ = 8.8 Hz, $J_{10\text{-}15'}$ = 9.0 Hz, H-10). ^{13}C NMR: 16.43 (q, C-11), 22.13 (q, C-13), 25.05 (t, C-4), 28.82 (q, C-12), 32.60 (q, C-14), 35.08 (t, C-3), 40.75 (s, *C-6), 41.50 (d, C-9), 44.41 (s, *C-8), 54.22 (t, C-15), 55.59 (d, C-5), 59.59 (t, C-7), 62.89 (d, C-2), 63.25 (d, C-10), 81.82 (s, C-1) (* assignments may be interchanged). Mass spectrum: *m/z* 258 (M^++2), 256 (7, M^+), 243 (2), 241 (8), 221 (25, M^+-Cl), 220 (37, M^+-HCl), 200 (66), 185 (26), 164 (35), 135 (49), 119 (52), 111 (79), 109 (83), 91 (75), 77 (55), 67 (48), 55 (100).

Treatment of 2β-Hydroxy-1-epiprobotrydial ***(19)*** *with $FSO_3H/ClFSO_2$.* Compound **19** (20 mg) was subjected to the same procedure described above for **18** yielding 2 mg of 1-epiprobotrydial **(4),** 10 mg of 2α,3-dihydro-1α-hydroxy-1-epiprobotrydial (**20,** 50%), and 2 mg of 10α-chloro-2α,3-dihydro-1α-hydroxy-1-epiprobotrydial (**21,** 8%).

Treatment of 1-Epiprobotrydial ***(4)*** *with SeO_2.* Compound **4** (Gollnick and Shade, 1970; Collado et al., 1994) (135 mg) dissolved in 10 ml of dry CH_2Cl_2, was treated with 0.25 ml of *t*-BuOOH (80%) and 52 mg of SeO_2. The reaction was followed by TLC. The solution was filtered and the solvent evaporated under reduced pressured. The crude material obtained was chromatographed (hexane–Et_2O), yielding 9 mg of unreacted starting material **(4),** 23 mg of 1α-hydroxy-1-epiprobotrydial (**5,** 16%), 64 mg of 11-hydroxy-1-epiprobotrydial (**6,**

44%), and 10 mg of 11-hydroxy-1α-hydroxy-1-epiprobotrydial (**7**, 6%). 1α-Hydroxy-1-epiprobotrydial (**5**): mp 123–124°C; $[\alpha]_D^{25}$ −22.3° ($CHCl_3$, c = 7.05 10^{-3}); ν^{KBr} 3339, 3209, 2972, 2933, 2882, 1634, 1429, 1357, 1293, 1242, 1173, 1126, 1015, 932, 855, 787, 706. ^{1}H NMR: 0.85 (s, 3H, H-13), 0.95 (s, 3H, H-12), 1.27 (s, 3H, H-14), 1.34 (m, 1H, H-5), 1.54 (d, 1H, $J_{7-7'}$ = 6.0 Hz, H-7), 1.58 (d, 1H, $J_{7-7'}$ = 6.0 Hz, H-7'), 1.66 (m, 1H, H-9), 1.68 (m, 1H, H-10), 1.72 (m, 1H, H-4), 1.73 (d, 3H, J_{11-3} = 1.5 Hz, H-11), 1.82 (m, 1H, H-10'), 1.86 (m, 1H, H-4'), 1.90 (m, 1H, H-15), 1.96 (m, 1H, H-15'), 5.44 (qd, J_{3-11} = 1.5 Hz, J_{3-1} = 5.8 Hz, 1H, H-3). ^{13}C NMR: 18.18 (q, C-11), 22.50 (q, C-12), 26.52 (t, C-4), 28.76 (q, C-13), 32.71 (q, C-14), 38.20 (t, C-15), 41.03 (s, *C-6), 42.44 (t, C-10), 46.41 (s, *C-8), 53.91 (d, C-5), 59.95 (t, C-7), 66.10 (d, C-9), 85.63 (s, C-1), 123.88 (d, C-3), 139.17 (s, C-2); (* assignments may be interchanged). Mass spectrum: *m/z* 220 (14, M^+), 202 (3, M^+-H_2O), 191 (5), 173 (1), 159 (3), 149 (7), 135 (17), 121 (22), 109 (100), 108 (36). 11-Hydroxy-1-epiprobotrydial (**6**): colorless oil, $[\alpha]_D^{25}$ −53.8° ($CHCl_3$, c = 6.5 10^{-3}); ν^{film} 3307, 2952, 1635, 1446, 1363, 1297, 1256, 991, 879, 796, 689. ^{1}H NMR: 0.84 (s, 3H, H-13), 0.99 (s, 3H, H-12), 1.09 (s, 3H, H-14), 1.35 (ddd, 1H, J_{5-9} = 12.2 Hz, J_{5-4} = 8.1 Hz, $J_{5-4'}$ = 4.4 Hz, H-5), 1.50 (br s, 2H, H-7 and H-7'), 1.51 (m, 3H, H-15, H-15', and H-10), 1.70 (m, 2H, H-9 and H-4), 1.93 (ddd, 1H, $J_{4-4'}$ = 16.6 Hz, $J_{4'-5}$ = 4.4 Hz, $J_{4'-3}$ = 5.6 Hz, H-4'), 2.02 (m, 1H, H-10'), 2.58 (br dd, 1H, $J_{1-10'}$ = 15.6 Hz, J_{1-9} = 7.6 Hz, H-1), 4.15 (br s, 2H, H-11 and H-11'), 5.73 (br d, $J_{3-4'}$ = 5.6 Hz, H-3). ^{13}C NMR: 22.22 (q, C-14), 25.74 (t, C-4), 28.61 (q, C-13), 30.19 (q, C-12), 31.02 (t, C-10), 40.40 (s, *C-6), 40.50 (d, C-1), 43.15 (t, C-7), 45.72 (s, *C-8), 48.68 (d, C-5), 57.50 (d, C-9), 58.85 (t, C-15), 66.84 (t, C-11), 124.34 (d, C-3), 142.26 (s, C-2); (* assignments may be interchanged). Mass spectrum: *m/z* 220 (28, M^+), 205 (32), 202 (22, M^+-H_2O), 187 (32), 159 (38), 133 (55), 105 (77), 91 (84). 11-Hydroxy-1α-hydroxy-1-epiprobotrydial (**7**); mp 51°C, ν^{KBr} 3344, 2951, 1851, 1443, 1362, 1034. ^{1}H NMR: 0.81 (s, 3H, H-13), 0.90 (s, 3H, H-12), 1.22 (s, 3H, H-14), 1.27 (ddd, $J_{5-4\alpha}$ = 12.1 Hz, J_{5-9} = 12.7 Hz, $J_{5-4\beta}$ = 4.3 Hz, H-5), 1.49 (d, 1H, $J_{7-7'}$ = 13.1 Hz, H-7), 1.54 (d, 1H, $J_{7-7'}$ = 13.1 Hz, H-7'), 1.7 (d, 1H, J_{9-5} = 12.7 Hz, H-9), 4.11 (d, 1H, $J_{11-11'}$ = 12.3 Hz, H-11), 4.26 (d, $J_{11-11'}$ = 12.3 Hz, H-11'), 5.74 (d, 1H, J = 4.7 Hz, H-3). ^{13}C NMR: 21.9 (q, C-13), 26.0 (t, C-4), 2.82 (q, C-12), 32.3 (q, C-14), 38.7 (t, C-10), 40.0 (s, C-6), 42.2 (t, C-15), 45.7 (s, C-8), 53.4 (d, C-5), 59.6 (t, C-7), 65.5 (t, C-11), 86.2 (s, C-1), 127.8 (d, C-3), 141.8 (s, C-2). Mass spectrum *m/z* 236 (5, M^+), 218 (M^+-H_2O), 207 (23), 203 (11), 189 (35), 161 (13), 147 (17), 134 (40), 125 (67), 121 (41), 119 (24), 95 (100).

Hydrogenation of 1α-Hydroxy-1-epiprobotrydial (**5**). Compound **5** (50 mg) was dissolved in a mixture of benzene–ethanol (3 : 1) and 450 mg of tris(triphenylphosphine)rhodium(I) chloride were added. The reaction mixture

was subjected to hydrogenation (1 kg/cm^2) for 80 hr. When starting material had disappeared by TLC, the mixture was filtered and the solvent removed. After purification, 42 mg (84%) of **20** was obtained.

Acetylation of 11-Hydroxy-1-epiprobotrydial **(6)** *and 11-Hydroxy-1α-hydroxy-1-epiprobotrydial* **(7).** Starting materials (**6**, 30 mg; and **7**, 20 mg) were separately dissolved in dry pyridine (2 ml) and acetic anhydride (6 ml) was added. The reaction mixtures were stirred for 14 hr. The solvent was removed and the crude chromatographed to give compounds **6a** (28 mg, 78%) and **7a** (15 mg, 68%), respectively. 11-Acetyloxy-1-epiprobotrydial **(6a),** oil; ν^{film} 2952, 2868, 1735, 1364, 1223, 1019. ^{1}H NMR: 0.78 (s, 3H, H-12), 0.93 (s, 3H, H-13), 1.04 (s, 3H, H-14), 1.29 (ddd, 1H, J = 12.1, 12.4, and 3.7 Hz, H-5), 1.50 (br s, 2H, H-7 and H-7′), 1.50–1.95 (m, H-9, H-10, and H-15), 2.00 (s, 3H, CH_3CO—), 2.46 (br dd, 1H, $J_{1-10'}$ = 15.8 Hz, J_{1-9} = 7.7 Hz, H-1), 4.43 (d, 1H, $J_{11-11'}$ = 12.2 Hz, H-11), 4.48 (d, 1H, $J_{11-11'}$ = 12.2 Hz, H-11′), 5.72 (d, 1H, $J_{3-4'}$ = 5.5 Hz, H-3). ^{13}C NMR: 21.09 (c, —CO$\underline{C}H_3$), 22.29 (q, C-14), 25.96 (t, C-4), 28.66 (q, C-13), 30.23 (q, C-12), 30.99 (t, C-10), 40.43 (s, *C-6), 41.04 (d, C-1), 43.11 (t, C-7), 45.80 (s, *C-8), 48.41 (d, C-5), 57.36 (d, C-9), 58.84 (q, C-15), 68.19 (t, C-11), 127.82 (d, C-3), 137.35 (s, C-2), 171.01 (s, —$\underline{C}OCH_3$); (* assignments may be interchanged). Mass spectrum: m/z 262 (0.4, M^+), 220 (15), 202 (98), 187 (42), 173 (30), 159 (45), 146 (60), 131 (83), 118 (60), 108 (100). 11-Acetyloxy-1α-hydroxy-1-epiprobotrydial **(7a);** mp 42–43°C, ν^{KBr} 3444, 2968, 2885, 1722, 1358, 1222, 1020. ^{1}H NMR: 0.95 (s, 3H, H-12), 1.27 (s, 3H, H-14), 1.35 (dt, 1H, J = 12.2 and 4.1 Hz, H-5), 1.54 (d, 1H, $J_{7-7'}$ = 13.1 Hz, H-7), 1.59 (d, 1H, $J_{7-7'}$ = 13.1 Hz, H-7′), 1.72 (d, 1H, J_{5-9} = 12.6 Hz, H-9), 4.54 (d, J = 12.4 Hz, H-11), 4.87 (br d, $J_{11-11'}$ = 12.4 Hz, H-11′), 5.80 (br d, J = 5.3 Hz, H-3). ^{13}C NMR: 21.9 (q, $\underline{C}H_3COO$—), 22.5 (q, C-13), 26.7 (t, C-4), 28.7 (q, C-12), 32.7 (q, C-14), 39.3 (t, C-10), 40.9 (s, C-6=, 42.7 (t, C-15), 45.9 (q, C-8), 53.2 (d, C-5), 59.7 (t, C-7), 65.3 (t, C-11), 65.8 (d, C-9), 84.1 (s, C-1), 129.0 (d, C-3), 137.8 (s, C-2), 170.0 (s, $CH_3\underline{C}OO$—). Mass spectrum: m/z 278 (0.2, M^+), 260 (2,, M^+-H_2O), 249 (2), 218 (17, M^+-AcOH), 107 (100).

Acetylation of 10α-chloro-2α,3-dihydro-1α-hydroxy-1epiprobotrydial **(21).** Compound **21** (5 mg) was dissolved in acetic anhydride (0.1 ml) and a catalytic amount of *p*-toluene sulfonic acid was added. The reaction mixture was stirred for 14 hr and then extracted with AcOEt. The organic layer was neutralized with saturated $NaHCO_3$ solution, washed with brine, and dried over anhydrous Na_2SO_4. Evaporation of the solvent furnished 4 mg (78%) of the acetyl derivative, colorless oil, $[\alpha]_D^{25}$ + 19.4° ($CHCl_3$, c = 1.6 10^{-3}); ν^{film} 2926, 2861, 1728, 1452, 1367, 1233, 1126, 1029, 955, 885, 839, 719, 664. ^{1}H NMR: 0.79 (s, 3H, H-12), 0.94 (s, 3H, H-13), 1.05 (d, 3H, J_{11-2} = 7.2 Hz, H-11), 0.95–1.15 (m, 3H, H-5, H-4 and H-3), 1.15 (s, 3H, H-14), 1.45–1.55 (m, 3H, H-4, H-7, and H-7′), 1.87 (m, 1H, H-3′), 2.01 (dd, 1H, J_{10-15} = 9.4 Hz, $J_{15-15'}$ =

14.0 Hz, H-15), 2.07 (s, 3H, CH_3—CO—), 2.36 (dd, 1H, $J_{10\text{-}15'}$ = 8.8 Hz, $J_{15\text{-}15'}$ = 14.0 Hz, H-15'), 2.93 (d, 1H, $J_{5\text{-}9}$ = 12.8 Hz, H-9), 2.98 (m, 1H, H-2), 4.59 (dd, 1H, $J_{10\text{-}15}$ = 9.4 Hz, $J_{10\text{-}15'}$ = 8.8 Hz, H-10). ^{13}C NMR: 16.67 (q, C-11), 22.26 (q, C-13), 23.35 (q, CH_3—CO—), 24.72 (t, C-4), 28.85 (q, C-12), 31.89 (q, C-14), 34.41 (d, C-9), 35.11 (t, C-3), 40.67 (s, *C-6), 43.73 (s, *C-8), 53.64 (t, C-15), 56.95 (d, C-5), 58.05 (t, C-7), 59.86 (2C d, C-2; d, C-10), 93.02 (s, C-1), 169.49 (s, CO-CH_3) (* assignments may be interchanged). Mass spectrum m/z 263 (2, M^+-Cl), 240 (8, M^+-AcOH), 238 (24, M^+-AcOH), 225 (2), 223 (1), 203 (18), 182 (11), 147 (30), 131 (15), 91 (11), 69 (12), 67 (13), 55 (16), 43 (30), 40 (100).

Rearrangement of 2α,3-Dihydro-1α-hydroxy-1-epiprobotrydial **(20)** *with $FSO_3H/ClFSO_2$.* Compound **20** (100 mg) was subjected to the procedure described above for **18** yielding 50 mg of 1-epiprobotrydial **(4)**, 10 mg of 10α-chloro-2α,3-dihydro-1α-hydroxy-1-epiprobotrydial **(21)**, and 22 mg of unreacted starting material.

Alcohol **(25)** (Figure 3). When kobusone **(23)** was treated with Zn in HCl as described (Kaiser et al., 1986) in addition to the *nor*-caryophyllene ketone **24**, alcohol **25** (16%) was obtained. This compound was identified as its acetyl derivative **25a**; oil, ν^{film} 2924, 1857, 1735, 1446, 1368, 1230. ^{1}H NMR: 0.91 (s, 6H, H-13 and H-14), 1.35 (br t, 1H, J = 10.26 Hz, H-2β), 1.52 (m, 1H, H-7β), 1.62 (m, 1H, H-2α), 1.62 (m, 2H, H-12), 1.75 (m, 1H, H-6), 1.86 (br dd, 1H, J = 9.65 and 116.98 Hz, H-1), 1.95 (m, 1H, H-7α), 2.04 (m, 1H, H-9), 2.10 (s, 3H, CH_3CO—), 2.25 (m, 1H, H-6), 4.70 (br d, 1H, J = 8.33 Hz, H-8), 5.39 (br t, 1H, J = 7.5 Hz, H-5). ^{13}C NMR: 15.87 (q, C-12), 20.98 (q, $\underline{C}H_3$CO—), 22.77 (t, C-6), 22.82 (q, *C-13), 28.71 (t, C-3), 29.99 (q, *C-14), 30.70 (t, C-2), 33.18 (s, C-11), 37.96 (t, C-10), 39.49 (t, C-7), 45.20 (d, C-1), 45.82 (d, C-9), 75.77 (d, C-8), 123.00 (d, C-5), 134.96 (s, C-4),

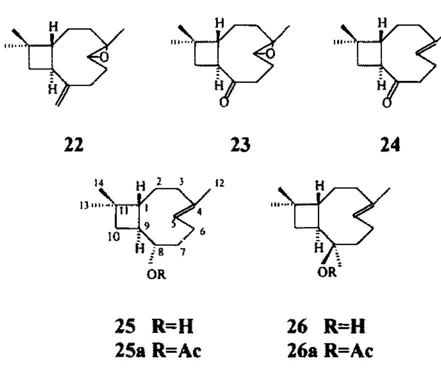

FIG. 3.

170.93 (s, $CH_3\underline{C}O$—) (* assignments may be interchanged). Mass spectrum: *m/z* 250 (0.17, M^+), 207 (2, M^+-Ac), 190 (13, M^+-AcOH), 175 (21), 147 (36), 134 (100), 119 (68).

RESULTS AND DISCUSSION

Biosynthetic studies carried out by Hanson (1981) suggest that botrydial (**1**) and dihydrobotrydial (**2**) are sesquiterpenes formed from farnesyl pyrophosphate by the folding showed in Scheme 1. The first stages in the biosynthesis involve the formation and cyclization of caryophyllene cation (**11**).

On the other hand, the tricyclic olefin **4** is an epimeric derivative at C-1 of the proposed intermediate carbocation **13** (Scheme 1), which we have named 1-epiprobotrydial (**4**), and it was used as starting material for our study. Compound **4** has previously been obtained only from isocaryophyllene (**3**) (Gollnick and Schade, 1970) and an improved method has recently been reported by us (Collado et al., 1994).

Compound **4** was subjected to the chemical transformation outlined in Fig. 2 in order to obtain analogues of the intermediates **13**, **14**, and **15** (Scheme 1).

Treatment of **4** with MCPBA gave oxiranes derivatives **16** and **17**, which were separated by chromatography. The structures of compounds **16** and **17** were determined by NMR spectroscopy; ^1H–^1H and ^1H–^{13}C COSY spectra allowed assignment of all the protons in both compounds. The orientation of the oxirane rings was easily assigned from the chemical shift of protons H-5 and H-1. So, in the ^1H NMR of **16**, the signals at 2.49 (m, 1H, H-1) and 1.18 (ddd, 1H, J = 12.8, 12.8, 4.8 Hz, H-5) were consistent with the α orientation of the epoxy group, while the signals at 2.25 (ddd, 1H, J = 6.8, 8.2, 9.2 Hz,

SCHEME 1.

H-1) and 1.38 (m, 1H, H-5) in compound **17** indicated a β orientation of the oxirane ring. The stereochemistry of **16** was confirmed by NOE experiments. Irradiation of the signal at 1.18 (H-5) led to the enhancement of those at 3.08 (H-3), 0.94 (H-12), while irradiation at 2.49 (H-1) enhanced the signal at 1.70 (H-9).

From **16** and **17,** the oxirane ring opening with $LiAlH_4$ afforded stereoselectively the alcohols **18** and **19,** respectively. Both had a molecular ion at *m/z* 222 and ^{13}C NMR consistent with a molecular formula of $C_{15}H_{26}O$. From the IR absorptions at 3373 and 3336 cm^{-1}, ions peaks at *m/z* 204 (M^+-18) in the MS and a quaternary carbon signal at 72.66 and 73.32 in the ^{13}C NMR spectra of **18** and **19,** respectively, the structures of epimeric alcohols were inferred. The stereochemistry of the hydroxyl group was assigned on the basis of the chemical shift observed for the proton H-9 and H-5 in both compounds. The signal corresponding to H-9 in α-alcohol **18** was clearly deshielded (+0.25) with respect to the same signal in **19,** while that the signal of H-5 in β-alcohol **19** was deshielded (+0.15 ppm) with respect to that in alcohol **18.**

In the proposed biosynthetic route (Scheme 1), the rearrangement led to a hydrogen atom appearing at C-2, thus generating the secondary methyl group in **14.** It has been reported that a 1,3-hydrogen shift occurs during the cyclization (Hanson, 1981).

Alcohols **18** and **19** were treated with $FSO_3H/ClFSO_2$ (−120°C) separately to study the hydrogen shift on the epimeric alcohols **18** and **19** and to obtain analogs of intermediates **14** and **15** (Scheme 1). When the reactions were quenched with acetone/H_2O, compounds **20** and **21** were obtained in both reactions.

The structure of **20** was confirmed by synthesis. 1-Epiprobotrydial **(4)** was subjected to oxidation with SeO_2 yielding compounds **5, 6,** and **7.** Hydrogenation of **5** with (triphenylphosphine)rhodium(I) chloride gave selectively a compound whose spectroscopic data were identical to compound **20.** On the other hand, the ^1H NMR spectra of compounds **6** and **7** showed clearly that an oxidation at C-11 and at C-11, C-1 had taken place, respectively. Hence, the signal corresponding to the methyl group on C-2, in both compounds, had disappeared. Instead, a typical signal of $-CH_2-OH$ group [4.15 (brd) **(6)** and 4.11 (d), 4.26 (d) **(7)**] was observed. Furthermore, the signal of the proton at C-1 was absent in the ^1H NMR of **7.** As expected, the signals assigned to H-11 were shifted downfield in the monoacetates **6a** and **7a,** respectively.

Compound **21** showed a molecular ion at *m/z* 256:258 and signals in its ^{13}C NMR corresponding to four methyl, four methylene, four methine, and three quaternary carbons, which were consistent with a saturated tricyclic sesquiterpene possessing a tertiary hydroxyl group and a chlorine atom with a molecular formula of $C_{15}H_{25}OCl$. When treated with acetic anhydride, compound **21** formed a monoacetate that lacked hydroxyl absorption in the IR. From

the study of ^{1}H and ^{13}C NMR spectra, structure **21** was proposed. It was supported by homo- and heteronuclear 2-D correlation experiments. The stereochemistry of chlorine and hydroxyl groups was assigned on the basis of the NOE experiment. Irradiation of the signal at 1.31 (H-14) led to enhancement of the signals at 2.01 (H-15) and 1.82 (H-9), indicating that the signal at 2.01 corresponded to the H-15 proton, which was α-oriented. Irradiation at 4.59 (H-10) enhanced the signals of protons H-11, β H-15, and H-5. These results indicated that the H-10 proton must be β-oriented confirming the structure proposed to compound **21.**

Obtaining **21,** from alcohols **18** and **19,** by treatment with superacid could be rationalized by dehydration of alcohol **20,** followed by syn addition of species Cl^+ and OH^- from the reaction mixture.

In order to confirm the formation of halohydrin **21** from alcohol **20,** this compound was treated with superacid. When **20** was subjected to treatment with $FSO_3H/ClFSO_2$, compounds **21** and **4** were obtained. Furthermore, when **20** was treated with sulfuric acid, only the olefin **4** was formed. These results confirm the formation of **21** from **20** and show that the 1,2-hydrogen shift is a reversible process in this skeleton.

On the other hand, we undertook some chemical transformation on caryophyllene oxide **(22)** (Fig. 3) in order to obtain analogs compounds of the intermediate caryophyllene cation **(11)** (Scheme 1). Hence, the ozonolysis of **22** yielded kobusone **(23)** (Kaiser and Lamparsky, 1976), which was treated with Zn in HCl to give the *nor*-caryophyllene ketone **24** and the alcohol **25.** The 8-β-hydroxy caryophyllene derivative **(26)** was obtained according to the procedure described previously (Kaiser and Lamparsky, 1976).

The antifungal properties of the synthesized analogs were determined against the growth of *B. cinerea* using the poisoned food technique (Patil et al., 1986). The commercial fungicide Euparen was used as a standard for comparison in this test. Several levels of inhibition were observed. The α-alcohol **18** exhibited the maximum percent of growth inhibition followed by alcohol **6.** Compound **18** (Figure 4) showed a total inhibition for four days and reduced the growth of the fungus (68%) after seven days. The analogs with a caryophyllene skeleton displayed a different degree of activity. Compounds **22–24** had no effect on the mycelial growth. Meanwhile alcohols **25** and **26** showed complete inhibition for three days, followed by a slow growth of fungus over seven days. These results showed that the fungus was able to overcome the inhibitory effect of these alcohols. On the other hand, the tricycle olefin **4** and its derivatives **5, 16, 17, 20, 21** were inactive.

The alcohol **6** was active (Figure 5), showing prominent zones of inhibition (35–40 mm) when it was applied at 90, 150, 250, and 300 ppm, with an apparent change of fungus morphology. However its derivatives **6a, 7,** and **7a** were inactive or showed quite weak activity.

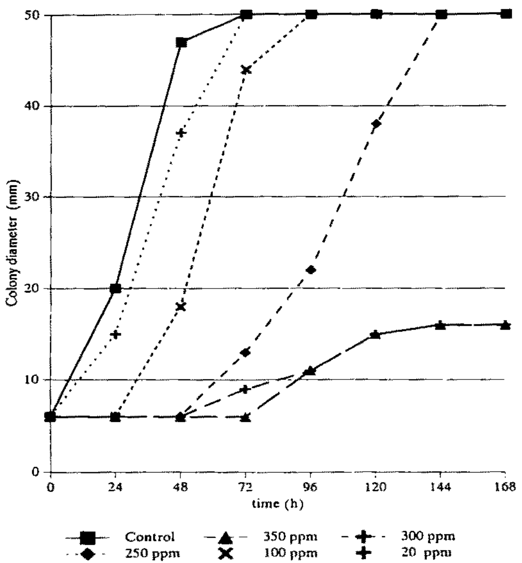

FIG. 4. Antifungal activity of alcohol **18.**

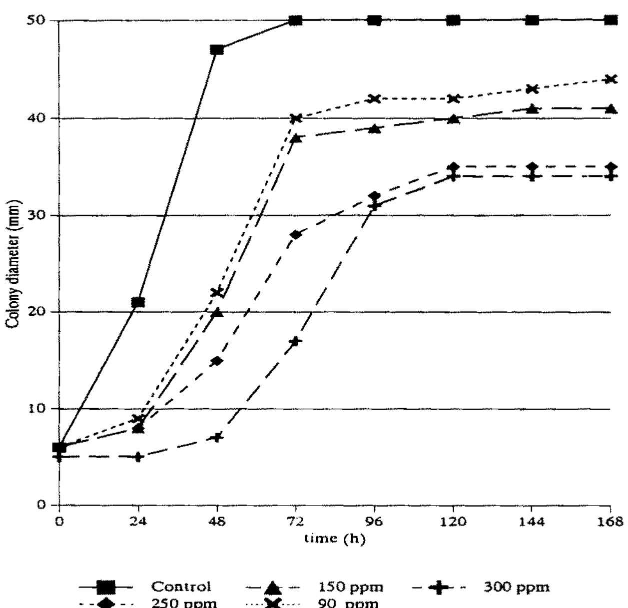

FIG. 5. Antifungal activity of compound **6.**

The study of the activity displayed by the 11-hydroxy analogs (**6** and **7**) and derivatives showed that the presence of a hydroxyl on C-1 is critical for antifungal activity of these molecules. On the other hand, it is worth noting that the acetyl derivatives of all active alcohols tested were inactive, which seems to indicate that the hydroxyl groups play an important role in the inhibition mechanism.

In conclusion, the active synthesized compounds showed fungistatic activity. The results described in this paper show that the fungus *B. cinerea* could be controlled by analogs of botrydial precursors. Work is in progress to study the mode of action of these compounds in the fungus metabolism.

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INTRASPECIFIC AND INTERSPECIFIC CHEMOATTRACTION IN *Biomphalaria glabrata* AND *Helisoma trivolvis* (GASTROPODA: PLANORBIDAE)

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Abstract—A Petri dish bioassay previously used to examine food preferences in planorbid snails was used to study intraspecific and interspecific chemoattraction in *Biomphalaria glabrata* (albino strain, M-line) and *Helisoma trivolvis* (Colorado strain) snails. *B. glabrata* snails showed significant intraspecific chemoattraction in the absence of visual cues and snail thigmotaxis. *H. trivolvis* snails also showed significant intraspecific chemoattraction. Interspecific chemoattraction between these species occurred in the bioassay, suggesting that the chemoattractants were not species specific. Artificial spring water conditioned by aqueous excretory-secretory products (snail-conditioned water) of *B. glabrata* elicited significant intraspecific chemoattraction. However, lipophilic excretory-secretory products of *B. glabrata* elicited significant chemorepulsion. Repellant factors in the lipophilic fraction were not characterized.

Key Words—Intraspecific chemoattraction, interspecific chemoattraction, *Biomphalaria glabrata*, *Helisoma trivolvis*, Gastropoda, Planorbidae, in vitro bioassay, chemorepulsion.

INTRODUCTION

Studies on the medically important planorbid *Biomphalaria glabrata*, have examined chemoattraction and dietary preferences of this snail in a Petri dish bioassay (Masterson and Fried, 1992). A recent study also examined chemoattraction of this planorbid to lipid standards and lipophilic factors in leaf lettuce

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and Tetramin fish food (Marcopoulos and Fried, 1993). Less information is available on how snails are attracted to other snails of the same or different species. Runham (1992) reviewed communicative behavior of gastropods and discussed visual cues, tactile stimuli, and chemoattractants involved in mating, feeding, and aggregation.

Information on factors associated with chemical communication in most gastropods is lacking. One purpose of our study was to examine possible factors involved in intraspecific and interspecific chemoattraction of two planorbid snails, *B. glabrata* (M-line or albino strain) and *Helisoma trivolvis* (Colorado strain). Previous studies (reviewed in Runham, 1992) suggested that snail-conditioned water (SCW), newly laid snail eggs, and snail mucus contained presumptive pheromones that mediate chemical communication in some gastropods. Therefore, another purpose of our study was to examine the role of SCW, eggs, and mucus of *B. glabrata* as chemoattractants.

METHODS AND MATERIALS

Stock cultures of *B. glabrata* and *H. trivolvis* were maintained in the laboratory on a mixed diet of iceberg leaf lettuce (*Lactuca sativa*) and Tetramin fish food (Tetra Werka, Melle, Germany) (Duncan et al., 1987). The bioassay chambers for the intraspecific and interspecific chemoattraction studies consisted of 10-cm Petri dishes (Masterson and Fried, 1992). Two parallel lines were drawn 2.8 cm apart on the bottom of each Petri dish to produce three zones (A, B, C). The side zones (A and C) each had an area of 14.1 cm^2, and the middle zone (B) had an area of 20.0 cm^2 (Masterson and Fried, 1992). Each Petri dish was filled with 55 ml of artificial spring water (Cohen et al., 1980). The dishes were kept at room temperature (21–23°C) on a level workbench under constant overhead diffuse fluorescent light. Ten Petri dishes were used for each trial.

Snails used in all trials were 11 $\pm$ 1 mm in shell diameter, unless otherwise stated. Snails that were allowed to migrate freely in the bioassay were termed migrators, while snails that were restrained in Hoffman open-jaw clamps (11- to 18-mm opening, catalog number 21715-020, VWR Scientific, Philadelphia, Pennsylvania), cheesecloth (purchased at a local supermarket, approximate 3-mm^2 mesh), or dialysis sacs (12,000 molecular weight exclusion) were termed releasers. A single migrator was placed in the center of zone B, whereas controls and releasers were placed at the edges of zones A and C, respectively.

Snails were maintained without food for approximately 2 hr prior to each trial. For each trial migrators were placed in the dishes 10 min after releasers to allow presumptive pheromones to diffuse.

Intraspecific chemoattraction studies were done by placing a single conspecific snail in a clamp in zone C and an empty clamp in zone A (see groups A, B and K, L in Table 1).

TABLE 1. INTRASPECIFIC CHEMOATTRACTION OF *Biomphalaria glabrata* (Bg) AND *Helisoma trivolvis* (Ht) SNAILS

Group	Experiment[a]	Percentage of snails in zones			Chi square	*P*
		A	B	C		
Releaser snails in clamps						
A	Bl vs. Bl	37	26	37		
B	Bl vs. Bg	27	16	57	17.4	0.0002
Releaser snails in clamps—experiment done in the dark						
C	Bl vs. Bl	38	28	34		
D	Bl vs. Bg	30	17	53	16.6	0.0003
Releaser snails wrapped in cheesecloth						
E	Bl vs. Bl	36	33	31		
F	Bl vs. Bg	36	16	48	18.1	0.0001
Releaser snails in perforated dialysis sacs						
G	Bl vs. Bl	39	24	37		
H	Bl vs. Bg	44	18	38	2.2	0.34
Releaser snails in nonperforated dialysis sacs						
I	Bl vs. Bl	39	24	37		
J	Bl vs. Bg	37	25	38	0.2	0.92
Releaser snails in clamps						
K	Bl vs. Bl	36	25	39		
L	Bl vs.Ht	42	13	45	7.7	0.02

[a]Bl = blank.

To observe chemoattraction of *B. glabrata* in the absence of visual cues, the above design was used except that the experiment was done in the dark. To record data, a fluorescent light was switched on for about 30 sec during each observation (group C and D in Table 1).

To avoid snail thigmotaxis, releasers were wrapped four times with cheesecloth and then clamped in place in zone C (groups E and F in Table 1).

To examine chemoattraction in the presence of excretory–secretory (ES) products, snails with a shell diameter of 8 ± 1 mm were placed in 1 × 0.5-cm^2 dialysis sacs (12,000 molecular weight exclusion; VWR Scientific, Plainfield, New Jersey) as described in Masterson and Fried (1992). Sacs were closed at both ends with cotton thread and perforated with a pin to allow presumptive pheromones of all molecular weights to be released (groups G and H in Table 1). Nonperforated sacs were closed as mentioned above and allowed for the release of substances with a molecular weight of less than about 12,000 daltons (Masterson and Fried, 1992) (groups I and J in Table 1).

Trials were done to test snail ES products. Ten *B. glabrata* snails were

placed in individual 17-mm-diameter wells of a multiwell plate (Falcon Tissue Culture Plates, Fisher Scientific, Pittsburgh, Pennsylvania) with 200 μl of spring water for 4 hr. The water containing ES products from each well was removed, filtered through glass wool, and used to impregnate a 1-cm^3 plug of filter paper (200 μl/plug). Each plug was secured with a paper clip in the bioassay, and trials were conducted as described.

Trials were also done to test the lipophilic and hydrophilic extracts of the filtered ES product (groups A–F in Table 2). To the filtered ES product obtained as described above, 400 μl of 2:1 chloroform–methanol was added and the mixture was filtered through glass wool. The supernatant was treated with 100 μl of the Folch wash (0.88 KCl) to separate the hydrophilic and lipophilic fractions. Each fraction was used to impregnate 1 cm^3 plugs of filter paper (200 μl/plug). Controls consisted of 1-cm^3 plugs impregnated with 200 μl of 2:1 chloroform–methanol and trials were conducted as mentioned above with these controls.

B. glabrata mucus was tested as a possible intraspecific chemoattractant (groups G and H in Table 2). To obtain mucus, 35 *B. glabrata* snails, 11–15 mm in shell diameter, were placed on filter paper in an 8.5-cm finger bowl

TABLE 2. INTRASPECIFIC CHEMOATTRACTION OF SNAILS TO EXCRETORY–SECRETORY PRODUCTS, MUCUS, AND EGG MASSES OF *Biomphalaria glabrata*

Group	Experiment[a]	Percentage of snails in zones			Chi square	*P*
		A	B	C		
ES products						
A	Bl vs. Bl	39	24	37		
B	Bl vs. ES	35	21	44	6.1	0.047
Lipophilic extract of ES						
C	Bl vs. Bl	39	20	41		
D	Bl vs. LES	50	22	28	7.4	0.024
Hydrophilic extract of ES						
E	Bl vs. Bl	39	24	37		
F	Bl vs. HES	32	30	38	2.8	0.25
Mucus						
G	Bl vs. Bl	38	24	38		
H	Bl vs. Mu	36	22	42	0.7	0.71
Egg masses						
I	Bl vs. Bl	39	24	37		
J	Bl vs. EM	38	20	42	1.4	0.50

[a] Bl = blank; ES = excretory–secretory products; LES = lipophilic extract of ES; HES = hydrophilic extract of ES; Mu = mucus; EM = egg masses.

(Carolina Biological Co., Burlington, North Carolina). The filter paper was moistened with 600 μl of spring water prior to placement in the bowl, and the snails were allowed to migrate in the bowl for 1 hr. The snails were then removed and the filter paper air dried. The mucus-containing filter paper was then cut into 1-cm^2 disks, and each disk was held in place during the bioassay with a paper clip (groups G and H in Table 2).

B. glabrata egg masses were tested to determine if they contained intraspecific chemoattractants (groups I and J in Table 2). Two newly laid masses of eggs, each mass containing 30–45 eggs, were wrapped four times in cheesecloth and held in place during the bioassay with a clamp.

For 50 min, at intervals of 5 min, the zone in which the migrator was located was recorded (a total of 10 observations per snail). The control (blanks) for each design consisted of a snail matched against either two clamps, two dialysis sacs, two 1-cm^3 plugs, or two 1-cm^2 filter paper disks. Random observations based on blank experiments were used as the expected values to calculate the chi-square value in each experimental design. $P < 0.05$ was considered significant.

A trial testing interspecific chemoattraction between *B. glabrata* and *H. trivolvis* was also done. Five bioassays consisted of five *B. glabrata* snails serving as the releasers while five *H. trivolvis* snails served as migrators, and another five bioassays consisted of five *H. trivolvis* releasers and five *B. glabrata* migrators.

The velocity of *B. glabrata* and *H. trivolvis* was determined in the bioassay. Ten dishes for each species were used each containing 55 ml of spring water. A single snail was placed in the center of each dish, the dish was covered, and the position of the snail was marked on the Petri dish cover. Every min for 10 min the position of the snail was recorded. To calculate the snail velocity in millimeters per minute, the total distance traveled by the snail in 10 min was divided by 10. Ten trials were performed for each snail species.

RESULTS

The results of the intraspecific chemoattraction studies of *B. glabrata* and *H. trivolvis* are presented in Table 1. There was significant intraspecific chemoattraction of *B. glabrata* (groups A and B) and *H. trivolvis* (groups K and L) in trials in which the releaser snails were contained in Hoffman clamps. *B. glabrata* showed significant intraspecific chemoattraction in the absence of visual cues (groups C and D) and in the absence of snail thigmotaxis (groups E and F). There was no significant intraspecific chemoattraction of *B. glabrata* snails when *B. glabrata* were maintained in either perforated (groups G and H) or nonperforated (groups I and J) dialysis sacs. The results of the intraspecific

TABLE 3. INTERSPECIFIC CHEMOATTRACTION BETWEEN *Biomphalaria glabrata* AND *Helisoma trivolvis* SNAILS

Group	Experiment[a]	Percentage of snails in zones			Chi square	P
		A	B	C		
Releaser snails in clamps						
A	Bl vs. Bl	39	24	37		
B	Bl vs. Sn	29	21	50	7.5	0.023

[a]Bl = blank; Sn = snails and refers to 5 trials in which *B. glabrata* was the migrator and *H. trivolvis* the releaser plus five trials in which *H. trivolvis* was the migrator and *B. glabrata* the releaser.

chemoattraction studies of *B. glabrata* to ES products, mucus, and egg masses of *B. glabrata* are presented in Table 2. *B. glabrata* snails were significantly attracted to ES products (groups A and B), but not to the hydrophilic fraction of the ES products (groups E and F), mucus (groups G and H), or egg masses (groups I and J). In the trial using *B. glabrata* lipophilic ES products (groups C and D), a significant number of snails were in zone A (the blank zone), suggesting that chemorepulsion occurred.

The results of the interspecific chemoattraction studies are presented in Table 3. There was significant interspecific chemoattraction between *B. glabrata* and *H. trivolvis* snails (groups A and B).

The velocity of *B. glabrata* was 17.6 ± 1.6 mm/min, compared to 9.6 ± 2.5 mm/min for *H. trivolvis*. The difference was significant (Student's t test, $P = 0.02$).

DISCUSSION

Results of our study showed that *B. glabrata* snails were attracted to each other in a Petri dish bioassay in the absence of snail phototactic or thigmotactic stimuli. Moreover, individual snails were attracted to ES products from cohorts in the bioassay. The results suggest that intraspecific attraction in *B. glabrata* is mediated by chemicals.

Chemoattraction was more significant in our bioassay using *B. glabrata* than *H. trivolvis* (see groups A and B versus K and L in Table 1). The reasons for this are probably related to the intrinsic velocity of each snail species in the bioassay, with *B. glabrata* being about twice as fast as *H. trivolvis*.

Pheromones associated with snail-mediated chemoattraction have not been elucidated, although Dinter (1974) used a Y-tube apparatus to show that *Litto-*

rina littorea females were attracted to water that had previously contained male littorines.

Whereas the eggs of the gastropod *Neptunea antiqua* appear to contain pheromones that attract conspecific males (Pearce and Thorson, 1967), we found no evidence of attractants in *B. glabrata* eggs. Although mucus trail-following in gastropods is a well-known phenomenon (see review in Runham, 1992), we found no evidence that *B. glabrata* would follow a cohort's mucus trail in our Petri dish bioassay. Townsend (1974) noted that *B. glabrata* snails were able to follow a cohort's mucus trail, but the response was lost within 30 min.

Evidence for chemorepulsion of *B. glabrata* was seen when snail lipophilic ES products were used in our bioassay. In helminth studies, phospholipids from the lipophilic ES products of the adult *Echinostoma trivolvis* (referred to as *E. revolutum* in that study) also elicited worm chemorepulsion in a bioassay designed to study intraspecific chemoattraction (Fried et al., 1980).

Evidence for chemical-mediated interspecific attraction of two planorbid snails from different genera has been presented in this paper for the first time. The results suggest that the chemoattractants are not species specific, a finding shared with other studies on interspecific chemical attraction in helminths (see Haseeb and Fried, 1988, for review). The fact that interspecific chemoattraction occurs between *B. glabrata* and *H. trivolvis* should interest malacologists who attempt to use various organisms as biological control agents against *B. glabrata*.

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PHEROMONE CHIRALITY OF AFRICAN PALM WEEVIL, *Rhynchophorus phoenicis* (F.) AND PALMETTO WEEVIL, *Rhynchophorus cruentatus* (F.) (COLEOPTERA: CURCULIONIDAE)

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Abstract—There are four stereoisomers of both 3-methyl-octan-4-ol, the aggregation pheromone of the African palm weevil, *Rhynchophorus phoenicis* (F.) and 5-methyl-octan-4-ol, the aggregation pheromone of the palmetto weevil, *Rhynchophorus cruentatus* (F.). Synthetic stereoisomers of 3-methyl-octan-4-ol and 5-methyl-octan-4-ol were baseline-separated on a Cyclodex-B fused silica column. Use of this column in gas chromatographic–electroantennographic detection (GC-EAD) and GC–mass spectrometric (GC-MS) analyses revealed that only one stereoisomer, (3*S*,4*S*)-3-methyl-octan-4-ol and (4*S*,5*S*)-5-methyl-octan-4-ol, is produced by male *R. phoenicis* and male *R. cruentatus*, respectively, and elicits good antennal responses by conspecific male and female weevils. In field trapping experiments, with *R. phoenicis* in Côte d'Ivoire and *R. cruentatus* in Florida, (3*S*,4*S*)-3-methyl-octan-4-ol and (4*S*,5*S*)-5-methyl-octan-4-ol strongly enhanced attraction of fresh palm tissue, whereas other stereoisomers were behaviorally benign. Stereoisomeric 3-methyl-octan-4-ol and 5-methyl-octan-4-ol may be utilized to monitor and/or manage populations of these two palm weevils.

Key Words—Coleoptera, Curculionidae, *Rhynchophorus phoenicis*, *Rhynchophorus cruentatus*, aggregation pheromone, pheromone chirality, (3*S*,4*S*)-3-methyl-octan-4-ol, (3*R*,4*R*)-3-methyl-octan-4-ol, (3*S*,4*R*)-3-methyl-octan-4-

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ol, (3*R*,4*S*)-3-methyl-octan-4-ol, (4*S*,5*S*)-5-methyl-octan-4-ol, (4*R*,5*R*)-5-methyl-octan-4-ol, (4*S*,5*R*)-5-methyl-octan-4-ol, (4*R*,5*S*)-5-methyl-octan-4-ol.

INTRODUCTION

Palm weevils in the Rhynchophorinae produce methyl-branched, secondary alcohols as aggregation pheromones: (2*E*)-6-methyl-hepten-4-ol (rhynchophorol) [American palm weevil, *Rhynchophorus palmarum* (L.) (Rochat et al., 1991)]; 3-methyl-octan-4-ol (phoenicol) [African palm weevil, *R. phoenicis* (F.) (Gries et al., 1993, 1994; Rochat et al., 1993]; 4-methyl-nonan-5-ol (ferrugineol) [Asian palm weevils, *R. ferrugineus* (Oliv.), *R. vulneratus* (Panz) Hallett et al., 1993; Rochat et al., 1993) and *R. bilineatus* (Montr.) (Oehlschlager et al., 1994)], and 5-methyl-octan-4-ol (cruentol) [Palmetto weevil, *R. cruentatus* (F.) (Weissling et al., 1994)]. Racemic (rhynchophorol) and stereoisomeric mixtures (phoenicol, ferrugineol, cruentol) of synthetic aggregation pheromones in combination with host material strongly attracted weevils in field experiments. Stereoselective production of and response to pheromone has been demonstrated in *R. palmarum* (Oehlschlager et al., 1992) and recently in the other *Rhynchorphorus* palm weevils (Perez et al., 1993). Male *R. palmarum* stereoselectively produce and both sexes respond to (*S*)-rhynchophorol, while the antipode is behaviorally benign (Oehlschlager et al., 1992). Male *R. phoenicis* produce one stereoisomer of phoenicol but electrophysiological and behavioral activity have not been investigated (Mori et al., 1993). In this study we report that *R. phoenicis* and *R. cruentatus* stereoselectively produce and respond to only one of the four possible stereoisomers of 3-methyl-octan-4-ol and 5-methyl-octan-4-ol, respectively.

METHODS AND MATERIALS

Laboratory Analysis

Male and female *R. phoenicis* were collected in oil palm plantations 40–50 km northeast of Abidjan, Côte d'Ivoire. Male and female *R. cruentatus* were collected in a 300-ha pasture interspersed with *Sabal palmetto* (Walter) and saw palmetto, *Serrenoa repens* (Bartr.), 12 km south of La Belle, Florida. Male-produced phoenicol and cruentol were captured (Gries et al., 1993; Weissling et al., 1994) and subjected to both gas chromatographic–electroantennographic detection (GC-EAD) (Arn et al., 1975) (Hewlett Packard 5890A) and GC–mass spectrometry (GC-MS) (Hewlett Packard 5985 B) on a fused silica, Cyclodex-B-coated column (30 m × 0.25 mm ID, J&W Scientific), which separates all four stereoisomers of phoenicol and cruentol. For GC-EAD recordings, a weevil antenna was removed from the rostrum and suspended between

two glass capillary electrodes with the antennal base being inserted into one and the olfactory club impaled by the other electrode. Chemical ionization (CI, isobutane) GC-MS analysis was conducted in both full-scan and selected-ion monitoring mode (SIM). A full-scan mass spectrum of synthetic phoenicol or cruentol was obtained to select diagnostic ions. For GC-MSCI-SIM, synthetic phoenicol and cruentol, hexane, and concentrated weevil-produced pheromone were injected in split mode and analyzed by scanning for diagnostic ions.

Instruments and General Procedures

Nuclear magnetic resonance (NMR) spectroscopy was conducted on a Bruker AMX-400 spectrometer at 400.13 and 100.62 MHz for ^{1}H and ^{13}CNMR spectra, respectively. ^{1}H chemical shifts are reported in parts per million (ppm, δ) and relative to TMS (0.00 ppm). ^{13}C spectra are referred to $CDCl_3$ (77.0 ppm). Gas chromatographic analyses were performed on Hewlett-Packard 5880A and 5890 instruments equipped with a flame ionization detector and a fused silica, DB-1 coated column (15 m × 0.25 mm ID; 0.25 μm film) (J&W Scientific). Elemental analyses were performed using a Carbo Erba model-1106 Elemental Analyzer. Diethyl ether (Et_2O), dichloromethane (CH_2Cl_2), and pentane were freshly distilled from sodium-benzophenone-ketyl, CaH, and P_2O_5, respectively. Chemicals obtained from commercial sources were used without further purification unless otherwise indicated. All moisture and air sensitive reactions were conducted under argon. Column chromatography refers to flash chromatography using Silica Gel 60 (230–400 mesh E Merck, Darmstadt) (Still et al., 1978). Thin-layer chromatography (TLC) was conducted on aluminum-backed plates precoated with Merck Silica Gel 60F-254 as the adsorbent, and visualized by treatment with an acidic solution of 1% $Ce(SO_4)_2$ and 1.5% molybdic acid followed by gentle heating.

Synthesis of Phoenicol Stereoisomers

(3*R*,4*R*)-, (3*S*,4*S*)-, (3*R*,4*S*)-, and (3*S*,4*R*)-3-methyl-octan-4-ol [(*R*,*R*)-, (*S*,*S*)-, (*R*,*S*)-, and (*S*,*R*)-phoenicol] were synthesized according to a method modified from Nakagawa and Mori (1984), which involved: (1) asymmetric epoxidation of (2*Z*-) or (2*E*)-2-penten-1-ol (Gao et al., 1987; Hill et al., 1985); (2) regioselective epoxide opening with trimethylaluminun (Pfaltz and Mattenberger, 1982; Suzuki et al., 1982; Takano et al., 1989; Vaccaro et al., 1992); (3) selective monotosylation; and (4) alkylation reaction using an organomagnesium cuprate reagent. Synthesis of (3*S*,4*S*)-3-methyl-octan-4-ol exemplifies the synthetic procedure (Figure 1):

(2S,3R)-2,3-Epoxy-pentan-1-ol ***(2a)***. Titanium(IV) isopropoxide (11.4 ml, 10.87 g, 38 mmol) in 250 ml of dry CH_2Cl_2 was mixed under argon with 1 g of 4A powdered, activated molecular sieves. After cooling to −78°C, diethyl

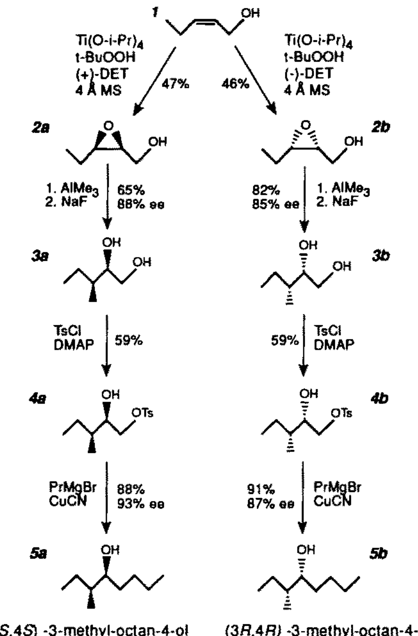

FIG. 1. Scheme for the synthesis of (3*S*,4*S*)-3-methyl-octan-4-ol, **5a,** and (3*R*,4*R*)-3-methyl-octan-4-ol, **5b.**

(2*R*,3*R*)-tartrate [L-(+)-DET, 7.8 ml, 9.9 g, 0.05 mol] was added *via* syringe followed by addition of (2*Z*)-2-penten-1-ol (8.21 ml, 7.0 g, 80 mmol, Aldrich Chemical Co., Milwaukee, Wisconsin). The mixture was stirred 15 min prior to dropwise addition of 25 ml (0.15 mol) of 5.7 M anhyd. *tert*-butyl hydroperoxide in CH_2Cl_2 (prepared as described by Gao et al., 1987) (precooled to −20°C). After the reaction had warmed up to −20°C it was stirred at this temperature for 48 hr. The reaction was monitored by TLC (2:8, pentane–Et_2O; R_f = 0.39). Nonaqueous work-up (Gao et al., 1987) followed by column chromatography (2:8, pentane–Et_2O) gave **2a** (3.79 g, 46% yield, 90% ee) as a colorless liquid [**2a:** ^{1}H ($CDCl_3$): δ 1.02 (3 H, t, J = 8.6 Hz), 1.52 (2 H, m), 2.04 (1 H, brs, D_2O exchangeable), 2.41 (2 H, t, J = 10 Hz), 3.15 (1 H, dd, J = 5, 10 Hz), 3.68 (1 H, dd, J = 4, 10 Hz); ^{13}C ($CDCl_3$) δ 61.82, 57.10, 56.98, 26.22, 13.91 ppm).] **2b** (3.93 g, 47% yield, 87% ee) was synthesized following the same procedure using diethyl (2*S*,3*S*)-tartrate [D-(−)-DET] as the epoxidation catalyst.

(2R,3S)-3-Methyl-pentane-1,2-diol ***(3a).*** A pentane (200 ml solution of **2a** (3.78 g, 37 mmol) was cooled to −50°C. Then 10.5 ml (0.11 mol) of

neat $AlMe_3$ (Aldrich Chemical Co.) was added dropwise followed by 8 ml of 2.49 M *n*-butyllithium (20 mmol). After stirring at −50°C for 20 min., the cooling bath was removed and the flask allowed to warm to room temperature. Monitoring the reaction by GC and TLC (1:9, pentane–Et_2O; R_f = 0.19) indicated reaction completion after 30 min. The reaction was quenched with NaF–H_2O (1:1) (Suzuki et al., 1982) at 0°C. The white precipitate was filtered and the obtained solid was washed with Et_2O. The ethereal layer was dried over anhyd. $MgSO_4$ and concentrated in vacuo to give a pale yellow liquid. Purification by column chromatography (1:9, pentane–Et_2O) afforded **3a** (2.93 g, 64.4% yield, 88% ee) as a colorless liquid [**3a**: ^1H ($CDCl_3$); δ 0.88 (3 H, t, *J* = 8 Hz), 0.90 (3 H, d, *J* = 8 Hz), 1.20 (1 H, m), 1.42 (2 H, m), 2.15 (2 H, brs, D_2O exchangeable), 3.45 (2 H, m), 3.62 (1 H, m); ^{13}C ($CDCl_3$) δ 75.52, 65.21, 37.40, 25.71, 14.11, 11.53 ppm; CI-MS *m/z* (isobutane, relative intensity): 119 (M^++1, 40).] **3b:** 3.59 g, 82% yield, 85% ee.

(2R,3S)-3-Methyl-1-tosyloxy-pentan-2-ol ***(4a)***. This was prepared from 2.92 g (25 mmol) of **3a** in dry pyridine, to which 0.73 g (6 mmol) of dimethyl aminopyridine (DMAP) was added. The flask was cooled to −20°C (ethylene glycol–water–Dry Ice) and 5.73 g (0.03 mol) of *p*-toluensulfonyl chloride added in one portion. After stirring 7 hr at −20 to −10°C and monitoring the reaction by GC and TLC (6:4, pentane–Et_2O, R_f = 0.27), the mixture was poured into ice-cooled NaCl solution and extracted (2 × 30 ml) with Et_2O. The organic layer was washed with 3 M HCl, saturated $NaHCO_3$, saturated NaCl, and dried over anhyd. $MgSO_4$. After concentration and column chromatography (6:4, pentane–Et_2O), solvent residues were removed under vacuum to give **4a** (4.0 g, 59 %) as a pale yellow oil. [**4a:** ^1H ($CDCl_3$); δ 0.85 (3 H, d, *J* = 8 Hz), 0.86 (3 H, t, *J* = 8 Hz), 1.20 (1 H, m), 1.45 (2 H, m), 1.90 (1 H, brs, D_2O exchangeable), 3.72 (1 H, dt, *J* = 8, 4 Hz), 3.98 (1 H, dd, *J* = 8, 2.5 Hz), 4.04 (1 H, dd, *J* = 8, 1.5 Hz); 7.34 (2 H, d, *J* = 8 Hz); 7.80 (2 H, d, *J* = 8 Hz); ^{13}C ($CDCl_3$); δ 144.95, 133.0, 129.89, 127.90, 72.93, 72.75, 36.97, 25.62, 21.57, 13.57, 11.41 ppm; CI-MS *m/z* (relative intensity): 273 (M^+ + 1, 100).] **4b:** 4 g, 59% yield.

(3S,4S)-3-Methyl-octan-4-ol ***(5a)***. This was prepared from propyl magnesium bromide (0.17 mol) in dry Et_2O [prepared by Grignard reaction between *n*-propyl bromide (Aldrich Chemical Co.) and magnesium turnings], which was cooled to −40°C and 1.58 g (17 mmol) of CuCN were added. After stirring the mixture for 30 min, the flask was cooled to −78°C and 4.89 g (17 mmol) of **4a** in 25 ml of dry Et_2O added *via* cannula. After 30 min of stirring, the cold bath was removed and the reaction allowed to warm to room temperature. The course of the reaction was followed by GC and TLC (9:1, pentane–Et_2O, R_f = 0.58). Upon completion, the reaction was quenched with 3 M HCl at 0°C. The aqueous layer was extracted with Et_2O (3 × 25 ml), washed with both saturated $NaHCO_3$ and NaCl, and then dried over anhyd. $MgSO_4$. Column

chromatography (9 : 1, pentane–Et_2O) afforded 1.97 g (91 %) of **5a** as a colorless liquid. [**5a:** ^1H ($CDCl_3$); δ 0.82 (3 H, d, J = 8Hz), 0.85-0.98 (6 H, m), 1.19 (2 H, m), 1.2–1.58 (7 H, m), 1.68 (1 H, s. D_2O exchangeable), 3.41 (1 H, m); ^{13}C ($CDCl_3$) δ 74.83, 39.94, 34.17, 28.42, 25.98, 22.76, 14.02, 13.11, 11.65 ppm. Anal. calcd. for $C_9H_{20}O$: C, 74.92; H, 13.98; found: C, 74.77; H, 13.88.] The use of D-(−)-DET in the asymmetric epoxidation followed by the same synthetic procedure renders the antipode, **5b** (1.97 g, 91% yield, 87% ee. Anal. calcd. $C_2H_{20}O$: C, 74.92; H, 13.98; found: C, 74.89; H, 13.75).

The corresponding *anti*-alcohols, **5c** and **5d,** were synthesized according to the same synthetic scheme except (2*E*)-2-pentenol **6** was used as starting material (Figure 2). The alkenol **6** was prepared by hydride reduction of 3-pentynol (Aldrich Chemical Co.) in 83% yield (Brandsma, 1988) [**6:** ^1H ($CDCl_3$): δ 1.0 (3 H, t, J = 7 Hz), 1.8 (1 H, brs, D_2O exchangeable), 2.05 (2 H, m, J = 8, 1.2 Hz), 4.05 (2 H, d, J = 8 Hz), 5.60 (1 H, dt, J = 13.8, 1.3 Hz), 5.75 (1 H, dt, J = 13.8, 1.3 Hz); ^{13}C ($CDCl_3$) δ 134.83, 127.94, 63.68, 25.14, 13.30 ppm].

(2S,3S)-2,3-Epoxy-pentan-1-ol ***(7a).*** This was prepared according to the procedure used for **2a.** Thus, 0.5 g of 4A powdered, activated molecular sieves and 5.7 ml (5.42 g, 19 mmol) of titanium(IV) isopropoxide in 150 ml of dry CH_2Cl_2 were cooled to −78°C in an acetone–Dry Ice bath. To this was added *via* syringe 3.9 ml (4.69 g, 23 mmol) of diethyl (2*R*,3*R*)-tartrate [L-(+)-DET] and 3.4 g (39 mmol) of (2*E*)-2-penten-1-ol. After stirring the mixture 15 min., 12 ml (68 mmol) of 5.7 M anhydrous *tert*-butyl hydroperoxide in CH_2Cl_2 (pre-cooled to −20°C) was added dropwise. After the reaction had warmed up to −20°C, it was stirred at this at this temperature for 4 hr and monitored by TLC (2:8, pentane:ether; R_f = 0.39). Nonaqueous work-up followed by column chromatography gave **7a** (1.70 g, 43% yield, 96% ee) as a colorless liquid [**7a:** ^1H ($CDCl_3$): δ 0.96 (3 H, t, J = 8 Hz), 1.52 (2 H, m), 2.80 (2 H, t, J = 8 Hz), 3.02 (1 H, brs, D_2O exchangeable), 3.45 (1 H, dd, J = 4, 8 Hz), 3.70 (1 H, dd, J = 8, 2 Hz); ^{13}C ($CDCl_3$) δ 62.05, 58.32, 57.10, 24.41, 13.91 ppm.] **7b** (1.64 g, 41% yield, 96% ee) was prepared following the same procedure using diethyl (2*S*,3*S*)-tartrate [D-(−)-DET].

(2R,3R)-3-Methyl-pentane-1,2-diol ***(8a).*** 1.18 g, 59% yield, 96% ee. ^1H

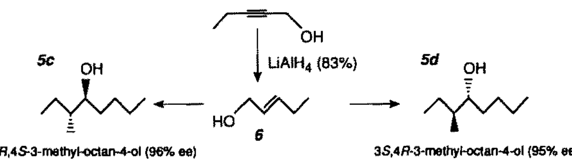

FIG. 2. Scheme for the synthesis of (3*S*,4*R*)- and (3*R*,4*S*)-3-methyl-octan-4-ol.

($CDCl_3$): δ 0.86 (3 H, t, J = 8 Hz), 0.91 (3 H, d, J = 8 Hz), 1.18 (1 H, m), 1.40 (2 H, m), 3.12 (2 H, brs, D_2O exchangeable), 3.40 (2 H, m), 3.55 (1 H, m); CI-MS m/z (relative intensity): 119 (M^++1, 45). **8b:** 1.28 g, 67% yield, 95% ee.

(2R,3R)-3-Methyl-1-tosyloxy-pentan-2-ol ***(9a).*** 1.52 g, 60% yield, ^1H ($CDCl_3$): δ 0.84 (3 H, d, J = 8 Hz), 0.86 (3 H, t, J = 8 Hz), 1.22 (1 H, m), 1.45 (2 H, m), 2.0 (1 H, brs, D_2O exchangeable), 3.64 (1 H, dt, J = 8, 4 Hz), 3.94 (1 H, dd, J = 8, 2.5 Hz), 4.01 (1 H, dd, J = 8, 1.5 Hz); 7.30 (2 H, d, J = 8 Hz); 7.75 (2 H, d, J = 8 Hz); CI-MS m/z (relative intensity): 273 (M^++1, 100). **9b:** 1.71 g, 62% yield.

(3S,4R)-3-Methyl-octan-4-ol ***(5d).*** 0.68 g, 85% yield, 96% ee. ^1H ($CDCl_3$): δ 0.81 (3H, d, J = 8.1 Hz), 0.84–1.0 (6H, m), 1.20 (2H, m), 1.22–1.60 (7H, m), 1.70 (1H, brs, D_2O exchangeable), 3.45 (1H, m); ^{13}C ($CDCl_3$) δ 75.71, 40.50, 33.07, 28.25, 24.55, 22.76, 14.70, 13.11, 11.82 ppm. Anal. calcd. for $C_9H_{20}O$: C, 74.92; H, 13.98, found: C, 74.76; H, 14.07. **5d:** 0.76 g, 84% yield, 95% ee. Anal. calcd. for $C_9H_{20}O$: C, 74.92; H, 13.98, found: C, 75.06; H, 14.01, Figure 2.

Synthesis of Cruentol Stereoisomers

(4*R*,5*R*)-, (4*S*,5*S*)-, (4*R*,5*S*)-, and (4*S*,5*R*)-5-methyl-octan-4-ol (*R*,*R*-, *S*,*S*-, *R*,*S*-, and *S*,*R*-cruentol) were synthesized according to a method modified from Nakagawa and Mori (1984), followed by Mitsunobu reaction (Mitsunobu, 1981) of the corresponding *anti*-isomers (Figure 3).

(2S,3S)-2,3-Epoxy-hexan-1-ol ***(11a).*** This was prepared according to the procedure employed for **2a.** Thus, 1g of 4A powdered, activated molecular sieves and 8.4 ml (8.79 g, 31 mmol) of titanium(IV) isopropoxide in 250 ml of dry CH_2Cl_2 were cooled to −78°C in an acetone–Dry Ice bath. Then *via* syringe was added 6.3 ml (5.23 g, 25 mmol) of diethyl (2*R*,3*R*)-tartrate [L-(+)-DET] and 6.1 ml (5.2 g, 52 mmol) of (2*E*)-2-hexen-1-ol **10** (Aldrich Chemical Co.). Stirring of the mixture was followed by dropwise addition of 18 ml (0.11 mol) of 6.2 M anhydrous *tert*-butyl hydroperoxide in CH_2Cl_2 (precooled to −20°C). The reaction was allowed to warm to −20°C with stirring and was stirred at this temperature for 3 hr while it was monitored by TLC (4:6, hexane–ether; R_f = 0.19). Ferrous sulfate/tartaric acid work-up (Gao et al., 1987) followed by column chromatography gave **11a** (4.82 g, 80% yield, 95% ee) as a colorless liquid, which crystallized as white needles at −20°C. [**11a:** ^1H ($CDCl_3$): δ 0.96 (3 H, t, J = 7.6 Hz), 1.48 (2 H, m), 1.54 (2 H, m), 1.80 (1 H, brs, D_2O exchangeable), 2.92 (2 H, m), 3.60 (1 H, dd, J = 5, 10 Hz), 3.90 (1H, dd, J = 10, 2.5 Hz); ^{13}C ($CDCl_3$) δ 61.76, 58.34, 55.81, 33.57, 19.23, 13.84 ppm.] **11b** (4.94 g, 82% yield, 95% ee) was prepared following the same procedure but employing diethyl (2*S*,3*S*)-tartrate [D-(−)-DET].

10 OH

80% 95% ee | 82% 95% ee

11a OH O | 11b OH O

78% 95% ee | 70% 98% ee

12a OH OH | 12b OH OH

76% | 78%

13a OH OTs | 13b OH OTs

78% 98% ee | 85% 98% ee

14a OH | 14b OH

1. $PhCOOH/Ph_3P/DIAD$
2. 15% KOH/MeOH

14c OH | 14d OH

98% ee | 96% ee

FIG. 3. Scheme for the synthesis of all four stereoisomers of 5-methyl-octan-4-ol.

(2R,3R)-3-Methyl-hexane-1,3-diol ***(12a)***. This was prepared according to the procedure employed for **3a.** Thus, to 4.80 g (0.04 mol) of **11a** in 250 ml of dry pentane cooled to −50°C was added dropwise 11.9 ml (8.64, 0.11 mol) of neat $AlMe_3$. This was followed by 16 ml of 2.49 M *n*-butyllithium (0.04 mol). After stirring 20 min, the cooling bath was removed and the flask allowed to warm to room temperature. The reaction was monitored by GC and TLC (2:8, hexane–ethyl acetate, R_f = 0.33) and was complete after 30 min. After quenching with 3 M HCl at 0°C and separation of the two phases, the aqueous layer was extracted with ether (3 × 40 ml), dried over anhyd. $MgSO_4$, and concentrated in vacuo. Purification by column chromatography afforded **12a** (4.26 g, 78% yield, 95% ee) as a colorless liquid, which crystallized as a white solid at −20°C. [**12a:** ^1H ($CDCl_3$): δ 0.88 (3 H, t, J = 10 Hz), 0.90 (3 H, d, J = 10 Hz), 1.14 (1 H, m), 1.25 (1 H, m), 1.46 (1 H, m), 1.60 (1 H, m), 2.10 (1 H, brs, D_2O exchangeable), 2.24 (1 H, brs, D_2O exchangeable), 3.50 (2 H, m), 3.70 (1 H, m); ^{13}C ($CDCl_3$) δ 76.28, 64.66, 35.94, 34.68, 20.06, 15.14, 14.26); CI-MS *m/z* (relative intensity): 119 (M^++1, 40.] **12b:** 3.91 g, 70% yield, 98% ee.

(2R,3R)-3-Methyl-1-tosyloxy-hexan-2-ol ***(13a)***. After purification by col-

umn chromatography (6:4, pentane–ether, R_f = 0.45), **13a** (6.54 g, 76% yield) was obtained as a pale yellow oil, ^{1}H ($CDCl_3$): δ 0.86 (6 H, m), 1.18 (2H, m), 1.40 (2H, m), 1.60 (1H, m), 1.90 (1H, brs D_2O exchangeable), 2.48 (3H, s), 3.64 (1H, m), 3.98 (1H, dd, J = 12, 8 Hz), 4.10 (1H, dd, J = 12, 4 Hz), 7.38 (2H, d, J = 8 Hz), 7.80 (2H, d, J = 8 Hz); ^{13}C ($CDCl_3$) δ 144.99, 132.5, 129.92, 127.93, 73.43, 72.66, 35.54, 34.15, 21.26, 19.96, 15.10, 14.16 ppm. **13b:** 6.09 g, 78% yield.

(4S,5R)-5-Methyl-octan-4-ol ***(14a).*** This was prepared by the route used for **5a** except that ethyl magnesium bromide (Aldrich Chemical Co.) (3 M solution in Et_2O) was used. After purification by column chromatography (9:1, pentane–ether, R_f = 0.08), **14a** (2.74 g, 78% yield, 98% ee), was obtained as a colorless liquid, which crystallized as a white solid at −20°C, ^{1}H ($CDCl_3$): δ 0.90 (3 H, t, J = 8 Hz), 0.92 (3 H, d, J = 8 Hz), 0.94 (3 H, t, J = 8 Hz), 1.10 (1 H, m), 1.24 (1 H, m), 1.32 (1 H, m), 1.40 (4 H, m), 1.50 (1 H, m), 1.70 (1 H, brs, D_2O exchangeable), 3.48 (1 H, m); ^{13}C ($CDCl_3$) δ 75.82, 38.61, 35.64, 34.17, 20.42, 19.28, 15.24, 14.34, 14.13 ppm; CI-MS *m/z* (relative intensity): 127 (100) ($M^+ - H_2O$): Anal. calcd. for $C_9H_{20}O$: C, 74.92; H, 13.98, found: C, 75.16; H, 14.11. **14b:** 2.73 g, 85% yield, 98% ee; Anal. calcd. for $C_9H_{20}O$: C, 74.92; H, 13.98, found: C, 73.87; H, 14.08.

[(4R,5R)-5-Methyl-4-octyl)]benzoate ***(15a).*** Triphenylphosphine (9.97 g, 38 mmol) and **14a** (2.74 g, 19 mmol) in 30 ml of dry benzene were added *via* cannula to diisopropyl azodicarboxyate (7.68 g, 7.5 ml, 38 mmol) (Aldrich Chemical Co.) and benzoic acid (4.64 g, 38 mmol) in 45 ml dry benzene. After stirring overnight at room temperature, pentane was added, at which point a white precipitate formed. The reaction mixture was filtered through a Florisil pad and concentrated under pressure. Purification by column chromatography (9:1, pentane–ether, R_f = 0.61) afforded **15a** (2.35 g, 50% yield) as a pale yellow liquid. Unreacted alcohol was recovered. [**15a:** ^{1}H ($CDCl_3$): δ 0.88 (3 H, t, J = 9 Hz), 0.98 (3 H, t, J = 9 Hz), 1.00 (3 H, d, J = 9 Hz), 1.10 (1 H, m), 1.38, (5 H, m), 1.58 (1 H, m), 1.70 (1 H, m), 1.80 (1 H, m), 5.10 (1 H, m), 7.40 (2 H, dd, J = 9, 2 Hz), 7.54 (1 H, ddd, J = 9, 2 Hz), 8.04 (2 H, dd, J = 9, 2 Hz); ^{13}C ($CDCl_3$) δ 166.37, 132.62, 130.98, 129.56, 128.29, 77.74, 36.27, 35.41, 33.77, 20.34, 19.07, 14.48, 14.22, 14.01 ppm; CI-MS *m/z* (relative intensity): 127 ($M^+ - C_6H_5$-CO, 100).] **15b:** 2.70 g, 57.3% yield.

(4R,5R)-5-Methyl-octan-4-ol ***(14c).*** To a 15% KOH solution of methanol was added **15a** (1.30 g, 52 mmol). After stirring the mixture overnight, it was quenched with water and extracted with Et_2O (3 × 30 ml). The ether extracts were washed with dilute HCl and saturated NaCl and then dried over anhyd. $MgSO_4$. Concentration *in vacuo* and column chromatography (9:1, pentane–ether, R_f = 0.13) gave **14c** (0.71 g, 95% yield, 98% ee) as a colorless liquid. [**14c:** ^{1}H ($CDCl_3$): δ 0.89 (3 H, d, J = 8 Hz), 0.92 (3 H, t, J = 8 Hz), 0.95 (3 H, t, J = 8 Hz), 1.12 (1 H, m), 1.24 (1 H, m), 1.33 (1 H, m), 1.39 (5 H,

m), 1.48 (1 H, m), 3.40 (1 H, m); ^{13}C ($CDCl_3$) δ 75.04, 38.70 36.73, 35.73, 20.51, 19.51, 15.33, 14.38, 13.65 ppm; Anal. calcd. for $C_9H_{20}O$: C, 74.92; H, 13.98, found: C, 74.74; H, 13.84.] **14d:** 0.75 g, 89% yield, 96% ee; Anal. calcd. for $C_9H_{20}O$: C, 74.92; H, 13.98; found: C, 74.69; H, 13.81.

The enantiomeric excesses of **5a** (93%), **5b** (87%), **5c** (96%), **5d** (95%), **14a** (98%), **14b** (98%), **14c** (98%), and **14d** (96%) and their corresponding intermediates except epoxides were determined by GC analyses on the Cyclodex-B column and by formation of the *O*-acetyllactyl methyl esters (Slessor et al., 1985). Enantiomeric excesses of epoxides **2a** (85%), **2b** (88%), **7a** (96%), **7b** (96%), **11a** (95%), and **11b** (95%) were determined by GC analysis of corresponding *O*-acetyllactyl methyl esters (Slessor et al., 1985) on a DB-23 column. Racemic 3-methyl-octan-4-ol and 5-methyl-octan-4-ol were synthesized as previously described (Gries et al., 1993; Weissling et al., 1994).

Field Experiments

African Palm Weevil. A six-replicate, five-treatment field experiment in a 10-year-old oil palm stand (La Me Research Station, Côte d'Ivoire) tested attraction of palm tissue (250 g) alone or in combination with either stereoisomeric, (*S*,*S*)-, (*R*,*R*)-, or (*S*,*S*)- plus (*R*,*R*)-phoenicol. Traps (Oehlschlager et al., 1993) were attached at breast height to oil palms in randomized blocks with traps at 27-m intervals and blocks 81 m apart. (*S*,*S*)- and (*R*,*R*)-phoenicol were released at 0.5 mg/day (at 25°C) from a 1.5-ml polyethylene centrifuge tube with two 2-mm holes below the top. Racemic phoenicol was dispensed at 2 mg/day (at 25°C) from four 1.5-ml polyethylene centrifuge tubes. Fresh palm tissue in each trap was treated with insecticidal (biodegradable) Evisect "S" (0.3% thiocyclamhydrogenoxalate in water) to retain captured weevils (Gries et al., 1993, 1994).

Trap catch data were subjected to analysis of variance followed by Scheffé test for comparisons of means (Zar, 1984).

Palmetto Weevil. A 12-replicate, four-treatment experiment in the same location as for weevil collection tested attraction of *Sabal palmetto* tissue (1.5 kg) alone or in combination with either stereoisomeric, (*S*,*S*)- or (*R*,*R*)-cruentol. Traps (Weissling et al., 1994) were secured on the ground in randomized complete blocks with traps at 20-m intervals and blocks at least 50 m apart. Unlike Weissling's trap, a tapered, inverted white plastic container (4.9 liter) with a screened lid was suspended in the mouth of the bucket by a capped PVC pipe (1.3 cm diameter) from which pheromone release devices were hung. (*S*,*S*)- or (*R*,*R*)-cruentol were released at 0.06 mg/day (at 25°C) from one and stereoisomeric cruentol from four bottom-sealed 1-μl microcapillary tubes (Drummond Scientific Co., Broomall, Pennsylvania) placed in bottom-sealed microhematocrit tubes (length 75 mm, ID 1.1–1.2 mm; Fisher Scientific, Pittsburgh, Penn-

sylvania). Hematocrit tubes were placed into polypropylene centrifuge tubes (Corning Glass Works, Corning, New York, with 6-mm holes drilled 1.8 cm from the top). Trap catch data were subjected to square root ($x + 0.5$) transformation and ANOVA (SAS Institute, 1990) followed by Waller-Duncan k-ratio t test to test differences between means ($P \leq 0.05$).

RESULTS AND DISCUSSION

Many coleopteran pheromones are optically active (Seybold, 1993; Leal and Mochizuki, 1993; Bestmann and Vostrowsky, 1988; Evershed, 1988; Borden, 1985, and literature cited therein). Enantioselective production of and response to pheromones contribute to species specificity of semiochemical communication (Borden et al., 1976, 1980; Brand et al., 1979; Birch et al., 1980; Payne et al., 1982; Oehlschlager et al., 1987; Pierce et al., 1987; Birch, 1984; Byers, 1989). The presence of nonnatural (non-beetle-produced) enantiomers in synthetic pheromones has been demonstrated to interfere with optimal attraction. For instance, the male-produced aggregation pheromone in the southern pine beetle, *Dendroctonus frontalis* Zimm., (1*R*,5*S*,7*S*)-(+)-*endo*-brevicomin, *endo*-7-ethyl-5-methyl-6,8-dioxa-[3.2.1]octane, markedly enhances the response by both sexes to female-produced frontalin (1,5-dimethyl-6,8-dioxa-[3.2.1]octane), whereas the presence of the antipode in racemic *endo*-brevicomin interferes with optimal attraction (Vité et al., 1985). In the Japanese beetle, *Popillia japonica* (N.), female-produced Japonilure, (*R*,*Z*)-(−)-5-(1-decyl)oxacyclopentan-2-one, strongly attracts males, whereas the antipode inhibits responses (Tumlinson et al., 1977). In the scarab beetle, *Anomala cuprea* only the (*R*,*Z*)-5-(−)-(oct-1-enyl)oxacyclopentan-2-one attracts conspecifics, while the presence of the nonnatural enantiomer reduced attraction (Leal and Mochizuki, 1993). Determination of insect-produced pheromone enantiomer(s) and/or stereoisomers is required to fully elucidate the chemical communication system for a target insect and to implement efficient pheromone-based monitoring and/or management. In this study, we confirmed the chirality of weevil-produced phoenicol (Gries et al., 1993; Perez et al., 1993; Mori et al., 1993), determined chirality of weevil-produced cruentol (Weissling et al., 1994), and field tested weevil attraction to natural and nonnatural stereoisomers.

Of several methods available to prepare the target chiral α-methyl secondary alcohols, the Sharpless asymmetric epoxidation combined with diastereoselective ring opening was the most appealing (Gao et al., 1987; Hill et al., 1985; for use of trimethylaluminum and organocuprates: Pfaltz and Mattenberger, 1982; Suzuki et al., 1982; Takano et al., 1989; Vaccaro et al., 1992; Miyashita et al., 1993). This strategy allowed the use of inexpensive reagents and the synthesis of all four stereoisomers from the same starting material.

Sharpless asymmetric epoxidation has been previously used for the synthesis of the stereoisomers of the elm bark beetle pheromone, 4-methyl-heptan-3-ol (Nakawaga and Mori, 1984). In contrast to this previous synthesis, preparation of phoenicol and cruentol used 0.5 equivalents of catalyst in the presence of a 4A molecular sieve coupled with addition of the oxidizing agent at −78°C to increase the optical purity of the initial epoxide product. Epoxidations were maintained at −20°C until 97–98% conversion was obtained for **2a** and **2b** (two days) as well as for **7a, 7b, 11a,** and **11b** (3–4 hr). Although reactions are not reported for diisopropropyl tartrate, higher enantioselectivities were achieved with diethyl tartrate. Nonaqueous work-up (Gao et al., 1987) followed by flash chromatography was used in the synthesis of the C-5 epoxides. Separation of the tartrate from the C-5 epoxides required two or more chromatographic cycles, whereas ferrous sulfate/tartaric acid work-up (Gao et al., 1987) followed by a single chromatography cleanly gave the C-6 epoxides. Diasteroselective epoxide ring-opening was conducted with neat $AlMe_3$ instead of a hexane solution of this reagent, as was employed by Nakawaga and Mori. This facilitated completion of the reaction in less than 1 hr compared to two to three days. Work-up *via* addition of saturated NaF at −40°C (Suzuki et al., 1982) for **3a, 3b, 8a,** and **8b,** and 3 M HCl for **12a** and **12b** afforded the corresponding diols after flash chromatography. Quenching with NaF rather than HCl improved isolated yields of 3-methyl-1,2-pentanediols, probably due to the high solubility of the diols in water. Products arising from breakage of the α-bond or retention of configuration during the cleavage of the β-epoxide bond were not detected by GC or ^{1}H NMR analysis. The *syn*-isomers of phoenicol and cruentol were obtained with moderate optical purities from asymmetric epoxidation of the requisite *Z*-alkenols. In contrast, Mori and Brevet (1991) and Mori and Harashima (1993) generated chirally pure epoxides through crystallization of derivatives, a process that leads to yields in the range of 40%. The *p*-nitrobenzoates or 3,5-dinitrobenzoates of **2a** and **2b** were oils at room temperature and below.

The *syn* isomers of 5-methyl-octan-4-ol were obtained in high enantiomeric excess through Mitsunobu (Mitsunobu, 1981; Hughes, 1992, and references cited therein) mediated inversion of configuration of the *anti*-isomers **14a** and **14b.** Use of *p*-nitrobenzoic acid–Ph_3P–diethyl azocarboxylate (DEAD) in THF yielded less than 25% of the corresponding *p*-nitrobenzoates. Successful Mitsunobu conditions (~51% yields) employed benzoic acid–Ph_3P–diisopropyl azocarboxylate (DIAD) and benzene as a solvent (Paquette and Sugimura, 1986; Dai et al., 1988; Dyer and Kishi, 1988). No epimerization or retention of configuration products were observed.

Both stereoisomeric phoenicol and cruentol elute from a polar SP-1000-coated, fused silica column in two resolved components. The shorter eluting component coincided with the male-produced pheromone of each weevil and was hypothesized to be the *syn* diastereoisomer, consisting of coeluting *S*,*S* and

R,R isomers. This assignment was made by analogy with the aggregation pheromone of the smaller European elm bark beetle, *Scolytus multistriatus* (Marsham), 4-methyl-heptan-3-ol, that also has two stereogenic centers and exists as two diastereoisomeric forms that are separable by GC on a polar Carbowax 20 M column (Pearce et al., 1975). Analysis of stereoselectively prepared *syn* and *anti* stereoisomers of phoenicol and cruentol confirmed the assignments. Synthetic (*R,R*)-, (*S,S*)-, (*R,S*)-, and (*S,R*)-phoenicol and cruentol were separated with baseline resolution on a fused silica, Cyclodex-B column. These analyses revealed that male *R. phoenicis* and male *R. cruentatus* produce the *S,S* stereoisomer of phoenicol (Figure 4) and cruentol (Figure 5), respectively. *S. multistriatus* also produces the *S,S* stereoisomer of 4-methyl-heptan-3-ol (Pearce et al., 1975), whereas the large European elm bark beetle, *Scolytus scolytus* (F.), produces both (3*S*,4*S*)- and (3*R*,4*S*)-4-methyl-heptan-3-ol (Blight et al., 1977, 1978, 1979; Wadhams et al., 1982).

Coupled GC-EAD of synthetic phoenicol (Figure 6) and cruentol (Figure 7) revealed strong antennal responses to weevil-produced (*S,S*)-phoenicol and (*S,S*)-cruentol. Lack of or reduced response to later-eluting stereoisomers can hardly be explained by an antennal refractory period. In GC-EAD recordings with the same Cyclodex-B column, antennae of two Asian palm weevils, *R. ferrugineus* (Oliv.) and *R. vulneratus* (Panz.), distinctively responded to both, closely eluting, (4*S*)- and (4*R*)-4-methyl-nonan-5-one (Perez et al., unpublished). Similarly, in GC-EAD analyses of oil palm volatiles, antennae of male

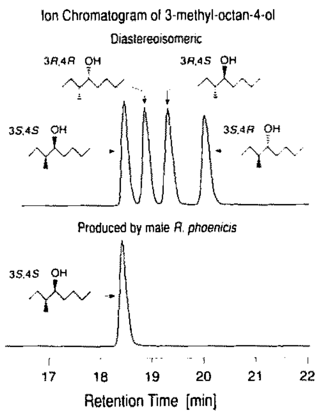

FIG. 4. Selected ion *m/z* 127 chromatogram (Hewlett Packard 5985B) of stereoisomeric and weevil-produced 3-methyl-octan-4-ol. *m/z* 127 was the parent ion [(M^+-H) 143, (M^+-H-OH) 127] of the full-scan mass spectrum in CI mode. (Cyclodex-B column; 90°C isothermal; linear flow velocity of carrier gas: 35 cm/sec; injector temperature: 220°C).

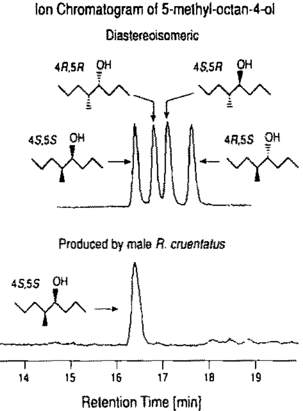

FIG. 5. Selected ion *m/z* 127 chromatogram of stereoisomeric and weevil-produced 5-methyl-octan-4-ol. *m/z* 127 was the parent ion [(M^+-H) 143, (M^+-H-OH) 127] of the full-scan mass spectrum in CI mode (instrument and chromatographic conditions as in Figure 4).

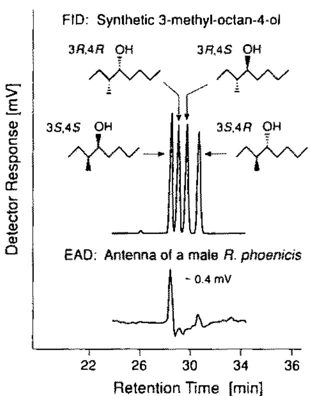

FIG. 6. Representative GC-EAD recording of a female *Rhynchophorus phoenicis* antenna responding to stereoisomers of 3-methyl-octan-4-ol (Hewlett Packard 5890A; split injection; column and chromatographic conditions as in Figure 4).

and female *R. phoenicis* responded within 2.5 min to four esters, two of which were barely baseline separated (Gries et al., 1994). Strong antennal activity of the *S,S*, and weak activity of *S,R* and *R,S* isomers of the pheromones (Figures 6 and 7) suggest that sensory recognition of the natural *S,S* stereoisomer is more dependent on the stereochemistry of the methyl than the hydroxy group.

In field experiments (*S*,*S*)-phoenicol and (*S*,*S*)-cruentol strongly synergized attraction of weevils to palm tissue (Figures 8 and 9). Because racemic, stereoisomeric mixtures were as synergistic as *S*,*S* isomers, the weakly EAD-active *S*,*R* isomers (Figures 6 and 7) neither enhanced nor reduced behavioral activity in the stereoisomeric mixtures (Figures 8 and 9). Lack of strong antennal (Figures 6 and 7) and any behavioral activity (Figures 8 and 9) of nonnatural isomers suggests that sympatric beetles are unlikely to utilize one or more stereoisomers

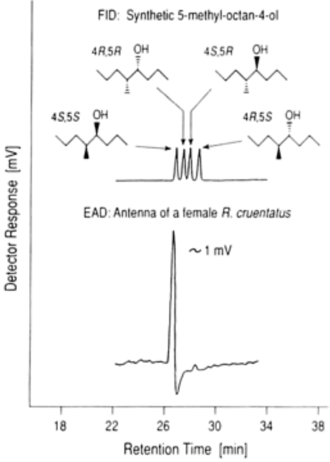

FIG. 7. Representative GC-EAD recording of a female *Rhynchophorus cruentatus* antenna responding to stereoisomers of 5-methyl-octan-4-ol (Hewlett Packard 5890A; split injection; column and chromatographic conditions as in Figure 4).

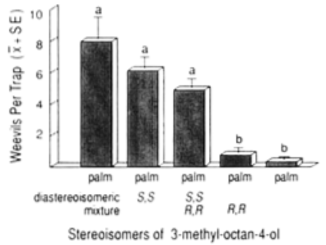

FIG. 8. Mean counts (+ standard error) of male and female *R. phoenicis* in traps baited with 250 g of chopped oil palm tissue alone and in combination with either stereoisomeric, (3*S*,4*S*)-, (3*R*,4*R*)- or (3*S*,4*S*)- plus (3*R*,4*R*)-phoenicol. La Me Research Station, Côte d'Ivoire; May 6–10, 1993; six blocks. Bars superscripted by the same letter are not significantly different. ANOVA followed by Scheffé test, $P < 0.05$.

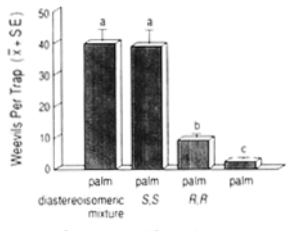

FIG. 9. Mean counts (+ standard error) of male and female *R. cruentatus* in traps baited with 1.5 kg of chopped *Sabal palmetto* palm tissue alone and in combination with either stereoisomeric, (4*S*,5*S*)- or (4*R*,5*R*)-cruentol. La Belle, Florida, USA, June 9–16, 1993; 12 blocks. Bars superscripted by the same letter are not significantly different. ANOVA followed by Waller-Duncan *k*-ratio *t* test on square root transformed data ($x + 0.5$), $P \leq 0.05$.

of phoenicol or cruentol as a pheromone. In practice, mixtures of all four stereoisomers of each pheromone could be used in combination with host materials to monitor and/or mass trap *R. phoenicis* and *R. cruentatus* populations.

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EVIDENCE FOR VOLATILE CHEMICAL ATTRACTANTS IN THE BEETLE *Maladera matrida* Argaman (Coleoptera: Scarabaeidae)

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Abstract—The *Maladera matrida* beetle (Coleoptera, Scarabaeidae, Melolonthinae), a relatively new species to science, was first identified in Israel in 1983. In the course of field observations it was found that adult *M. matrida* beetles emerged from the soil at sunset to feed and mate. During the first 20 min of flight, most of the beetles were males. The females emerged shortly afterwards, and aggregations numbering 20–30 individuals with equal proportions of males and females were eventually formed on peanut plants. Laboratory olfactometer bioassays showed that peanut leaves (food) attracted both males and females. Field-trapping experiments and olfactometer studies showed that *M. matrida* beetles were highly attracted by live virgin females in the presence of food (cut-up peanut leaves). Another set of field trapping experiments indicated that airborne volatiles produced by live virgin females plus food had the same attracting ability as live virgin females plus food. The attraction exerted by the combination of live virgin females and peanut leave volatiles suggests a synergism effect. Accordingly, we propose a two-stage mechanism of chemical communication in the *M. matrida* beetles: first, the males cause mechanical damage to the host plant to attract both sexes; later, the females emit attractants (sex pheromone) while eating or shortly thereafter.

Key Words—*Maladera matrida*, Coleoptera, Scarabaeidae, collection of volatiles, field trapping, olfactometer, attractants, host plant volatiles, synergism, aggregation, sex pheromone.

INTRODUCTION

The *Maladera matrida* Argaman beetle (Coleoptera, Scarabaeidae, Melolonthinae), a relative newcomer to Israel, was first detected by Klein and Chen

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(1983) and later described as a new species by Argaman (1986, 1990). This species has recently appeared in Saudi Arabia as well (D. Ben-Yakir, personal communication).

The *M. matrida* beetles, like most other Scarabaeidae (Leal et al., 1993), is considered to be a serious polyphagous pest, affecting many agricultural crops. Adult beetles feed on foliage and flowers in orchards, irrigated fields and ornamental gardens. The grubs develop in the soil and cause damage to underground crops such as peanuts and potatoes (Gol'berg et al., 1986, 1989; Harari et al., 1994).

Because *M. matrida* is a new species, basic biological information, mainly about aggregation and mating behavior and possible bioassay, is scanty. By the end of the '80s only three scarab pheromones have been identified (Henzell and Lowe, 1970; Tumlinson et al., 1977; Tamaki et al., 1985), the main problem being the lack of uniform and consistent bioassays (Domek and Johnson, 1987; Bestmann and Vostrowsky, 1988; Leal et al., 1992, 1993).

A variety of systems for the collection of airborne volatiles from insects have been reported (e.g., Gaston, 1984, and references therein; Golub and Weatherston, 1984, and references therein; Shani and Lacey, 1984; Jursik et al., 1990; Heath and Manukian, 1992). In these methods attention is paid to a number of factors: protection against contamination, prevention of stress to the insect, avoidance of disturbing the natural environment of the insect, efficiency and speed of the collection from a single insect or from a large number of insects, and simplicity and low cost of the construction and operation of the collection system.

The role of host plant volatiles and/or food-type lures with pheromones and their synergistic effect in the attraction of insects has been investigated (Klein et al., 1981; Ladd et al., 1981; Domek and Johnson, 1988; Domek et al., 1990; Bartelt et al., 1993; Campbell et al., 1993; Weissling et al., 1993). Semiochemical management of *M. matrida* populations may require identification of both the insect and host plant volatiles and the synergism between them.

The aim of our research was to collect and characterize the volatiles produced and emitted by live virgin beetles as a means of studying the chemical communication in the species and subsequently to apply the findings to pest control. In this paper we present the results of field observations, field-trapping experiments with live beetles and their volatiles, and laboratory olfactometer bioassays. We also describe an all-glass system for the collection of insect volatiles. Finally, we propose a possible mechanism for chemical communication among *M. matrida* beetles.

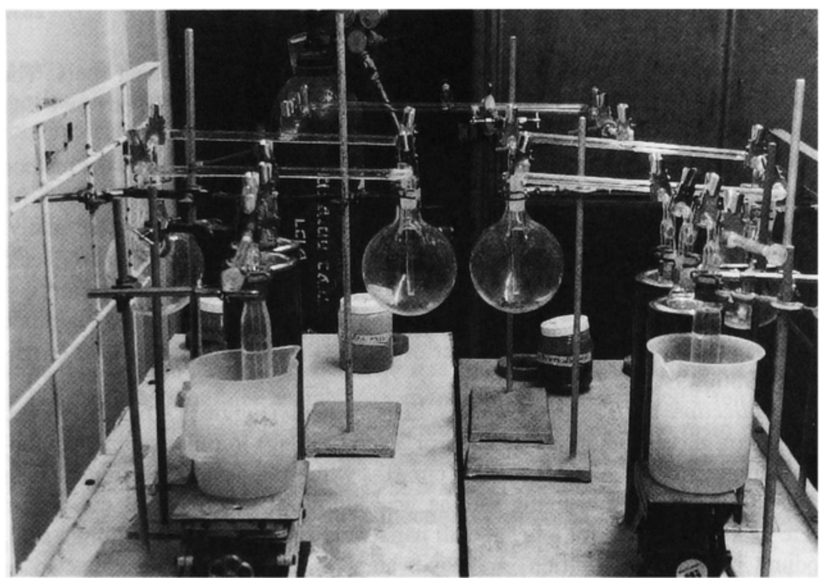
METHODS AND MATERIALS

Rearing of Beetles. Third-stage grubs of *M. matrida* were collected in the field and reared individually in 10-ml plastic boxes containing humid sand. The boxes were kept in a climate-controlled room at 27 ± 3°C, 60 ± 15% relative

humidity, and a 14L:10D regime. The grubs were fed with wheat roots. The adult beetles were reared in groups (up to 100 beetles of the same sex in 1-liter plastic boxes) and fed with rose flowers.

Sexing was performed with the aid of a dissecting microscope by the method described by Gerling and Hefez (1990). Adult beetles were frozen at 0°C for about 3 min and then inspected after the pigydium had been opened gently. Females are distinguished by the presence of two elastic chitinic plates located on each side of the pigydium aperture across the abdomen tip. Males are distinguished by a sex organ, which widens into a funnel-like asymmetric chitinic appendage, reminiscent of the stinger of a bee, for grasping the female (Argaman, 1986, 1990).

Collection System for Volatiles. The system (Figure 1A) is composed entirely of glass (without any plastic or Tygon piping) in order to prevent contamination. All the tubes are 0.83 cm ID, with spherical S-29 ground glass connections. A strict cleaning routine was followed: new parts of the system were heated at 550°C for 4 hr, and all parts were rinsed with sulfochromic acid, sodium bicarbonate solution, and tap water. The volatiles were collected as follows: By means of an airflow regulator (Mego-Afek, Israel) and air flowmeter (model 10A1190, Fisher and Porter Co., Warminster, Pennsylvania, capable of discharging 10–117 ml/min), clean dry compressed air (Maxima, Israel) was passed through Poropak Q (Millipore Co., Milford, Massachusetts) and silica blue (Merck Co., Darmstadt, Germany) filters into 2-liter glass flasks containing live beetles. The conditions in the flasks were similar to those in the field, i.e., light intensity of 0.01–0.1 lux (darkness), temperature of 24–30°C (ambient temperature), airflow rate of 0.1 liter/min (wind velocity of 0.1 m/sec), and collection of the volatiles over 150 min (flight time in nature). Volatiles were collected separately from male and female virgin, laboratory-reared, and wild beetles from the field whose sexual maturity and status was not known. The volatiles liberated by the beetles were carried over by the airflow and captured in a number of traps connected in series. In most cases three traps were used, the first two being empty tubes cooled with liquid nitrogen and the third containing an organic solvent, but in some cases the volatiles were trapped in two tubes, both containing organic solvents (cyclohexane or hexane, HPLC grade, Aldrich Chemical Co. Inc., Milwaukee, Wisconsin). After collection of volatiles was completed, the traps were cooled by liquid nitrogen, and the flasks containing the beetles were rinsed twice with 5 ml of solvent each time. The rinsing solutions were collected for each container separately, dried over calcium chloride if necessary (mainly the traps), and then concentrated with the aid of a slow nitrogen stream up to a final volume of 0.5 ml. The same drying and concentration process was also applied to the solvents in the traps. The concentrates were stored under an inert atmosphere in 1-ml conical tubes stoppered with S-14 ground glass stoppers at 4°C.



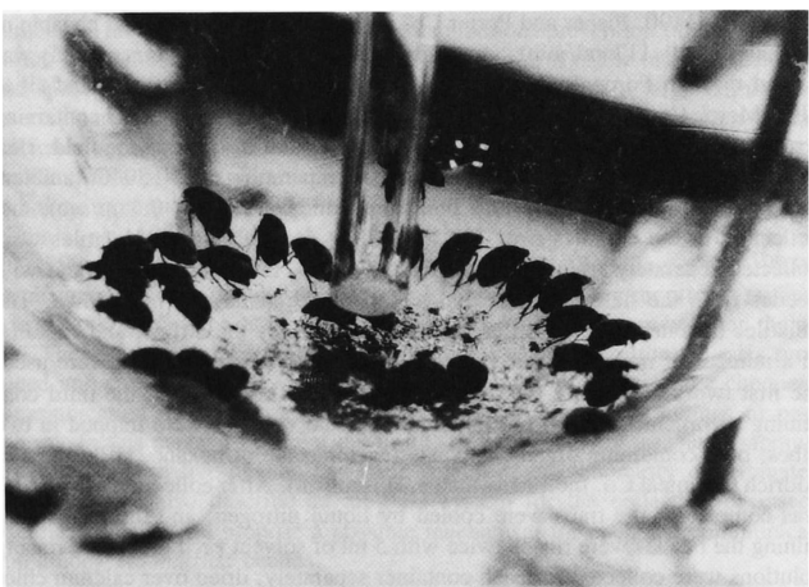


FIG. 1. Collection system for volatiles: (A) general view; (B) close-up. The airborne volatiles of the beetles were collected separately from four large groups (up to 150 in each flask) of beetles. The air, cleaned by passing it through Poropak Q and silica blue filters, was passed over live beetles kept in 2-liter glass flasks under conditions imitating those in the field as closely as possible. The volatiles liberated by the beetles were carried over by the airflow and captured by three traps connected in series, two empty tubes cooled with liquid nitrogen, and a tube containing cyclohexane.

Field Observations of Aggregations. Observations were conducted in peanut fields in the south of Israel at the Kibbutzim Sufa, Holit, and Ze'elim (biological field, where no pesticides were applied) during the summers of 1991–1993. Flying, eating, and/or mating beetles on the foliage were collected by hand at 5-min intervals during the first 25 min after sunset. Data analysis was performed according to the G-test procedure for replicated goodness of fit, at the 5% level of significance for the deviation from expectation (1 : 1) (Sokal and Rohlf, 1981).

Field-Trapping Experiments. The bait comprised three adult *M. matrida* virgin beetles with or without food. Another set of baits contained a concentrate of volatiles that was collected from hundreds of beetles over a number of 150-min periods (each period being defined as a day of collection). We may thus estimate that 10 μl of cyclohexane solution is about 300 beetle equivalents (BE) per day of collection. The concentrate was applied to Whatman No. 1 filter paper (4.25 cm circle) (the solvent was evaporated for 10 min outside the trap) with or without food (cut-up peanut leaves from a biological field) placed in a perforated 25-ml plastic box connected to the top of a trap. The traps were the type used for trapping Japanese beetles (Ringer Co., Minneapolis, Minnesota); they were washed with detergent and water and then dried in the sun for three days before each field study. The trapping tests were arranged in a randomized complete block design; the distance between traps in a replicate and between replicates was 20 m. The traps were positioned 40 cm above the foliage.

Field experiments involving trapping with live virgin beetles as attractants were conducted on May 14, 1992 (29–25°C, 40–50% relative humidity, 0.2–0.01 lux) and on May 28, 1992 (27–24°C, 50–55% relative humidity, 0.2–0.01 lux) at Kibbutz Sufa. These experiments comprised the following five treatments (six replications): live virgin females with or without food; live virgin males with or without food, and food alone. No empty traps were used (see below). The attracted adults were sexed under a dissecting microscope. Data were analyzed according to the procedure of Sokal and Rohlf (1981), three-way mixed model ANOVA (days, treatments, and blocks) for the two variables (males and females of *M. matrida* that were trapped), and the Student-Newman-Keuls multiple-range test at the 5% level of significance.

Field experiments involving trapping with volatiles produced by live virgin beetles as the attractant were conducted on May 15, 1993 (32–28°C, 40–45% relative humidity, 0.3–0.01 lux) at Kibbutz Holit and consisted of the following six treatments (seven replications): live virgin females with food, volatiles produced by live virgin females with or without food; volatiles produced by live virgin males with or without food, and cyclohexane with food alone. Data were analyzed by a two-way mixed ANOVA (treatments and blocks) model for the two variables and the Student-Newman-Keuls multiple-range test at the 5% level of significance.

Laboratory Olfactometer Bioassays. A Y-shaped glass olfactometer system (2.64 cm ID) was constructed from two 10-cm-long glass tubes and one 15-cm tube joined with a spherical S-35 ground glass connection that allowed an insect to crawl and fly freely through the Y tube. The three ends of the Y tube were closed with plugs that enabled fast insertion and removal of the beetle. A different bait was placed at the end of each of the two arms of the Y tube, and an insect that had been deprived of food for 24 hr prior to the start of the study was inserted at the tail of the tube. The insect then chose to move into one of the arms against a stream of clean air flowing from the ends of the arm. The air, cleaned by passing it through Poropak Q and silica blue filters, was pumped (pump model 9100, TIF Co., Chicago, Illinois) via flowmeters (9232 Cole Palmer Co., Chicago, Illinois) located at the air inlet and outlet. (By means of smoke, it was verified that the air flow is not turbulent). Each pass lasted 5 min.

The olfactometer system was cleaned the same way and maintained under the same conditions as those pertaining to the collection system. The baits were: three virgin females or three males with or without food, food alone, or 10 μl of a cyclohexane solution of female volatiles with food. Each type of bait was tested versus the other baits. The results were examined by the G-test procedure at the 5% level of significance (Sokal and Rohlf, 1981).

RESULTS AND DISCUSSION

Collection System for Volatiles. The method we report here for the collection of volatiles fulfills the criteria required for such a system: protection from contamination; simplicity, rapidity, and flexibility of construction; operation of the system for a large number and wide diversity of insect populations; and low cost (Figure 1A). The airborne volatiles of the beetles were collected separately from four large groups of beetles (up to 150 in each flask—virgin males and females reared in the laboratory, and males and females from the field) (Figure 1B) for comparisons of biological activity and chemical profile. These comparisons are necessary in order to determine the efficacy of the method of collection of the volatiles and the possibility of mass collection of volatiles from beetles in the field. Collection in empty traps cooled with liquid nitrogen was found to be superior, since it reduced the amount of contaminants (that are present in the solvents) and obviated the need to evaporate large volumes of solvent, thus preventing the loss of components of the volatiles. This technique will thus be used in the future to collect additional quantities of volatiles for structural studies and other analyses using GC-MS, EAG, GC-EAD, preparative separation, NMR, and other spectroscopic techniques.

Field Observations of Aggregations. We focused our interest on the sex ratio and time dependence of the formation of aggregations in peanut fields.

This aggregation behavior was studied in peanut fields where we conducted our trapping experiment. Such aggregations are not unique to the peanuts and were also found on roses, potatoes, and wild grass surrounding the field. At sunset, mature *M. matrida* beetles emerged from the ground to search for food and to mate (Figure 2). Most of the beetles captured on the peanut plants during the first 20 min of flight were males (in the first 10 min 85% of the captured beetles were males, in the next 5 min 67% were males, in the final 5 min 59% were males), but afterwards equal numbers of males and females were captured. Soon thereafter, aggregations of 20–30 beetles were formed on the peanut plants, the aggregations being composed of the two sexes in equal numbers. This 1 : 1 sex ratio was kept in the aggregations throughout the activity period, until feeding and mating was over. Mating was observed in the period from 15 to 25 min after emergence and lasted for at least for 15–20 min while the females continued feeding. These observations indicate that aggregation takes place at the same time as feeding and mating and provides support for the existence of a chemical communication mechanism based on volatiles liberated by the beetles or the

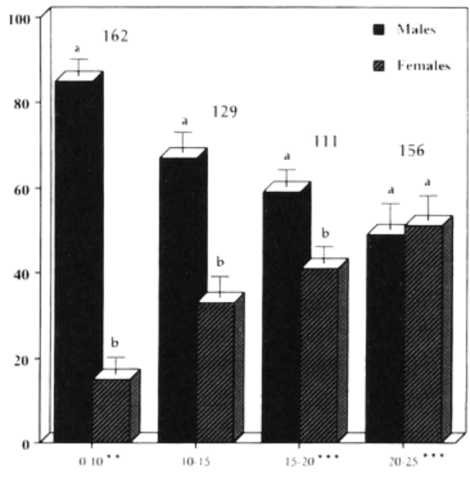


FIG. 2. Field observations of aggregations. The sex ratio and time dependence of the formation of aggregations were examined ($P \ll 0.05$) in peanut fields according to the G-test procedure for replicated goodness of fit, at the 5% level of significance. Values followed by the same letter are not significantly different in each time interval.

plants or a combination of both, which act synergistically as sex and/or aggregation pheromone.

Field-Trapping Experiments. The attraction of wild *M. matrida* beetles to different combinations of live virgin beetles (Table 1) or volatiles produced by live virgin beetles (Table 2) with or without the addition of food (cut-up peanuts leaves) was investigated. In the first trapping experiments in the field, the strongest attractant for wild males and females was the combination of three live virgin females with cut-up peanut leaves as food (127 captured males and 86 females) (Table 1).

More males than females were attracted, even though the ratio between the two sexes was 1 : 1 in the field. This preferential attraction by the combination of virgin females and food might indicate that during the time that the *M. matrida* female eats, or shortly thereafter, it excretes volatile chemical attractants—sex and/or aggregation pheromones—at the same time that plant volatiles are released as a result of mechanical damage to the leaves.

In the second set of trapping experiments in the field (Table 2), the strongest attractants for wild males and females were the combination of three live virgin females with cut-up peanut leaves (244 captured males and 200 females) and the combination of volatiles from virgin females with cut-up peanut leaves (225 captured males and 183 females).

In preliminary tests we found that baits of cyclohexane alone or empty traps attract very low numbers of beetles. In an independent monitoring study, empty traps were hung on posts along the edges of the peanut fields (where we con-

TABLE 1. FIELD-TRAPPING EXPERIMENTS WITH LIVE BEETLES AS BAIT[a]

Treatment	Attracted males/trap[b]	Attracted females/trap[b]
3 live females with food	127 ± 31 a	86 ± 31 a
3 live females without food	55 ± 18 b	30 ± 8 b
3 live males with food	30 ± 8 bc	30 ± 8 b
Food alone[c]	14 ± 4 c	15 ± 4 b
3 live males without food	9 ± 3 c	12 ± 4 b

[a] The attraction of wild *M. matrida* beetles to different combinations of live virgin beetles with or without the addition of food (cut-up peanut leaves) as attractants ($P < 0.001$) was investigated on May 14, 1992 (29–25°C, 40–50% relative humidity, 0.2–0.01 lux) and on May 28, 1992 (27–24°C, 50–55% relative humidity, 0.2–0.01 lux) at Kibbutz Sufa (five treatments, six replications).

[b] Values are means ± SE. Values followed by the same letter are not significantly different according to a three-way mixed ANOVA model for the two variables and the Student-Newman-Keuls multiple-range test at the 5% level of significance.

[c] An independent monitoring study, in the same fields in which our experiments were conducted empty traps were set, trapped on May 14, 1992, 12 beetles/trap and on May 28, 1992, 10 beetles/trap.

TABLE 2. FIELD-TRAPPING EXPERIMENTS WITH VOLATILES PRODUCED BY LIVE VIRGIN BEETLES[a]

Treatment	Attracted males/trap[b]	Attracted females/trap[b]
Live females with food	244 ± 47 a	200 ± 38 a
Female volatiles with food	225 ± 41 a	183 ± 40 a
Cyclohexane with food[c]	122 ± 13 b	95 ± 10 b
Male volatiles with food	105 ± 24 b	92 ± 14 b
Female volatiles without food	88 ± 31 b	67 ± 22 b
Male volatiles without food	48 ± 13 b	44 ± 13 b

[a]The attraction of wild *M. matrida* beetles to different combinations of virgin beetles and volatiles produced by live virgin beetles with or without the addition of food (cut-up peanut leaves) as attractants ($P < 0.001$) was investigated on May 15, 1993 (32–28°C, 40–45% relative humidity, 0.3–0.01 lux) at Kibbutz Holit (six treatments, seven replications).

[b]Values are means ± SE. Values followed by the same letter are not significantly different according to two-way mixed model ANOVA for the two variables and the Student-Newman-Keuls multiple-range test at the 5% level of significance.

[c]An independent monitoring study, in the same fields in which our experiments were conducted empty traps were set, trapped on May 15, 1993, six beetles/trap.

ducted our experiments and where high populations of beetles exist) for the whole growing season of peanuts, and the numbers of beetles caught were very low (5–12/trap/night) (A. Harari, personal communication). The rinsing solutions from flasks containing virgin females also attracted beetles, but to a lesser degree than volatiles of virgin females. These trapping experiments reinforce the conclusions of the first set of experiments that the attraction is due to a combination of volatiles from the females and from the mechanically damaged plants, in accordance with the findings of Harari et al. (1994). The experiments also showed that virgin females and their volatiles trapped more males than females (ratio in percentage 55–60:45–40) and, no less important, that the collection system is effective in trapping volatiles with biological activity.

Laboratory Olfactometer Bioassays. In preliminary olfactometer experiments we found that food alone was more attractive to both females and males than the empty arm. For the females the results were 71 ± 4% for food versus 29 ± 4% for the empty arm ($N = 120$) and for the males 73 ± 3% versus 27 ± 3% ($N = 130$).

The results of the laboratory bioassays were similar to those from the field trapping experiments (Table 3). The strongest attractants for the males and females were females eating peanut leaves, i.e., 59% of the females were attracted by eating females vs. eating males; 78%of the males were attracted by eating females vs. eating males. When we looked at the attraction by female volatiles plus food as bait, we found that 85% of experimental females were

TABLE 3. LABORATORY OLFACTORY BIOASSAYS TESTING PREFERENCE OF INDIVIDUAL MALE OR FEMALE BEETLE FOR ONE OF TWO BAITS IN Y-SHAPED GLASS TUBE OLFACTOMETER[a]

	Experimental beetles		Bait 1		Bait 2	
Entry	Type	Total	Type	% attracted[b]	Type	% attracted[b]
1.	Virgin females	124	Females + food[c]	59 ± 2 b	Males + food	41 ± 2 a
2.	Virgin females	60	Female volat. + food	60 ± 3 b	Male volat. + food	40 ± 3 a
3.	Virgin females	60	Food	15 ± 2 a	Female volat. + food	85 ± 2 b
4.	Virgin males	92	Females + food	78 ± 3 b	Males + food	22 ± 3 a
5.	Virgin males	66	Female volat. + food	75 ± 3 b	Male volat. + food	25 ± 3 a
6.	Virgin males	70	Food	10 ± 2 a	Female volat. + food	90 ± 2 b

[a] The baits were: three virgin females or three males with or without food, food alone, or 10 μl of a cyclohexane solution of female volatiles (volat.) with food. The control was an empty container.

[b] Values are means ± SE for beetles tagged in two to four replications. Values are given as percentages according to G-test procedure for replicated goodness of fit, at the 5% level of significance for the deviation from expectation (1 : 1). Values followed by the same letter are not significantly different in each experiment.

[c] Cut-up peanut leaves were used as food.

attracted by volatiles from virgin females plus food vs. food alone and 60% vs. volatiles from virgin males plus food; 90% of experimental males were attracted by volatiles from virgin females plus food vs. food alone and 75% vs. volatiles from virgin males plus food. These results indicate that attraction by live females plus food was similar to attraction by volatiles from virgin females plus food, in keeping with the findings of the field trapping experiments (Tables 1 and 2). An interesting point is that females or female volatiles plus food were more attractive to males (78–75%) than to females (59–60%) (Table 3, entries 4 and 5 vs. 1 and 2). This may indicate the presence of a sex pheromone released by the females to attract males, in addition to the attraction by the food volatiles.

In general, our findings that in the field and in the olfactometer female volatiles have the same effect as live female beetles on the behavior of the tested beetles are in keeping with the olfactometer study of Harari et al. (1994). The only exception lies in the preference of virgin females toward females and food vs. males and food (entry 1): while we found a small preference of females towards females and food, Harari et al. (1994) reported no preference in such an experiment.

The findings of Harari et al. (1994) are based solely on olfactometer studies, whereas our conclusions are based on field trapping, which is a completely different environment in terms of ambient conditions and other factors distinguishing a natural from an artificial state. Although Harari et al. (1994) did not find evidence for the existence of an aggregation pheromone released either by males or females, we cannot, at this stage, ignore or exclude such a possibility under field conditions.

Biological Behavior. The findings of our field and laboratory experiments indicate the operation of a possible two-step mechanism of chemical communication in *M. matrida*. The first step is the liberation of plant volatiles as a result of the mechanical injury caused by the males, which emerge first. These volatiles attract both sexes until a 1 : 1 sex ratio is reached. The second step is the excretion of an attracting mixture (perhaps a sex or aggregation pheromone) by the females while they eat the leaves of the plant or just after eating. The attraction may be due to a combination of insect pheromone(s) and plant volatiles, which are liberated during the process of eating, and the two agents may act synergistically.

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COMPARATIVE ANALYSIS OF STEAM DISTILLED FLORAL OILS OF CACAO CULTIVARS (*Theobroma cacao* L., Sterculiaceae) AND ATTRACTION OF FLYING INSECTS: IMPLICATIONS FOR A *Theobroma* POLLINATION SYNDROME

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Abstract—Steam-distilled floral fragrance oils from nine distinctive cultivars of *Theobroma cacao* L. (Sterculiaceae) in Costa Rica were examined with GC-MS to determine whether or not major differences existed among these cultivars for volatile constituents comprising 50% or more of the samples. The cultivars selected for floral oil analyses were chosen to represent diverse cultivars having supposedly different genetic backgrounds and histories of artificial selection for agronomic purposes. Cluster analysis revealed two major groupings of cultivars: those with higher molecular weight dominant compounds, and those having lower molecular weight compounds. Additionally, one cultivar, Rim-100, selected from criollo or ancestral-type cacao in Mexico and resembling criollo in the appearance of flowers and fruits, formed an extreme group having the highest molecular weight profile for major volatile compounds. Based upon these analyses, bioassays using McPhail traps were performed in an abandoned cacao plantation in northeastern Costa Rica during rainy and dry seasons to determine the relative attraction of these oils to flying insects. Bioassays revealed that the Rim-100 cultivar attracted by far the greatest numbers of cacao-associated midges (Diptera: Ceratopogonidae and Cecidomyiidae), as well as stingless bees (Hymenoptera: Apidae: Meliponinae), suggesting that a floral fragrance having high-molecular-weight volatiles is more potent as an attractant to flying insects than floral oils having lower-

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molecular-weight compounds. It is suggested that Rim-100 more closely resembles an ancestral or wild-type cacao than the other cultivars examined, and therefore it is more effective in attracting opportunistic dipteran floral visitors and pollinators than other cultivars in plantation settings. Several of the major volatile compounds found in the floral oils of *T. cacao* and other species of *Theobroma* occur in mandibular and other exocrine glands in various bees, including stingless bees and halictids, known visitors of *Theobroma* flowers. These compounds are particularly present in noncultivated species of *Theobroma* and have much more noticeable fragrances than the seemingly scentless flowers of cultivated *T. cacao* selected for agriculture. It is hypothesized that the floral attraction system of ancestral or wild (noncultivated) *T. cacao* and other species of *Theobroma* may have evolved to attract certain bees as their principal pollinators in natural habitats in the Neotropics, with a floral reward hypothesized as being sociochemicals needed by bees for mating, foraging, territorial defense, etc. Because of the many generations of extensive selection by cloning for desired cultivars, *T. cacao* might have lost much of its original floral attraction system for bees, instead being pollinated opportunistically by dipterans in plantation habitats. This may help to explain why natural pollination in cultivated *T. cacao* is generally very poor relative to observed levels of fruit-set in wild or noncultivated species of *Theobroma*.

Key Words—Floral oils, cacao, *Theobroma cacao*, Sterculiaceae, attraction, Diptera, Ceratopogonidae, Cecidomyiidae, Hymenoptera, Apidae, pollination, 1-pentadecene, *n*-pentadecene.

INTRODUCTION

The pollination of *Theobroma cacao* L. (Sterculiaceae), cocoa or cacao, is mediated in cultivated stands or plantations by various species of midges belonging to the dipteran families Ceratopogonidae and Cecidomyiidae (Dessart, 1961; Glendenning, 1962; Saunders, 1959; Soria, 1970; Posnette, 1944; Kaufman, 1973, 1975a,b; Young, 1983, 1985a,b, and many other references). While various bees, especially Meliponinae (Apidae) and Halictidae, are regular visitors to the often brightly colored and highly fragrant flowers of various noncultivated species of the exclusively Neotropical *Theobroma* (e.g., Aguiar-Falcao and Lieras, 1983; A.M. Young, unpublished observations), the smaller, less conspicuous, and weakly fragrant flowers of cultivated *T. cacao* in plantations are only occasionally visited by bees (e.g., Soria, 1975; Kaufman, 1975a; Young, 1985b). Because the basic floral design is very similar among the 22 recognized species of *Theobroma* (Cautrecasas, 1964), it has been suggested that floral habits in the genus, including cacao, may have originally evolved as a bee pollination system (Erickson et al., 1987). In this context, an ancestral form, or wild-type, cacao may be more effective in attracting flying insects, including bees, than cultivars derived from extensive cloning and artificial selection for agricultural purposes.

However, there is a dearth of observations on the natural pollinators of wild or noncultivated *T. cacao*, especially in its native locality on the eastern, lower slopes of the Andes Mountains. Floral structure in *T. cacao* and related species of *Theobroma* accommodates visitation and effective pollination by medium to small-bodied flying insects (Young et al., 1987a). Steam-distilled whole floral oils from fresh flowers of these species, when bioassayed in the field, attract both dipterans (Ceratopogonidae and Cecidomyiidae) and meliponine (stingless) and halictid bees, albeit in low numbers (Young et al., 1987b, 1988; Young, 1989a). Synthetic analogs of various major volatile constituents of *Theobroma* floral oils attract midges in very low numbers (Young, 1989b). Thus, floral fragrances derived from floral oils undoubtedly function as cues in attracting most visitors and pollinators to flowers of *Theobroma* species. Because cultivated *T. cacao* in plantations consists of many distinctive cultivars or horticultural races, floral oil chemistry and intensity of pollinator attraction may vary considerably among different cultivars, depending upon individual genetic history, degree of cloning, and degree of closeness to a wild-type or ancestral form. In this paper we report for the first time distinctive groupings of several cultivars of *T. cacao*, as determined by patterns of floral fragrance chemistry, and differential attractiveness for flying insects, especially Ceratopogonidae and Cecidomyiidae (Diptera) and stingless bees (Hymenoptera: Apidae: Meliponinae), as assessed in a Costa Rican cacao plantation during rainy and dry season bioassays. These studies suggest that considerable differences exist in the degree to which steam-distilled floral oils from different cultivars of *T. cacao* attract flying insects in a plantation habitat. Further, the steam-distilled floral oil from one cacao cultivar derived from and resembling an ancestral-type cacao from Mexico and Central America is more effective in attracting insects than the other, more derived cultivars examined. Finally, even a relatively primitive cacao cultivar is less attractive to bees relative to reported observations for completely noncultivated species of *Theobroma*. The extensive cloning or asexual propagation of cacao cultivars over long periods of time may have resulted in a breakdown of the original floral attraction-reward system designed to attract flying insects, including effective pollinating insects.

METHODS AND MATERIALS

Analyses of Floral Oils. Fresh flowers for volatile oil extractions were obtained from nine cultivars of *T. cacao* at C.A.T.I.E. in Turrialba (600 m elevation), Cartago Province, Costa Rica. Each of these cultivars was sampled during June of 1988 and 1990. One cultivar, UF613, was sampled at two different time periods, early morning and mid-afternoon, during both years. Time of sample collection for the other cultivars was generally random with respect

to years. Single samples from *T. speciosum* and *T. pentagona* were collected during 1988 and 1990, respectively. About 400 freshly opened flowers were collected for each sample.

Immediately following collection, flowers were subjected to vacuum steam distillation using a 500-ml short-path vacuum distillation unit (KONTES). Flowers were distilled for 1 hr at 100°C, the distillate extracted 3× with dichloromethane, and dried over sodium sulfate. The oil obtained was then frozen.

Frozen samples were transported on ice to the State Hygiene Laboratory at the University of Wisconsin (Madison) and subjected to gas chromatography–mass spectrometry (GC-MS) as previously described (Erickson et al., 1987). Individual components of each sample were qualified by peak areas with normalization to 100. Specific identification of the individual components was determined in a previous study (Erickson et al., 1987).

To examine variability, both between cultivars and species, we elected to compare the relative amounts of the 12 most abundant compounds from the previously described volatile spectra for UF613 (Erickson et al., 1987). The mean contribution for each of these compounds over both years was calculated. Relationships among cultivars and species based upon relative amounts of selected volatile components were analyzed using Euclidean distance and cluster analysis (SAS Institute, 1990). A cluster diagram was constructed using the average linkage clustering algorithm.

Field Bioassays. Based upon the results of the GC-MS analyses of the distilled floral oils, three groupings of these oils, based upon cluster analysis were revealed, providing a rationale for the design of field bioassays. Two field bioassays were established in order to evaluate the comparative attractiveness of representative cultivars from each grouping or cluster to flying insects, including either known or suspected pollinators of cacao. This locality generally experiences annually one moderately intense dry season between January and April (and shorter dry spells at other times) and a lengthy rainy season, which may have profound effects on the population cycles of cacao-pollinating midges (Young, 1983). Therefore, bioassays were scheduled for both rainy and dry seasons in order to obtain an adequate assessment of insect attraction to cacao floral oils. Field bioassays using 100 ppm serial dilution by volume of floral oil in spectral-grade chloroform were conducted in a semiabandoned cacao plantation, Finca La Tigra, La Virgen (10°23′N, 84°07′W; 220 m elevation), Sarapiqui District, Costa Rica, during the dry season (March 7–11) and rainy season (September 14–18) in 1991. The site within this cacao plantation where traps were placed is about 25 m from mixed primary and secondary premontane tropical wet or rain forest (Holdridge, 1966).

Inoculations of McPhail traps were done using carefully sealed and refrigerated samples of the same floral oils used for the analysis described above. Each McPhail Trap (Bennett, 1972) was labeled, using white tape, to identify

the treatment (floral oil type used) and preparation of the traps and subsequent observations on their contents followed that of previous studies (Young et al., 1987a,b, 1988; Young, 1989a,b). Floral oils from three distinct cacao cultivars, Rim-100, Catongo, and UF613, representing the three major groupings detected by the GC-MS analysis, were used in both bioassays. Inoculation of each trap consisted of dripping 2 ml of floral oil solution directly on a 100% cotton ball, using a pipete, suspended inside the McPhail trap. Inoculated traps were suspended in cacao trees at heights between 1.0 and 1.5 m from the ground (Figure 1). In both bioassays, traps were reinoculated on the third day of each five-day bioassay period. The design of each bioassay consisted of six replicates for each of the three floral oils being tested, and two blank or control traps, each of the latter inoculated with 2 ml of 10% chloroform–distilled water mixture. Traps were arranged in the cacao trees in moderate proximity to one another, with a range of 5–10 m of space between adjacent traps.

The 20 traps in each bioassay were emptied each morning by pouring the soapy water from each through a metal strainer lined with a coffee filter. Trapped insects were picked from soaked filter paper using fine forceps and placed into vials of 70% ethanol. Traps were refilled with water daily. Collected insect samples were kept separate for each treatment and date, and later sorted in the laboratory and identified using previously determined voucher specimens.

RESULTS

Analyses of Floral Oils. The mean relative abundance of the major *T. cacao* volatile components, consisting of saturated and unsaturated hydrocarbons, is shown in Table 1. The relative composition of these compounds in UF613 is within the range of previously reported values for this cultivar (Erickson et al., 1987). The most abundant compounds observed in this cultivar were 1-pentadecene and *n*-pentadecane. The mean level of 1-pentadecene was increased in samples of UF613 collected in mid-afternoon (46.9%) compared to samples collected in early morning (26.0%). Relatively high levels of 1-pentadecene were observed in four other *T. cacao* cultivars (SCA6, UF29, UF221, UF668). We were unable to detect 1-pentadecene in samples from Rim-100 and *T. speciosum*. Low levels of 1-pentadecene were observed in the remaining *T. cacao* cultivars and in *T. pentagona*.

Given the relatively high levels of lower-molecular-weight compounds observed in UF613 in this study (Table 1) and in previous studies (Erickson et al., 1987), we elected to conduct a cluster analysis based on the summation of the relative contribution of the five lowest-molecular-weight compounds (tridecane, 1-tetradecene, *n*-tetradecane, 1-pentadecene, *n*-pentadecane). This analysis separated all samples into two major groups, those with relatively high

FIG. 1. Abandoned cacao plantation in Costa Rica used for bioassays of cacao floral oils (above), and McPhail trap used in bioassays (below).

TABLE 1. MEAN RELATIVE COMPOSITION OF MAJOR VOLATILE COMPOUNDS OBTAINED BY STEAM DISTILLATION OF FLOWERS FROM NINE *Theobroma cacao* CULTIVARS AND FROM *T. pentagona* AND *T. speciosum*, 1988 AND 1990[a]

	Cultivar or species											
Compound	Catongo	EET400	Pound7	Rim-100	SCA6	UF29	UF221	UF613a[b]	UF613b	UF688	*T. pentagona*[c]	*T. speciosum*[c]
Tridecane	0.4	0.1	0.1	0.0	0.5	0.5	2.3	2.9	1.5	2.3	0.1	0.1
1-Tetradecene	2.2	0.2	0.3	0.1	0.4	0.8	1.8	5.9	2.3	0.4	0.0	0.0
n-Tretradecene	0.6	0.5	0.2	0.0	0.3	0.5	1.1	1.5	1.8	0.2	0.1	0.0
1-Pentadecene	8.2	6.0	7.0	0.0	32.3	26.9	41.3	46.9	26.0	24.3	11.8	0.0
n-Pentadecane	3.8	9.0	1.8	0.5	6.8	6.2	13.2	10.0	12.4	9.0	6.4	0.7
1-Hexadecene	1.4	3.7	0.4	0.0	0.4	3.1	0.9	1.9	0.8	0.4	0.1	0.0
1-Heptadecene	22.6	24.5	27.0	0.8	19.1	0.3	8.2	13.8	0.5	6.7	8.8	0.0
Henicosane	0.9	1.3	0.7	2.6	0.5	0.5	0.4	0.1	0.4	1.0	1.7	1.0
Tricosane	6.0	5.2	1.9	9.9	2.2	0.1	5.2	1.9	2.9	4.9	1.7	9.1
Tetracosane	0.7	0.7	2.5	5.4	0.4	0.6	0.4	0.2	0.6	0.2	0.7	14.1
Pentacosane	7.6	5.1	4.4	14.3	3.8	6.0	4.6	1.4	2.0	4.6	3.1	16.2
Hexacosane	0.0	0.4	0.1	5.4	0.2	0.2	1.0	1.0	0.0	0.0	0.0	14.2

[a] Relative percentages listed as zero represent observed levels of less than 0.1%.
[b] UF613a samples collected during early morning; UF613b samples collected during mid-afternoon.
[c] Single sample of *T. pentagona* collected in 1990; single sample of *T. speciosum* collected in 1988.

levels of the lower-molecular-weight compounds and those with relatively low levels of those same compounds (Figure 2).

Variability among the group exhibiting high levels of these compounds probably relates to time of sample collection, as the cultivars with the highest levels represented samples collected in mid- to late afternoon (UF613a and UF221), while cultivars with slightly lower levels represented samples collected from early to late morning (SCA6, UF29, UF613b, UF668).

Variability among the group exhibiting low levels of these compounds appeared to be independent of time of sample collection. This group was, however, separated into two distinct subgroups. One subgroup consisted of those samples in which essentially none of the lower-molecular-weight compounds was observed (Rim-100 and *T. speciosum*). The second subgroup consisted of those samples in which each of the lower-molecular-weight compounds was observed (Catongo, Pound7, EET400, *T. pentagona*), albeit at greatly reduced levels when compared to the high level group.

Field Bioassays of Midges. About four times the number of midges belonging to the Cecidomyiidae were retrieved from all traps combined over the bioassays than midges belonging to the Ceratopogonidae (Table 2). The frequency at which midges are attracted to the Rim-100 floral oil is significantly different (3 × 11 contingency test, $\chi^2 = 40.50$, $df = 20.00$, $P < 0.01$) (Table 2) from the attraction of midges to the other floral oils bioassayed. Thus, only 23.46% of all midges attracted to floral oils in this study were Ceratopogonidae, and these were represented by only a few species. For Ceratopogonidae and Cecidomyiidae combined, more than three times the number of midges were attracted to Rim-100 cacao floral oil than to Catongo, and more than nine times the number attracted to UF613 (Table 2). Although sample sizes of trapped

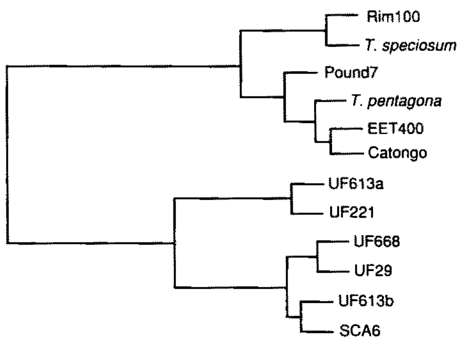

FIG. 2. Cluster analysis based on lower-molecular-weight volatiles representing major compounds observed in steam-distilled volatiles from *Theobroma cacao*, cultivar UF613 (Erickson et al., 1987).

TABLE 2. PATTERN OF ATTRACTION OF MIDGES (DIPTERA: CERATOPOGONIDAE AND CECIDOMYIIDAE) TO MCPHAIL TRAPS INOCULATED WITH DISTILLED FLORAL OILS[a] FROM THREE CACAO CULTIVARS IN NORTHEASTERN COSTA RICA[b]

Midge	Total numbers of midges by treatments[c]				
	Rim-100	Catongo	UF613	Blanks	Total
Ceratopogonidae					
Forcipomyia spatulifera Saunders	4 (f)	0	0	0	4
F. genualis (Loew)	8 (f)	3 (f)	0	0	11
Dasyhelea sp.	2 (f)	0	0	0	2
Atrichopogon sp.	2 (f)	0	0	0	2
Total	16	3	0	0	19
Cecidomyiidae					
Mycodiplosis ligulata Gagné	22 (17f, 5m)	7 (7f)	3 (f)	0	32
Aphodiplosis triangularis (Felt)	10 (8f, 2m)	3 (2f, 1m)	1 (m)	0	14
Clinodiplosis sp.	8 (7f, 1m)	3 (f)	0	0	11
Ledomyia sp.	0	1 (f)	0	0	1
Bremia sp.	0	1 (f)	1 (f)	0	2
Chrybaneura sp.	0	0	1 (f)	0	1
Micromyini	0	0	1 (f)	0	1
Total	40	15	6	0	61
Combined totals	56	18	6	0	80

[a]Steam distilled oils from collected *Theobroma cacao* L. flowers in Costa Rica during 1988–1990.
[b]Two field bioassays performed at Finca La Tigra, La Virgen, Sarapiqui District, Heredia Province, Costa Rica, March 7–11, and September 14–18, 1991.
[c]Six replicate traps of each cultivar floral oil at a dilution of 100 parts/million, and two blanks or control traps inoculated with similarly diluted chloroform solvent only. Traps suspended over moderate distances on adjacent cacao trees in a semiabandoned plantation adjacent to tropical rain forest.

midges are relatively small, the data exhibit fairly marked patterns of differential attraction of midges to floral oils belonging to three distinctive cacao cultivars, especially evident for the more abundant Cecidomyiidae in the samples. Blank traps in this study lured no midges at all (Table 2).

Almost twice as many species of cecidomyiids were attracted to the lures than ceratopogonids (Table 2), even though the total numbers of both groups of midges trapped in this study remained the same between rainy and dry season bioassays at this locality (Table 3). For the three most attracted species of Cecidomyiidae, 82.46% of the total catches were females (Table 4). For all of these species, the sex ratio for trapped midges was skewed towards females, a pattern especially noticeable in the most attracted species, *Mycodiplosis ligulata* Gagné (Table 4). In addition to midges, a total of four individuals of an un-

TABLE 3. GENERAL PATTERNS OF ATTRACTION OF MIDGES (DIPTERA: CERATOPOGONIDAE AND CECIDOMYIIDAE) TO MCPHAIL TRAPS INOCULATED WITH FLORAL OILS OF *Theobroma cacao* L. CULTIVARS (STERCULICEAE), AND CONTROL (BLANK) TRAPS DURING RAINY AND DRY SEASONS IN A NORTHEASTERN COSTA RICA CACAO PLANTATION[a]

	Numbers of midges in traps			
Ranked abundance of midge species	Floral oils	Controls	Rainy season	Dry season
Ceratopogonidae				
Forcipomyia genualis (Loew)	11	0	6	5
F. spatulifera Saunders	4	0	2	2
Dasyhelea sp.	2	0	0	2
Atrichopogon sp.	2	0	1	1
Cecidomyiidae				
Mycodiplosis ligulata Gagné	32	0	18	14
Aphodiplosis triangularis (Felt)	14	0	9	5
Clinodiplosis sp.	11	0	5	6
Bremia sp.	2	0	0	2
Ledomyia sp.	1	0	0	1
Chrybaneura sp.	1	0	0	1
Micromyini (undetermined species)	1	0	0	1
Total catches	81	0	41	40

[a] Rainy season census: September 14–18, 1991; dry season census: March 7–11, 1991.

identified species of stingless bee, *Trigona* sp., were found in the Rim-100 traps.

DISCUSSION

The steam-distilled floral fragrance oil from freshly collected flowers of *T. cacao* cultivar UF613, as determined in a previous study (Erickson et al., 1987), revealed a predominance of the straight-chain hydrocarbons pentadecene and pentadecane. Our cluster analysis of the major volatile constituents, arbitrarily based upon cultivar UF613, for the series of other cultivars examined in this present study, revealed some interesting differences among these cultivars for the five or six most abundant compounds in our samples. Although it cannot be determined from our study, it is likely that the basis for midge attraction to

TABLE 4. PATTERNS OF SPECIFIC ATTRACTIVENESS OF THREE SPECIES OF CECIDOMYIIDAE (DIPTERA) MOST ATTRACTED TO FLORAL OILS OF *Theobroma cacao* L. (STERCULIACEAE) CULTIVARS IN MCPHAIL TRAPS IN A CACAO PLANTATION IN NORTHEASTERN COSTA RICA[a]

	Numbers of midges in traps						
	Rainy season			Dry season			
Midge species	Female	Male	Total	Female	Male	Total	Total
Mycodiplosis ligulata Gagné	15	3	18	12	2	14	32
Aphodiplosis triangularis (Felt)	6	3	9	4	1	5	14
Clinodiplosis sp.	5	0	5	5	1	6	11
Total catches	26	6	32	21	4	25	57

[a] Data shown are for floral oil-inoculated traps only, since no midges were found in the control (blank) traps. In each of two field bioassays, a total of 20 McPhail traps were used, with six replicates for each of the three types of floral oil evaluated for midge attractiveness, and two blanks. Rainy season bioassay: September 14–18, 1991; dry season bioassay: March 7–11, 1991.

distilled floral oils of *T. cacao* is a mixture of several chemical constituents, perhaps a combination of major and minor compounds. Examining the distribution of the major volatiles among the cacao cultivars is a logical way to begin looking for differences among cultivars, but does not comprise a complete analysis. Aside from these abundant compounds, our distilled floral oils contained many minor components (Erickson et al., 1987) comprising less than 1% of the estimated 80 compounds found in *T. cacao* floral oils. The compounds selected for analyses represent 50% or more of the total spectrum of floral oil constituents revealed in our samples.

In spite of steam distillation being more prone to yielding artifacts and possibly underrepresenting lower-molecular-weight compounds, data from our bioassays show that midges are attracted to steam-distilled floral oils of *T. cacao*. Higher-molecular-weight hydrocarbons revealed in our analyses (e.g., pentacosane and hexacosane) are not very volatile and are probably culticular waxes on the surface of flowers. Thus the chemical composition of these steam-distilled floral oils is almost certainly not what the midges perceive from intact cacao flowers.

The cacao cultivars examined fall into two major clusters based upon the relative abundance of high- and low-molecular-weight compounds. What is particularly interesting about the Rim-100 cultivar is the complete absence of pentadecene, a major component of the floral oils in several other cultivars, especially those in the UF series, in which lower-molecular-weight compounds prevail. Upon examination of the presence or absence of genetic self-compati-

bility, self-incompatibility, and general regions of derivation for the cultivars examined (i.e., Amazonian Ecuador cultivars vs. Central American-derived cultivars), we could find no definite correlation with the results of this cluster analysis based upon floral oil chemistry (Enriquez and Soria, 1967).

Several of the major floral oil constituents available as synthetic analogs, when bioassayed in the field, attract low numbers of both Ceratopogonidae and Cecidomyiidae (Young, 1989a,b), the principal groups of Diptera visiting cacao flowers through the cacao-growing regions of the world (Entwistle, 1972). Differences among *T. cacao* cultivars in floral oil composition may influence the ability of cacao flowers to attract insect visitors, including pollinators. While we were not equipped to perform EAG analysis as a means of screening major volatile constituents for olfactory reception activity (e.g., Gabel et al., 1992), our results nonetheless reveal some interesting differences in levels of insect attraction among cultivars.

Floral oils of *T. cacao*, as examined with UF613 and various species of *Theobroma* in previous field bioassays, attract low numbers of Ceratopogonidae and somewhat higher numbers of Cecidomyiidae (Young et al., 1987a, 1988), as well as other insects (Young et al., 1987b). Although cecidomyiid midges are most likely cacao pollinators (Kaufman, 1973; Young, 1985a), they are not considered as effective pollinators in spite of their relatively high abundance as cacao flower visitors (Brew, 1986; Ibrahim and Jussein, 1987). It is generally considered that ceratopogonid midges, especially *Forcipomyia* (*Euprojoannisia*) sp., are effective pollinators of *T. cacao* in plantation settings (e.g., Saunders, 1959; Hernandez, 1965; Posnette, 1944; Soria, 1970; Winder, 1977, Bystrak and Wirth, 1978).

Our field bioassays confirmed the well-known observation that cecidomyiid midges, especially females, belonging to several genera and species, are attracted to not only cacao flowers, but distilled floral oils from them. Ceratopogonid midges are generally much less abundant at cacao flowers, with only a few species represented, and our bioassay data confirms the same pattern with floral oil traps. Although twice the number of cecidomyiids were trapped during the dry season than in the rainy season in a previous bioassay in Costa Rica (Young et al., 1988), the numbers of these midges remained about the same between dry and rainy seasons in the present study. Because midges are very sensitive to moisture, year-to-year variation in seasonal rainfall patterns, typical for this particular locality, will bring about differences in abundance levels and population dynamics between years. The high abundance of female midges in the traps most likely indicate a behavioral preference in which females seek some reproductive or mating resource from cacao flowers. Female cecidomyiids, especially in the most abundant species such as *M. ligulata* Gagné, are far more numerous at cacao flowers (Young, 1985a–c).

Our data show that cecidomyiid midges, especially *M. ligulata* Gagné, a

probable opportunistic pollinator of cacao (Young, 1985a–c), are strongly attracted to Rim-100 cacao floral oil, moderately attracted to Catongo, and only weakly attracted to the UF series floral oils. Exactly 70% of all midges trapped in the two bioassays combined were obtained from the Rim-100 traps, and about 40% of these belonged to *M. ligulata* Gagné. The three most abundant cecidomyiids, *M. ligulata* Gagné, *Aphodiplosis triangularis*, and *Clinodiplosis* sp., were most frequently trapped with Rim-100 floral oil and were found in previous studies in Costa Rica to be the most abundant species in other bioassays (Young et al., 1987a,b, 1988) and visiting cacao flowers (Young, 1985). About 84% of the ceratopogonids trapped were found trapped with Rim-100 floral oil, and all four species are known or suspected pollinators of *T. cacao* (e.g., Bystrak and Wirth, 1978; Winder, 1977; Young, 1983). In a previous bioassay comparing the attractiveness of Rim-100 floral oil with that of Catongo in another abandoned cacao plantation, more than twice the number of ceratopogonid midges were found in the Rim-100 traps (Young, 1989a).

While our study does not allow us to determine precisely why Rim-100 floral oil strongly attracted cacao-associated midges, the unique chemical composition of this floral oil, with higher-molecular-weight components prevailing, may be a key factor in pollinator attraction. Rim-100 was selected in Mexico from a criollo-type cacao, and the flowers and fruits closely resemble criollo (G.A. Enriquez, personal communication). Criollo is considered to be the original wild-type or near wild-type cacao endemic to Mexico and Central America (Cuatrecasas, 1964). If it is assumed that the Rim-100 cultivar of *T. cacao* is closely akin to Central American wild-type or criollo cacao, its floral fragrance composition may resemble that of wild *T. cacao*. Such a floral fragrance might have been characterized by higher-molecular-weight volatile components that provided a stable fragrance cue for pollinators, but with the extensive selective breeding of *T. cacao* cultivators, and their propagation by cloning over many generations, there could have occurred a relaxation of selection pressures favoring a functional floral fragrance. The result would be the production of many cultivars with nonfunctional or only partially functional floral fragrances, since sexual reproduction has essentially been uncoupled and replaced with clonal propagation or asexual reproduction. If true, this could help explain why natural pollination in commercial cacao plantations is generally very poor (Dessart, 1961), since plantations contain an assortment of cultivars propagated chiefly by vegetative means.

Various species of stingless bees (Hymenoptera: Apidae: Meliponinae), and other wild bees (Halictidae), have been trapped with *Theobroma* distilled floral oils, especially *T. simiarum* Donn. Smith. (Young et al., 1987a,b). Cacao floral oils, as seen in previous studies and in the present study, weakly attract stingless bees. The floral oil of *T. simiarum* is rich in terpenoid compounds, unlike the hydrocarbons predominating in *T. cacao* (Erickson et al., 1987).

While large-sized *Trigona* bees routinely visit the flowers of *T. simiarum* and *T. cacao*, smaller-size species function as "pollen thieves" on the small whitish and scentless *T. cacao* flowers (Young, 1985c). The role of *Trigona* and other bees as pollinators of the large, red, highly fragrant flowers of *T. simiarum* remains unstudied. Interestingly, hydrocarbons such as pentadecane and terpenoids such as geraniol and citronellol, major volatile constituents of floral oils among *Theobroma* species (Erickson et al., 1987), are important mandibular gland exudates in various species of bees, including some stingless bees (Blum et al., 1970; Crewe and Fletcher, 1976; Duffield et al., 1984). Whether or not stingless bees and other bees such as halictids may collect these compounds from floral hosts remains to be studied.

Because most cultivars of *T. cacao* have been artificially selected, including Rim-100, they may be largely dysfunctional in attracting the most effective pollinating insects. It has been argued elsewhere (Erickson et al., 1987) that certain bees could be the most effective pollinators or *Theobroma* species, perhaps even *T. cacao*. Certain unusual features of cacao floral morphology in some cultivars (UF613), suggest a dysfunctional condition for attracting pollinators. Such a condition is absent in the flowers of noncultivated species of *Theobroma*, which also tend to have highly fragrant flowers (Young et al., 1984).

Depending upon their genetic histories, which are largely unknown, and the degree of selection, cultivated cacao varieties may represent a wide range of functional to dysfunctional flowers for natural pollination. Even in a cultivar such as Rim-100, which is relatively far more attractive to insects than others bioassayed, as indicated in the present study, some level of dysfunctionality for pollination may still exist. Midge-mediated pollination in cacao plantations is most likely opportunistic and effective primarily over small distances (Yamida, 1991), even though cacao trees in these settings are spaced closely together. Yet purportedly wild *T. cacao* in the Ecuadorian Amazon occurs as small clumps scattered over large areas of tropical rain forest (Allen, 1982 and personal communication). Under these conditions, long-distance movement of pollen among different clumps of *T. cacao* may necessitate strong flying insects such as bees.

Our data suggest that wild *T. cacao* would very likely possess a floral fragrance profile dominated by higher-molecular-weight volatile compounds (given phenotypic similarities of the Rim-100 cultivar to presumed wild cacao), even more so than that what was found in the Rim-100 cultivar examined in the present study. It is predicted that the floral fragrance profile of ancestral or wild *T. cacao*, therefore, would more closely resemble that of the criollo-derived Rim-100 than that of cultivars having low-molecular-weight constituents such as the UF series examined. Thus, what might once have been an ancestral bee-pollination syndrome in wild *T. cacao* centered around the collecting of fragrance compounds needed for mating, territorial defense, and foraging by bees

(Duffield et al., 1984), as a result of artificial selection, has been broken down in cultivated *T. cacao* in which midges prevail as opportunistic pollinators in plantation habitats.

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MOTH RESPONSES TO SELECTIVELY FLUORINATED SEX PHEROMONE ANALOGS

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Abstract—Partially fluorinated analogs of the European corn borer (*Ostrinia nubilalis*) female sex pheromone, 11-tetradecenyl acetate (97 : 3 *Z*:*E*), having mono- and trifluorosubstitutions at the terminal carbon of the pheromone chain, mimicked the biological activity of the pheromone, while analogs with fluorine at either side of the double bond and a pentafluoro analog were essentially inactive. Comparison of the pheromonal activity of these analogs with the previously reported activity of similarly fluorinated pheromones in five other species of moths revealed an unpredictable relationship between fluorine substitution pattern and pheromone-mimicking activity. Fluorine substitution patterns that rendered pheromonal analogs biologically inactive in the European corn borer had no detrimental influence upon pheromonal activity in other species and the converse was also true. This is evidence that the relative importance of electronic qualities of sites within a pheromone molecule differ from species to species. Furthermore, it indicates that the biochemical components (pheromone receptor proteins, binding proteins, and enzymes) that make up moth olfactory chemosensory systems must also vary structurally from species to species, despite the fact that they are involved in olfactory sensing of compounds having very similar chemical structure.

Key Words—Lepidoptera, Pyralidae, *Ostrinia nubilalis*, 11-tetradecenyl acetate, (*Z*)-14-fluoro-11-tetradecenyl acetate, (*Z*)-14,14,14,-trifluoro-11-tetradecenyl acetate, pheromone analogs, fluorinated analogs.

INTRODUCTION

Sex pheromones mediate behavioral aspects of moth reproduction. Knowledge of how male moths detect pheromone is a topic of considerable interest because

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it might lead to development of ways to interfere with normal sex pheromone communication and suppress reproductive potential of pests. Recent hypotheses (Prestwich, 1993) concerning mechanisms involved in moth olfactory sensing of pheromones propose that the lipophilic pheromone enters olfactory sensilla on the male's antenna through pores in the cuticular wall of the sensilla and that the pheromone is solubilized in the hydrophilic lumen of the sensilla by binding with pheromone-binding protein (Vogt et al., 1991). The bound pheromone is transported across the sensillar lumen and transferred to a membrane-bound pheromone receptor protein (Vogt et al., 1988) on sensory dendrites within the sensillum. The binding of the pheromone with the receptor causes generation of an electrophysiological response in the dendrites that make direct input into the olfactory glomeruli of the moth central nervous system (Hansson et al., 1992) and result in the display of complex behavioral reactions by the male. The receptor-bound pheromone is then removed from the receptor and catabolized to ready the system for a fresh in-coming stimulus (Klun et al., 1992; Klun and Schwarz, 1993).

Organofluorine compounds have proven useful as probes in studies of the reactivity and modes of action of biologically important agonists such as pheromones and hormones (Welch, 1991). Due to its electronegativity, fluorine has a strong inductive electron-withdrawing effect that makes the chemical reactivity of bonds involving fluorine different from those involving hydrogen. Depending upon the extent and position of substitutions of hydrogen with fluorine in a molecule, fluorinated analogs can have dipole moments, solubilities, volatilities, and stability characteristics that are vastly different from compounds containing the usual carbon–hydrogen bonds. However, the fluorine atom is only 10% larger than the hydrogen atom, with a Van der Waals radius of 1.65 Å vs. 1.50 Å for hydrogen (Wenqi et al., 1993). Thus, fluorinated analogs are expected to have electronic characteristics that are different from the natural molecule but that possess space-filling qualities closely resembling the natural product. Substitution of fluorine for hydrogen in a biologically active substance and assay of the influence of such substitutions on activity can provide insight into the relative importance of electronic qualities of sites in the bioactive molecule as it interacts with charged sites within its complementary receptor.

Our long-standing interest in structure–activity relationships (Klun et al., 1992; Schwarz et al., 1990) in the female sex pheromone, 11-tetradecenyl acetate (11–14:OAc) (Figure 1, **1**), of the European corn borer (ECB), *Ostrinia nubilalis*, prompted us to investigate selectively fluorinated analogs of the pheromone. The purpose of this study was to learn how ECB males would respond when fluorine was substituted for hydrogen at specific sites in the pheromone molecule and to compare these responses with assay results obtained by other researchers who had evaluated the biological activity of similarly fluorinated sex pheromone analogs in other species of moths.

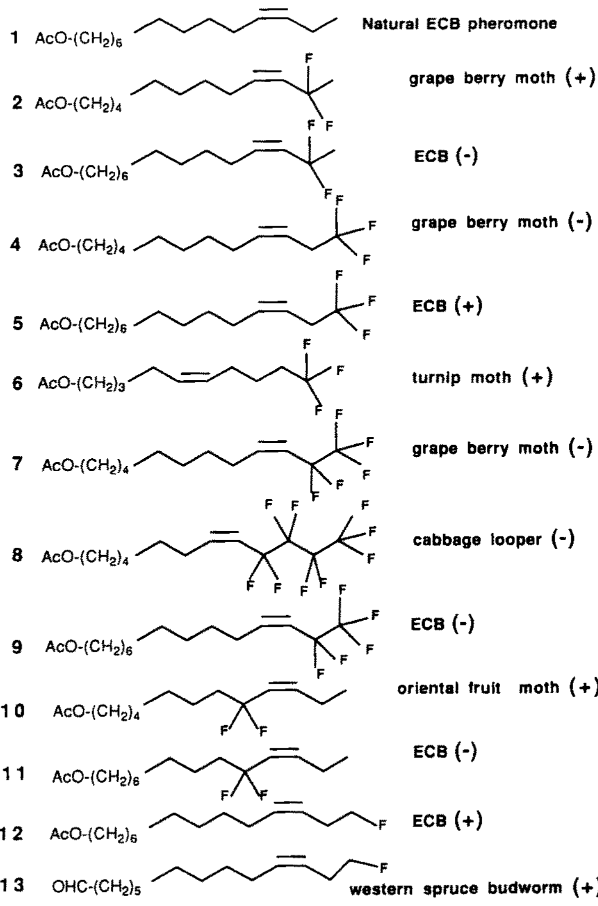

FIG. 1. The structure of partially fluorinated pheromone analogs of five species of moths and the European corn borer (ECB). The biological activities of analogs in the five species were determined and published previously by other researchers referred to in the text. (+) = analog mimicked natural pheromone. (−) = analog was not a pheromonal mimic. Structure **1** represents the natural ECB pheromone.

In the last few years many studies involving selectively fluorinated analogs of moth sex pheromones have been reported; however, previously no attempt had been made to develop a comparative overview of the effect of specific fluorine substitution patterns upon moth response across several species of moths. We surmised that such an overview might provide insight into whether the olfactory system of various species of moths that use chemically homologous

pheromones were structurally similar or dissimilar. We report results that show that these systems vary considerably among the moths.

METHODS AND MATERIALS

Rationale for Selecting Specific Fluorinated Pheromone Analogs

Five fluorinated ECB pheromone analogs (Figure 1; compounds **3**, **5**, **9**, **11**, and **12**) were selected for study. The fluorine substitutions in the analogs were designed to be identical to substitution patterns in compounds that had been previously tested by others as pheromone mimics in species of moths that use acetate esters of monounsaturated long-chain fatty alcohols as pheromones. In general, fluorine was substituted for hydrogen atoms at either side of the double bond and on the terminal carbon atoms. Pheromone analog **2**, which had been studied (Bengtsson et al., 1990) in the grape berry moth (*Eupoecilia ambiguella*), had substitutions identical to our analog **3**. Our trifluoroanalog, **5**, was similar to pheromone analogs **4** and **6** tested against the grape berry moth (Bengtsson et al., 1990) and turnip moth (*Agrotis segetum*) (Wenqi et al., 1993), respectively. Pentafluoro-analog **9** was fluorinated like pheromone analogs **7** and **8**, which were the subjects of study with the grape berry moth (Bengtsson et al., 1990) and the cabbage looper moth (*Trichoplusia ni*) (Linn et al., 1992). ECB analog **11**, having two fluorine atoms internalized in the carbon chain next to the olefinic site was similar to **10**, which had been studied (Masnyk et al., 1989) in the Oriental fruit moth (*Grapholitha molesta*). The ECB analog, having a single fluorine atom on the terminal carbon, **12**, had a counterpart, **13**, that was studied in the western spruce budworm (*Choristoneura occidentalis*) (McLean et al., 1989). Analogs **1–7**, **9**, **11**, and **12** were evaluated for biological activity in field trapping tests. Some of them were also assayed using electrophysiological and/or flight tunnel techniques that confirmed their biological activities. Compounds **8**, **10**, and **13** were assayed using flight tunnel or electrophysiological methods alone. In Figure 1, a positive sign follows the name of the insect if the corresponding fluorinated pheromone analog mimicked the species' natural pheromone. A negative sign is an indication that an analog lacked pheromonal activity.

Synthesis of Analogs

General. Gas chromatography on two Hewlett-Packard 5880A chromatographs fitted, respectively, with a polar 60-m × 0.25-mm DB-WAX (J & W Scientific, Folsom, California) and a nonpolar 50-m × 0.32-mm Ultra-1 columns (Hewlett-Packard, Palo Alto, California 94304) were used to determine the retention indices (Kováts et al., 1965) and chemical–geometrical purity of

the compounds. Infrared (IR) spectra were obtained on a Perkin-Elmer model 1320 spectrophotometer. Nuclear magnetic resonance (NMR) spectra were obtained using a GE QE300 with $CDCl_3$ as solvent and trimethylsilane for internal standard. The results are reported in δ values. Electron impact–mass spectra (EI-MS) were obtained using a Finnigan Mat INCOS 50 mass spectrometer.

Figure 2A shows the synthetic route we used to prepare (Z)-10,10-difluoro-11-tetradecenyl acetate **(11)**. Details of the synthesis and spectral data for intermediates and the end product are as follows.

10-Undecenyl Tetrahydropyranyl (THP) Ether. To a stirred solution of 10-undecenyl-1-ol (40.0 g, 0.235 mol) and K-10 Montmorillonite clay (4.0 g) in diethyl ether (40 ml) was added dihydropyran (DHP) (23.6 g, 0.281 mol) during 45 min while the reaction temperature was kept below 30°C. After 2 hr, the reaction mixture was centrifuged. Most of the solvent was removed on a rotary evaporator and the residue was distilled at 106–110°C (0.14 mm); the yield was 50.16 g (84% of theory). IR 3069 (m), 2929 (s), 2853 (s), 1639 (m), 1464 (m), 1454 (m), 1443 (m), 1347 (m), 1320 (w), 1258 (w), 1199 (m), 1118 (s), 1073, 1027 (s), 988 (m), 902 (m), 866 (s), 809 (w).

10-(Tetrahydropyranyloxy)decanal. To a mechanically stirred mixture of 10-undecenyl THP ether (30 g, 0.12 mol), osmium tetroxide (250 mg, 1.2 mmol in 10 ml *t*-butyl alcohol), dioxane (360 ml), and water (120 ml) was added sodium periodate (60 g, 0.28 mol) during 45 min, while keeping the reaction

FIG. 2. Schemes for synthesis of pheromone analogs **11** and **3**: (A) preparation of **11** and (B) preparation of **3**.

temperature below 30°C. The mixture was stirred for another 1.5 hr, water (200 ml) and petroleum ether (50 ml) were added, swirled vigorously, and the ether phase was removed. Extraction was repeated three more times; the combined extract was extracted once with aqueous Na_2SO_3, twice with water, and dried over anhydrous $MgSO_4$. The product was purified by flash chromatography using petroleum ether–methyl tertiary butyl ether (MTBE)–$CHCl_3$ (80:10:10). The yield was 16.6 g (57% of theory). IR 3004 (s), 2921 (s), 2851 (s), 2706 (w), 1722 (s), 1464 (m), 1453 (m), 1443 (m), 1350 (m), 1199 (m), 1118 (s), 1072 (s), 1029 (s), 968 (m), 899 (m), 865 (w), 750 (s). ^{1}H NMR 9.765 CHO (t, J = 1.5, H1), 2.423 (t, J = 7.2; d, J = 1.5, H2), 1.305 [$-(CH_2)_x-$].

14-Tetrahydropyranyloxy-3-tetradecyn-5-ol. To a stirred solution of 1-butyne (3.2 g, 50 mmol) in 70 ml tetrahydrofuran (THF), cooled in an ice–salt bath, was added methyllithium (25 ml of 1.4 M in ether, 35 mmol) at such a rate that the reaction temperature was kept below 0°C. The mixture was stirred for 30 min, then cooled to −78°C and a solution of 10-(tetrahydropyranyl-oxy)decanal (7.48 g, 29 mmol) in THF (20 ml) was added while the reaction temperature was kept below −70°C. After 1.5 hr, the reaction mixture was poured into water, acidified, and worked up to yield a pale green oil that was purified by flash chromatography using petroleum ether–MTBE–$CHCl_3$ (75:15:10) to obtain a clear, colorless oil in a yield of 3.40 g (38% of theory). IR 3394 (s), 2915 (s), 2860 (s), 1460 (m), 1355 (m), 1320 (m), 1260 (m), 1120 (s), 1070 (s), 1020 (s), 900 (m), 865 (m), 785 (s), 630 (m). ^{1}H NMR 1.238 (t, J = 7.4, H1), 2.226 (t, J = 7.4, d, J = 1.5, H2), 4.340 (t, J = 6.6, further small splitting, H5), 1.302 [$-(CH_2)_x-$].

14-Tetrahydropyranyloxy-3-tetradecyn-5-one. A mixture of 14-tetrahydro-pyranyloxy-3-tetradecyn-5-ol (4.0 g, 12.9 mmol) and pyridinium chlorochromate (PCC) (11 g, 51 mmol) in methylene chloride (60 ml) was allowed to stand for 1 hr, and then it was diluted with diethyl ether (80 ml). The solution was decanted from the gummy black precipitate and passed through a column of Florisil (60 g), followed by a mixture of methylene chloride–ether (40:60) (200 ml). Solvent was removed and product was purified by flash chromatography using hexane–MTBE–(90:10). The yield of clear, colorless oil was 2.03 g (50% of theory). IR 2920 (s), 2852 (s), 2205 (s), 1665 (s), 1453 (m), 1349 (m), 1313 (m), 1254 (m), 1233 (m), 1198 (m), 1160 (m), 1134 (s), 1117 (s), 1072 (s), 1024 (s), 986 (m), 892 (w), 865 (w), 808 (w).

14-Acetoxy-3-tetradecyn-5-one. A mixture of 14-tetrahydropyranyloxy-3-tetradecyn-5-one (1.73 g, 5.6 mmol), acetyl chloride (0.5 ml), acetic anhydride (Ac_2O) (5 ml), and acetic acid (5 ml) was allowed to stand for two days at room temperature, then poured into stirred aqueous $NaHCO_3$ containing ice. After work-up the product was purified by flash chromatography using petroleum ether–MTBE (85:15). Impure fractions were combined and rechromatographed to yield a total of 0.60 g (40% of theory) of colorless oil. IR 2924 (s), 2853

(s), 2204 (s), 1732 (s), 1663 (s), 1458 (m), 1381 (m), 1361 (s), 1311 (m), 1235 (s), 1159 (s), 1030 (m). ^{1}H NMR 1.212(t, J = 7.5, H1), 2.378(q, J = 7.5, H2), 2.523(t, J = 7.5, H6), 1.298(br.s, H7–H13), 4.049(t, J = 6.6, H14), 2.049(s,CH_3COO—).

10,10-Difluoro-11-tetradecynyl Acetate. To a stirred solution of diethylaminosulfur trifluoride (DAST) (3.6 g, 22 mmol) in 1,2-dichloroethane (20 ml) at room temperature was added 14-acetoxy-3-tetradecyn-5-one (600 mg, 2.3 mmol). The reaction flask was placed in a bath at 60°C. At 6 hr and at 9 hr, 1.2 g (1 ml) portions of DAST were added. After 30 hr, the cooled reaction mixture was poured onto an aqueous solution of $NaHCO_3$ containing ice and worked up. Flash chromatography with petroleum ether–$CHCl_3$ (70:30) yielded 120 mg (18.5%) of yellow oil. IR 2925 (s), 2853 (s), 2249 (m), 1729 (s), 1460 (m), 1361 (m), 1317 (m), 1231 (s), 1163 (m), 1029 (m). ^{1}H NMR 1.183 (t, J = 7.5, H14), 2.287 (m, H2), 1.90–2.02 (m, H9), 1.311 (s, H2–H8), 4.050 (t, J = 6.76, H1), and 2.050 (s,CH_3COO—).

(Z)-10,10-Difluoro-11-tetradecenyl Acetate **(11)**. 10,10-Difluoro-11-tetradecynyl acetate (100 mg, 0.35 mmol) in pyridine (2 ml) was hydrogenated at atmospheric pressure in the presence of 5% Pd on $BaSO_4$ (300 mg) until the theoretical amount of hydrogen was taken up. The reaction mixture diluted with petroleum ether was filtered through a column of Florisil (5 g) and the pyridine removed by aqueous washing. Flash chromatography on silica gel with petroleum ether–MTBE (85:15) followed by short-path distillation at 0.05 mm pressure and bath temperature of 130°C yielded 80 mg (80%) of clear colorless liquid. Gas chromatography showed that it was a 97:3 mixture of (Z)- and (E)-10,10-difluoro-11-tetradecenyl acetate. IR 2926 (s), 2855 (s), 1734 (s), 1658 (w), 1463 (m), 1408 (w), 1362 (m), 1233 (s), 1163 (w), 1029 (m). ^{1}H NMR 1.009 (t, J = 7.5, H14), 2.246 (d, J = 9.1, q, J = 7.5, H13), 5.646 (t, J = 9.1, d, J = 10.8, H12), 5.417 (t, J = 16.2, d, J = 10.8, H11), 1.889 (m, H9), 1.303 (br.s, H2–H8), 4.051 (t, J = 6.6, H1), 2.044 (s, CH_3COO—). EI-MS 250 (0.5, M^+-2HF), 210 (0.4 M^+-60), 181 (0.6), 167 (1), 161 (2), 153 (3), 147 (2), 139 (3), 133 (4), 132 (2), 128 (6), 122 (4), 121 (5), 119 (3), 113 (9), 112 (16), 111 (7), 108 (7), 105 (10), 100 (12), 98 (14), 97 (17), 95 (15), 94 (41), 85 (16), 82 (12), 81 (20), 77 (22), 69 (23), 67 (25), 61 (14), 55 (54), 43 (100).

Synthesis of (Z)-13,13-Difluoro-11-tetradecenyl acetate **(3)** is shown schematically in Figure 2B. Details for synthesis of the compound are as follows.

11-Dodecyn-1-ol. To NaH (12.5 g, 0.52 mol) was added 1,3-diaminopropane (150 ml, freshly distilled from CaH_2) and the bath temperature was raised to 75°C. When hydrogen evolution subsided after 2 hr, 7-dodecyn-1-ol (29 g, 0.16 mol) was added and after 40 min the mixture was heated to 100°C for 2 hr. The reaction was worked up by partitioning between petroleum ether and

water and used for the preparation of 11-dodecynyl THP ether without further purification.

11-Dodecynyl THP Ether. To the stirred red solution of crude 11-dodecyn-1-ol, obtained above, was added K-10 Montmorillonite clay (64 g) and DHP (17 g, 0.20 mol). After 10 hr, the clay was removed by centrifugation and rinsed twice with petroleum ether. The combined petroleum ether solution was concentrated and distilled. 11-Dodecynyl THP ether had a bp 119–125°C (0.60–0.10 mm). The yield was 25.65 g (58% of theory). IR 3309 (m), 2916 (s), 2853 (s), 2112 (w), 1451 (m), 1347 (m), 1320 (w), 1256 (w), 1116 (s), 1071 (s), 1020 (s), 978 (m), 898 (w), 865 (w), 809 (w), 616 (m). ^{1}H NMR 1.949 (t, J = 2.9, H12), 2.180 (t, J = 7.2, d, J = 2.9, H9), 1.207 (br.s, H2–H8).

14-Tetrahydropyranyloxy-3-tetradecyn-2-ol. To a solution of 11-dodecynyl THP ether (15.0 g, 56 mmol) in THF (100 ml), cooled in an ice–salt bath, was added methyllithium (52 ml of 1.4 M in ether, 73 mmol) during 10 min while keeping the reaction temperature below 0°C. The mixture was allowed to stand at −10°C for 30 min, cooled to −78°C, and acetaldehyde (3.7 g, 84 mmol) was added during 15 min while the temperature was kept below −60°C. When addition was completed, the cooling bath was removed and the reaction mixture was allowed to stand at room temperature for 20 hr. The reaction was worked up in the usual manner and the product was purified by flash chromatography using petroleum ether–MTBE–$CHCl_3$ (70:20:10). The yield of product was 12.37 g (71% of theory). IR 3418 (m), 2921 (s), 2853 (s), 2239 (w), 1443 (m), 1348 (m), 1320 (m), 1275 (w), 1258 (w), 1131 (m), 1116 (m), 1071 (m), 1020 (m). ^{1}H NMR 1.429 (d, J = 6.6, H1), 4.514 (q, J = 6.6, t, J = 1.9, H2), 2.188 (t, J = 7.0, d, J = 1.9, H5), 1.234 (br.s, H6–H15).

14-Tetrahydropyranyloxy-3-tetradecyn-2-one. A mixture of 14-tetrahydropyranyloxy-3-tetradecyn-2-ol (8.0 g, 25.8 mmol) and PCC (22 g, 102 mmol) in methylene chloride (120 ml) was stirred for 2 hr while the reaction temperature was kept below 30°C. The reaction mixture was diluted with diethyl ether and the gummy precipitate washed with additional ether. The combined organic layer was passed through Florisil (60 g) followed by a mixture of methylene chloride–MTBE (40:60) (400 ml). After the solvent was removed, the residue was purified by flash chromatography using petroleum ether–MTBE (85:15). The yield of clear colorless oil was 5.45 g (68% of theory). IR 3332 (w), 2920 (s), 2850 (s), 2200 (s), 1666 (s), 1453 (m), 1352 (s), 1320 (s), 1220 (s), 1200 (s), 1118 (s), 1072 (s), 1020 (s), 964 (m), 899 (m), 864 (m), 807 (m), 713 (w), 650 (w). ^{1}H NMR 2.323 (s, H1), 2.350 (t, J = 6.9, H5). 1.287 (br.s, H6–H13).

14-Acetoxy-3-tetradecyn-2-one. 14-Tetrahydropyranyloxy-3-tetradecyn-2-one (5.0 g, 16.2 mmol), acetyl chloride (0.5 ml), acetic anhydride (15 ml), and acetic acid (15 ml) were mixed and heated in an oil bath at 60°C for 1.5 hr. The deep red reaction mixture was poured into a stirred mixture of aqueous $NaHCO_3$ and ice and worked up with petroleum ether. The product was purified

by flash chromatography using petroleum ether–MTBE (90:10) to yield 2.74 g (64% of theory) of a clear, colorless oil. IR 3331 (w), 2918 (s), 2857 (s), 2202 (s), 1726 (s), 1669 (s), 1458 (m), 1426 (m), 1359 (s), 1226 (s), 1034 (s), 960 (m), 926 (m). ^{1}H NMR 2.323 (s, H1), 2.353 (t, J = 6.9, H5), 1.290 (br.s, H6–H12), 1.587 (m, H13), 4.052 (t, J = 6.8, H14), 2.049 (s, CH_3COO—).

13,13-Difluoro-11-tetradecynyl Acetate. To a stirred solution of 14-acetoxy-3-tetradecyn-2-one (2.3 g, 9.6 mmol) in methylene chloride (50 ml) was added DAST (13.9 g, 86 mmol, 11.4 ml) and kept at 60°C for 25 hr. The reaction mixture was poured into aqueous $NaHCO_3$ containing ice, worked up, and purified by flash chromatography using petroleum ether–MTBE (92:8) to yield a pale yellow oil (0.82 g, 33% of theory). IR 2917 (s), 2853 (s), 2243 (s), 1726 (s), 1639 (w), 1443 (m), 1380 (s), 1363 (s), 1228 (s), 1159 (s), 1126 (s), 1030 (m), 908 (m). ^{1}H NMR 1.842 (t, J = 20.1, H14), 2.51 (m, H10), 1.288 (br.s, H2–H9), 4.056 (t, J = 6.8, H1), 2.048 (s, CH_3COO—).

(Z)-13,13-Difluoro-11-tetradecenyl Acetate **(3).** 13,13-Difluoro-11-tetradecynyl acetate (300 mg, 1.0 mmol) and 5% Pd on $BaSO_4$ (600 mg) were placed in pyridine (Py) (5 ml) and hydrogenated at atmospheric pressure. The reaction mixture was centrifuged to remove catalyst and worked up to yield 280 mg (93% of theory) of clear, colorless oil. GC analysis indicated that it was a 94:6 mixture of (Z)- and (E)-13,13-difluoro-11-tetradecenyl acetate. IR 2924 (s), 2853 (s), 1733 (s), 1656 (m), 1447 (m), 1381 (m), 1362 (m), 1228 (s), 1152 (s), 1123 (s), 1031 (s), 904 (s). ^{1}H NMR 1.702 (t, J = 18.0, H14), 5.504 (q, J = 12.0, H12), 5.678 (m, H11), 2.241(m, H10), 1.276 (br.s, H2–H9), 4.050 (t, J = 6.8, H1), 2.047 (s, CH_3COO—). EI-MS 251 (0.7), 250 (2, M^+-2HF), 207 (0.6), 175 (0.6), 161 (2), 153 (1), 150 (1), 139 (2), 135 (3), 133 (3), 125 (3), 124 (4), 123 (2), 121 (4), 112 (8), 109 (7), 108 (6), 99 (8), 96 (9), 95 (18), 86 (30), 83 (14), 82 (23), 81 (25), 73 (20), 69 (27), 67 (30), 55 (45), 43 (100).

14,14,14-Trifluoro-11-tetradecenyl Acetate **(5).** The synthesis of **5** is shown schematically in Figure 3A. 3,3,3-Trifluoropropyltriphenylphosphonium bromide (Ullmann and Hanack, 1989) (1.1 g, 2.5 mmol in 20 ml THF; 2.7 mmol)

A: $O{=}CH(CH_2)_8CH_2OAc$ + $F_3C(CH_2)_2P^+\Phi_3\ Br^-$, NaBTMSA/THF → F_3C–CH=CH–$(CH_2)_9OAc$ (5); ΦSH/benzene, AIBN → 78:22 (Z:E)

B: HC≡C–$(CH_2)_8$–CH_2OH; 1. F_3C-CF_2I/AIBN, 2. ODS Hplc → F_3C-CF_2–CH=CI–$(CH_2)_9$–CH_2OH; 1. BuLi 2. MeOH, 3. Ac_2O/Py → (9)

FIG. 3. Schemes for synthesis of **5** and **9:** (A) preparation **5** and (B) preparation of **9.**

was added to 2.7 ml of a 1 M THF solution of sodium bistrimethylsilylamide (2.7 mmol). After 0.5 hr, the solution was cooled to −78°C and 11-oxoundecyl acetate (Schwarz et al., 1986) (0.625 g, 2.7 mmol) was added. The reaction mixture was warmed to 0°C and held at that temperature overnight. Work-up in the usual manner and flash chromatography (ethyl acetate–hexane, 7.5:92.5) yielded 0.4 g (50% of theory) product. GC analysis on the polar and nonpolar capillaries showed a single compound that was presumed to be the *Z* isomer. Isomerization of 0.1 g of the product using 2,2′-azobis(2-methylpropionitrile) (AIBN)–thiophenol in benzene (Schwarz et al., 1986) gave rise to a 80:20 *E*:*Z* mixture. The two peaks had identical mass spectra. IR for *Z* 3015 (m), 2915 (s), 2880 (s), 1740 (s), 1650 (w), 1480 (m), 1370 (m), 1350 (s), 1250 (s), 1130 (s). ^{1}H NMR 1.28 [br. s, $-(CH_2)_x-$], 1.603 (m, H2), 2.044 (br. s, H10 and CH_3COO-), 2.839 (m, H13), 4.051 (t, J = 6.6, H1), 5.377 (t, J = 10.8, t, J = 6.9, d, J = 1.5, H12), 5.723 (m, H11). EI-MS 284 (1), 214 (4), 213 (2), 200 (4), 199 (3), 173 (1), 145 (1), 138 (10), 127 (3), 124 (2), 123 (2), 116 (4), 111 (1), 110 (3), 109 (9), 103 (3), 98 (3), 97 (8), 96 (11), 95 (15), 85 (2), 84 (6), 83 (21), 82 (19), 81 (18), 79 (2), 77 (4), 73 (6), 71 (5), 70 (11), 69 (37), 68 (14), 67 (21), 65 (2), 61 (37), 59 (2), 58 (3), 57 (13), 56 (17), 55 (52), 54 (13), 53 (3), 44 (3), 43 (100), 42 (14), 41 (37).

14,14,14,13,13-Pentafluoro-11-tetradecenyl Acetate **(9)**. Figure 3B shows the preparation of **9**. The synthesis was carried out by a reaction sequence that was identical to the one reported by Sun and Prestwich (1990). The (*Z*)-iodide resulting from the addition of pentafluoroethyl chloride to 11-dodecyn-1-ol was separated from the minor *trans*-isomer by reverse phase HPLC. The desired (*Z*)-14,14,14,13,13-pentafluoro-11-tetradecen-1-ol was obtained after metal–halogen exchange with *n*-butyl lithium and subsequent quenching with methanol. The crude reaction product was acetylated using acetyl chloride–pyridine and purified by flash chromatography with a 96.5:3.5 hexane–ethyl acetate mixture. A portion of the compound was isomerized using thiophenol–AIBN in benzene (Schwarz et al., 1986) to yield an 80:20 *E*:*Z* mixture which was used to make the appropriate 97:3 *Z*:*E* mixture for bioassay purposes. IR 3315 (w), 2915 (s), 2845 (s), 1740 (s), 1660 (s), 1555 (w), 1505 (w), 1465 (s), 1455 (s), 1410 (w), 1385 (m), 1365 (s), 1335 (s), 1220 (s), 1100 (s), 1050 (s), 740 (s), 635 (m), 605 (m). ^{1}H NMR 1.28 [br. s, $-(CH_2)_x-$], 1.419 (m, H9), 1.619 (m, H2), 2.045 (s, CH_3COO-), 2.321 (br. s, H10), 4.052 (t, J= 6.6, H1) 5.460 (m, H12), 6.107 (t, J = 9.9, t, J = 7.8, d, J = 2.1, H11). EI-MS 284 (1), 214 (4), 213 (2), 200 (4), 199 (3), 173 (1), 145 (1), 138 (10), 127 (3), 124 (2), 123 (2), 116 (4), 111 (1), 110 (3), 109 (9), 103 (3), 98 (3), 97 (8), 96 (11), 95 (15), 85 (2), 84 (6), 83 (21), 82 (19), 81 (18), 79 (2), 77 (4), 73 (6), 71 (5), 70 (11), 69 (37), 68 (14), 67 (21), 65 (2), 61 (37), 59 (2), 58 (3), 57 (13), 56 (17), 55 (52), 54 (13), 53(3), 44 (3), 43 (100), 42 (14), 41 (37).

14-Fluoro-11-tetradecenyl Acetate. The synthesis of the geometrical iso-

mers of this compound was reported previously (Schwarz et al., 1990). The isomers were mixed to make a 97:3 Z:E mixture for the field testing. ^{1}H NMR 1.276 [br. s, $-(CH_2)_x-$], 1.615 (m, H2), 2.045 (br. s, H10 and CH_3COO-), 2.462 (m, H13), 4.052 (t, J = 6.9, H1), 4.418 (t, J = 6.6, d, J = 47.1, H14), 5.368 (m, H12), 5.538 (m, H11). EI-MS: 212 (M^+-60; 6), 192 (3), 164 (1), 163 (1), 150 (1), 149 (3), 141 (2), 138 (2), 137 (1), 136 (3), 135 (7), 128 (3), 127 (3), 124 (5), 123 (4), 122 (4), 121 (10), 115 (1), 114 (9), 113 (3), 111 (2), 110 (13), 109 (10), 108 (5), 107 (5), 101 (3), 100 (34), 99 (3), 98 (1), 97 (6), 96 (24), 95 (22), 94 (10), 93 (8), 91 (2), 87 (2), 86 (4), 85 (15), 84 (2), 83 (3), 82 (41), 80 (16), 79 (11), 77 (2), 73 (6), 72 (2), 71 (2), 70 (3), 69 (24), 68 (25), 67 (44), 66 (5), 65 (2), 61 (11), 59 (7), 57 (7), 56 (8), 55 (52), 54 (25), 53 (8), 47 (2), 44 (4), 43 (100), 42 (14), 41 (68).

Field Tests

We used insect traps to bioassay the analogs. Tests were conducted in corn fields near Beltsville, Maryland, during summer flights of the ECB. Three pheromonal forms of ECB (Klun and Huettel, 1988) exist in the Beltsville area, and they communicate by using geometric blends of 11-14:OAc; ca. 97:3, 40:60, or 3:97 Z:E. The form using 97:3 Z:E is most abundant in the area. Therefore, the isomeric composition of the analogs was adjusted to target that form of the moth. Analogs **3** and **11** had 94:6 and 97:3 Z:E proportions, respectively, as result of the Lindlar reduction, and they were used as such. Exact 97:3 Z:E geometric mixtures of the other analogs were prepared for the field tests by mixing purified isomers or by mixing Z:E with pure Z isomer. All compounds used in the field tests were purified (95–98%) by flash chromatography (Still et al., 1978), reverse-phase chromatography using a 31-cm × 10-mm-ID column packed with 5-μm ODS Spherisorb (Rainin Instrument Co., Inc., Emeryville, California 94608) eluted with acetonitrile–water (9:1) at 2.5 ml/min or by argentation chromatography using two 31-cm × 10-mm-ID columns packed with 20% $AgNO_3$-impregnated (w/w) 5-μm Spherisorb silica plumbed in series and eluted with toluene pumped at 5 ml/min. The first field test compared responses of males to the natural pheromone (**1**) versus a monofluorinated analog (**12**). The second test compared male responses to the pheromone and four analogs (**3, 5, 9,** and **11**). For all tests, heptane solutions of the compounds (ca. 10 μg/μl) were prepared and 100 μg of each compound was applied individually to red rubber serum bottle stoppers (#1780J07, Thomas Scientific, Swedesboro, New Jersey 08085), which were placed randomly in cone-shaped traps (Webster et al., 1986) positioned at the perimeter of corn fields with 30 m between traps. Nightly ECB male captures in traps baited with analogs were compared to captures in traps baited with the natural pheromone. New lures were placed in the traps nightly, and all tests were conducted using a randomized complete

block design with replication within and over nights. Trap capture data were analyzed as a three factor model: treatment, night, and replicate. Treatment was a fixed effect, while night and replicate were random effects. To meet the assumptions of the general linear model, the In($y + 1$) transformation was used on the dependent variable (number of moths captured). Statistical analysis was done on the transformed data using the SAS PROC GLM (SAS Institute, 1989).

RESULTS AND DISCUSSION

Gas chromatography of the ECB pheromone and its analogs showed (Table 1) that only the polyfluorinated analog **9** was significantly more volatile than the pheromone **1.** This was consistent with the observation of Prestwich et al. (1990) that perfluoroalkyl compounds were always more volatile than their corresponding hydrocarbon analogs. The volatilities of the difluorosubstituted **11** and **3** and the trifluorosubstituted **5** were only slightly different from the pheromone **1.** The monofluorinated compound **12** was least volatile of all compounds in the set based upon the 2408 retention index on the Ultra 1 column. At the same time, the compound displayed significantly greater polarity than all other compounds based upon its 1871 retention index on DB-WAX. The chromatographic data reflect the physical-chemical effects caused by substitution of hydrogen with fluorine in a molecule.

Bioassay results reported in Table 2 show that traps baited with 14,14,14-trifluoro-11-14:OAc **(5)** and 14-monofluoro-11-14:OAc **(12)** proved to be equivalent to the natural pheromone in causing capture of ECB males. This was true although the volatility and polarity of **5** and **12** were different from each another and the pheromone **1.** On the other hand, 13,13-difluoro-11-14:OAc **(3),** 10,10-difluoro-11-14:OAc **(11),** and 13,13,14,14,14-pentafluoro-11-

TABLE 1. RETENTION INDICES FOR EUROPEAN CORN BORER SEX PHEROMONE AND FLUORINATED PHEROMONE ANALOGS ON POLAR (DB-WAX) AND NONPOLAR (ULTRA 1) CHROMATOGRAPHIC COLUMNS

Compound	Retention index	
	DB-WAX	Ultra 1
13,13,14,14,14-Pentafluoro-Z-11-14:OAc **(9)**	1656	1964
14,14,14-Trifluoro-Z-11-14:OAc **(5)**	1733	2141
Z-11-14:OAc **(1)**	1786	2154
10,10-Difluoro-Z-11-14:OAc **(11)**	1802	2296
13,13-Difluoro-Z-11-14:OAc **(3)**	1794	2303
14-Monofluoro-Z-11-14:OAc **(12)**	1871	2408

TABLE 2. MEAN NIGHTLY MALE ECB TRAP-CAPTURE RATES OF TRAPS BAITED WITH SYNTHETI(ECB FEMALE SEX PHEROMONE AND FLUORINATED ANALOGS[a]

Test	Compound	Mean males/trap/nigl
1 (*N* = 24)	11-14:OAc (**1**)	7.5a
	14-monofluoro-11-14:OAc (**12**)	7.3a
2 (*N* = 25)	11-14:OAc (**1**)	19.4a
	14,14,14-trifluoro-11-14:OAc (**5**)	16.7a
	10,10-difluoro-11-14:OAc (**11**)	1.1b
	13,13-difluoro-11-14:OAc (**3**)	1.5b
	13,13,14,14,14-pentafluoro-11-14:OAc (**9**)	0.7b

[a] Means reported within tests followed by the same letter are not significantly different from one anoth(according to Sidak *t* tests (Sidak, 1967). Alpha = 0.05. *N* = number of replicates.

14:OAc (**9**) were essentially inactive as pheromone mimics. The potency of **5** and **12** as pheromone mimics shows that these analogs fulfilled a requirement for a 14-carbon chain in the pheromone molecule and that the fluorination of the terminal carbon did not interfere with a productive interaction with the ECB chemoreceptive system. Comparison of the biological activity of **5** with identically substituted analogs of the pheromones of the grape berry moth (**4**) and the turnip moth (**6**) (Figure 1) shows that trifluoro substitution in the terminal carbon of the pheromone destroyed activity in case of the grape berry moth but not in case of the turnip moth. Retention of pheromonal activity in the monofluorinated ECB analog **12** was like the result obtained with the western spruce budworm pheromone analog (**13**), where pheromonal activity was retained with monofluorination of the terminal carbon. Substitution of hydrogen on either side of the double bond with fluorine (compounds **3** and **11**) resulted in loss of ECB biological activity. However, substitution of the hydrogen on either side of the double bond in pheromones of the grape berry moth (**2**) and oriental fruit moth (**10**) was permissible. It did not adversely affect pheromonal activity in those species. Multiple fluorination of carbon atoms beyond the double bond in the ECB pheromone (**9**) caused a loss of pheromonal activity for the analog. This effect was the same as was observed when all hydrogen beyond the double bond in pheromones of the grape berry (**7**) and the cabbage looper (**8**) moths were substituted with fluorine. Previous studies with fluorinated methanes (Welch, 1991) have shown that C—F bond length shortens and bond strength increases with increased fluorination, and it is reasonable that these effects and increased electron density associated with polyfluorination contribute to a loss of pheromone activity in grape berry, cabbage looper, and ECB moths.

Clearly, introduction of the same or similar fluorination patterns into a pheromone molecule have unpredictable effects on moth responses from one

species to another. This is evidence that the importance of the electronic qualities at specific sites within a pheromone molecule differs from one species to another. Correspondingly, the complementary sites for interaction of pheromone with binding proteins, receptor proteins, and/or catabolic enzymes that comprise the olfactory chemosensory systems of the moths must also differ structurally from species to species. This idea is reinforced by the work of Chapman et al. (1978) and Bestmann et al. (1992). Chapman et al. found that despite the fact that the red-banded leaf roller (*Argyrotaenia velutinana*) and the ECB use an identical compound as sex pheromone, their chemoreceptors for it have different chirality. Bestmann et al. (1992) have developed evidence to show that the pheromonal receptor systems of *Bombyx mori* and *Manduca sexta* are structurally different, although they use the same compound as pheromone. Their research also showed that a similar situation exists in *Antherea pernyi* and *A. polyphemus*. Thus, there is compelling evidence to conclude that the components of the olfactory systems of moths are structurally dissimilar notwithstanding that they are responsible for detection of similar or identical pheromonal compounds.

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MAJOR COMPONENT IN MALE SEX PHEROMONE OF CEREAL PEST *Eurygaster integriceps* PUTON (HETEROPTERA: SCUTELLERIDAE) IDENTIFIED AS A HOMOSESQUITERPENOID

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Abstract—A homosesquiterpenoid, (4*Z*,4′*E*)-4-(1′,5′-dimethyl-4′-heptenylidene)-1-methylcyclohexene, has been identified as a major component of the scent emitted by calling males of *Eurygaster integriceps*. Minor components of the scent included vanillin. The *E. integriceps* male homosesquiterpenoid is an addition to the list of sesquiterpenoids identified as components of male attractant sex pheromones in pentatomoid Heteroptera.

Key Words—*Eurygaster integriceps*, Heteroptera, Scutelleridae, wheat pest, homosesquiterpenoid, male pheromone, vanillin.

INTRODUCTION

The Sunn bug *Eurygaster integriceps* is a major pest of wheat in several countries in southeast Europe and the Near East (Paulian and Popov, 1980; Lodos, 1981). Experimental evidence that mature males signal to females by the pro-

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duction of a volatile sexual attractant was first reported by Litvenkov (1971). He observed that unmated females are capable of responding to males at distances greater than 2 m and that, in the laboratory, they reacted anemotactically by their approach to a jet of air containing the pheromone. In mated females the strength of this anemotactic response was much reduced. Calling males emit a strong odor resembling that of vanillin and Ubik et al. (1975) presented evidence that the odorous substance is vanillin and that it is produced and released by males only during the period of sexual activity. However, biological tests for a behavioral response by receptive females to authentic vanillin were inconclusive (Litvenkov, 1973), suggesting that the problem of the identity of the male pheromone was more complex. Vrkoc et al. (1977) subsequently isolated and identified ethyl acrylate as a second component of the male pheromone and reported that the stimulatory effect of a mixture of vanillin and ethyl acrylate on receptive females was greater than that of either compound when tested alone. However, no practical application so far seems to have arisen from the chemical findings, possibly because as yet further sex-specific compounds remain to be identified.

Thanks to assistance received from the Iranian Ministry of Agriculture, we have been able to examine the volatiles emitted by sexually mature male *E. integriceps*. We confirm the presence of vanillin, as a minor component, and report the isolation and identification of a homosesquiterpenoid as the major component of the volatiles produced by mature calling male *E. integriceps*.

METHODS AND MATERIALS

E. integriceps adults were collected on two days (April 9 and 10) in the spring of 1993 from hibernation sites at two locations: Kabatoor-Ahang, in west Iran, and Qasvine in central Iran. In the laboratory, the sexes were segregated and maintained on whole wheat grain at 26°C for seven days prior to analysis. The airborne volatile extraction apparatus had two 25-cm × 4.5-cm sample chambers for the simultaneous collection of volatiles from the two sexes separately. Air was drawn through an activated charcoal scrubber into each sample chamber at 600 ml/min. A roll of clean chicken wire provided the enclosed insects with support. The volatiles from each chamber were trapped in a glass tube (7 cm × 0.5 cm ID) containing 0.45 g of 20–40 mesh activated carbon (Aldrich Chemical Company Inc.). The trapped analytes were desorbed with 1.5 ml of HPLC grade dichloromethane (Aldrich) and the resulting sample stored at −20°C. Four successive extractions were carried out over a period of 20 days. In the first extraction run (24 hr in total) 440 males and 290 females were used. Parasitization by tachinids and death from other causes reduced these numbers in subsequent runs. During each trapping period the temperature was maintained approximately constant at 24°C.

Combined gas chromatography–mass spectrometry (GC-MS) was carried out in the electron impact (EI) mode using a DB-225 column (0.25 μm film, 30 m × 0.25 mm ID). The mass spectra were recorded on a INCOS 50 mass spectrometer interfaced to a Varian 3400 gas chromatograph. Spectra were recorded at 70 eV with the injector at 200°C, separator 168°C, and ion source 150°C. The column was maintained at 70°C for 5 min and then programmed at 6°C/min to 200°C.

Hydrogenation was carried out using 10% palladium on carbon catalyst in ethanol under 1 atmosphere of hydrogen.

The nuclear magnetic resonance (NMR) spectral data were obtained using a Bruker 400AM 400 PFT spectrometer operating at 400 MHz for ^{1}H spectra and at 100 MHz for ^{13}C spectra. Tetramethylsilane was used as internal standard and coupling constants (J) are quoted in hertz.

RESULTS

The results (GC-MS) of the first paired run made on collections of male and female *E. integriceps* are compared in Figure 1. Peaks have been numbered in the two traces considered jointly on the basis of retention time (R_t) and MS. Thus, peaks with same number share the same R_t and MS. With the exception of peak 15, which was not examined, MS were recorded for all twenty-four peaks. Peaks 4 and 5 were not identified.

Impurities. Peaks 2, 7, and 10 were assigned from their EI-MS to column bleed and need be considered no further. Peak 3, a monoterpene with MS similar to that of limonene, was present in both male and female extracts but was also detected in the activated charcoal scrubber. Thus peak 3 is probably an impurity. The MS of peak 6 was similar to that of a methyl-branched alkane of MW 156. The MS of peak 8 was similar to that of a methyl-branched alkane of MW 170. It is presumed that peaks 6 and 8 are impurities, since peaks with similar retention times and MS were detected in samples of the activated charcoal used in the traps.

Scent Gland Volatiles. The metathoracic scent gland as a possible source of volatiles was briefly examined by EI-GC-MS. Five peaks in the gland extract were readily identified from their MS as typical scent gland volatiles. In order of elution, they were 2-hexenal, 4-oxohex-2-enal. 2-octenal, *n*-tridecane, and *n*-tetradecane. The EI-MS of two additional late eluting peaks were superimposable on those recently reported for two isomeric dimers of 4-oxohex-2-enal (Aldrich et al., 1993). Three peaks remained unidentified.

It was now possible to identify scent gland volatiles in the airborne volatile extracts. The EI-MS of peak 1 in Figure 1 was superimposable on that of 2-hexenal. The MS of peak 9 was similar to that of *n*-tridecane, and the MS of

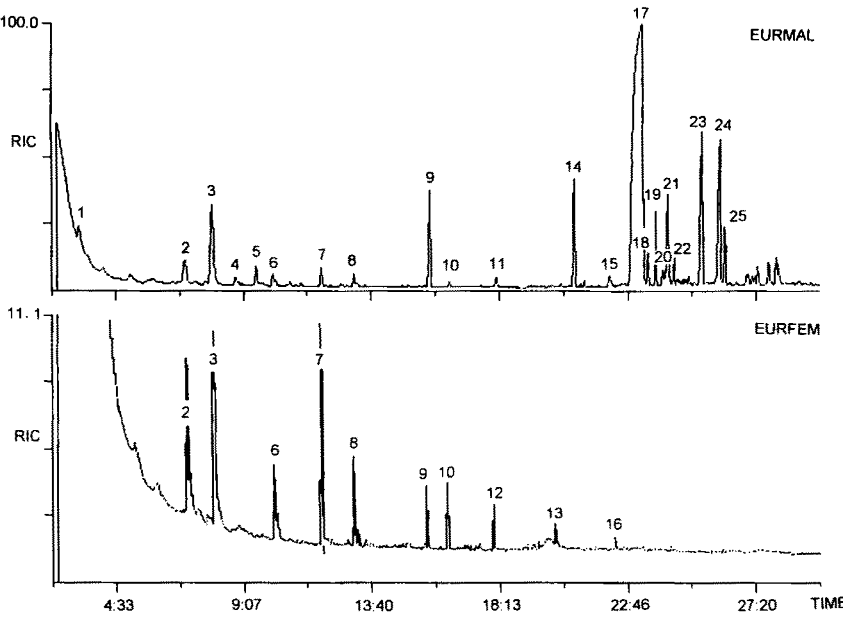

FIG. 1. Reconstituted total ion current traces. EURMAL, above, *Eurygaster integriceps* male volatile extract. EURFEM, below, simultaneously collected *Eurygaster integriceps* female volatile extract. DB-225, 0.25 μm film, 30 m × 0.25 mm ID; 5 min at 70°C then temperature programmed at 6°/min to 200°C.

peak 12 superimposable on that of *n*-tetradecane. Thus peaks 1, 9, and 12 in Figure 1 probably originate in the metathoracic scent gland. Although it was not detected in the first airborne volatile extracts, 4-oxohex-2-enal was detected in the second male extract, but the dimers of 4-oxohex-2-enal (M^+, *m/z* 224) were not detected in either. Peaks corresponding to the three unidentified scent gland peaks were not detected in the airborne volatile extracts.

Male-Specific Volatiles: EI-MS Data. Peaks 11, 14, and a series of peaks from 17 to 24 were recorded uniquely in the male extract (Figure 1; EURMAL). The very minor peak 11 was identified as vanillin. Peaks 14, 17 (the major component), 18, 23, and 24 were initially assigned to unidentified sesquiterpenoids. Peaks 19–21 were assigned to unidentified isomeric aromatics of M^+ *m/z* 216. Peak 22 was a mixture (sesquiterpenoid and aromatic). The aromatics were not detected in subsequent male extracts and have therefore not been investigated further.

Peak 11 was identified as vanillin (4-hydroxy-3-methoxybenzaldehyde) from its EI-MS and by comparison of its GC retention time with authentic material

(Aldrich). MS: *m/z* 152 (M^+, 91%), 151 (100), 123 (18), 109 (22), 81 (40), 55 (36), 41 (32).

Peak 14 is a sesquiterpene. MS: *m/z* 204 (M^+, 36%), 189 (4), 175 (11), 161 (12), 148 (5), 147 (10), 134 (30), 133 (17), 121 (27), 119 (72), 109 (4), 107 (78), 93 (100), 79 (38), 69 (26), 67 (12), 55 (36), 41 (70).

The MS of peak 17, the major peak, was similar to that of peak 14, but the presence of 14 additional mass units suggested that it was a homosesquiterpenoid. MS: *m/z* 218 (M^+, 36%), 203 (2), 175 (1), 162 (14), 147 (14), 134 (41), 119 (88), 107 (77), 93 (100), 79 (42), 67 (12), 55 (68), 41 (53). High-resolution mass spectrometry of this peak confirmed the expected composition to be $C_{16}H_{26}$ (*m/z* found, 218.2042; *m/z* expected, 218.2034).

Peak 18 is a homosesquiterpene oxide. MS: *m/z* 232 (M^+, 9%), 200 (22), 185 (12), 175 (12), 157 (88), 149 (48), 135 (53), 121 (90), 107 (67), 93 (59), 79 (26), 69 (77), 55 (50), 43 (97), 41 (100). The molecular formula given by high-resolution mass spectrometry was $C_{16}H_{24}O$ (*m/z* found, 232.1838; *m/z* expected, 232.1827).

Peak 22 was not homogeneous but could be resolved into two components, the second an unidentified aromatic. The MS obtained by MS subtraction of this second component suggested a sesquiterpene of MW 234. Mass spectral similarities suggested that peaks 22, 23, and 24 are isomeric sesquiterpenoids.

Peak 23 is a sesquiterpenoid. MS: *m/z* 234 (M^+, 3%), 216 (33), 201 (5), 187 (7), 173 (1), 159 (43), 157 (28), 151 (16), 133 (100), 119 (30), 105 (70), 91 (60), 77 (22), 67 (15), 55 (68), 43 (45), 41 (61).

Peak 24 is a sesquiterpenoid. MS: *m/z* 234 (M^+, 11%), 216 (33), 201 (6), 187 (7), 173 (1), 159 (26), 157 (33), 151 (20), 133 (100), 119 (20), 105 (60), 91 (55), 77 (20), 65 (10), 55 (77), 43 (52), 41 (52).

Female Volatiles. Only two peaks were uniquely present in the female extract. As minor components, they might have been present but overlooked in the male extract. Peak 13 (Figure 1; EURFEM) was identified from its matching EI-MS as *n*-pentadecane. From its EI-MS, peak 16 was identified as *n*-hexadecane. Vanillin was searched for by single-ion monitoring but not found, in agreement with the finding of Ubik et al. (1975) that vanillin is a male-specific volatile.

EI-MS Data: Hydrogenated Male Sample. The EI-GC-MS trace obtained from a hydrogenated sample of male extract is shown in Figure 2 (EURMALH2). Peaks 1–7 in the sesquiterpenoid region supplied the following MS data (peak 8 was not examined):

Peak 1(H) MS: *m/z* 210 (M^+, 13%), 195 (1), 167 (1), 125 (23), 112 (14), 97 (87), 81 (39), 69 (45), 55 (100), and 41 (47), matching that of bisabolane [1-(1,5-dimethylhexyl)-4-methylcyclohexane].

Peak 2(H) MS showed only minor quantitative differences from that of peak 1, suggesting that this compound is a second diastereoisomer of bisabolane.

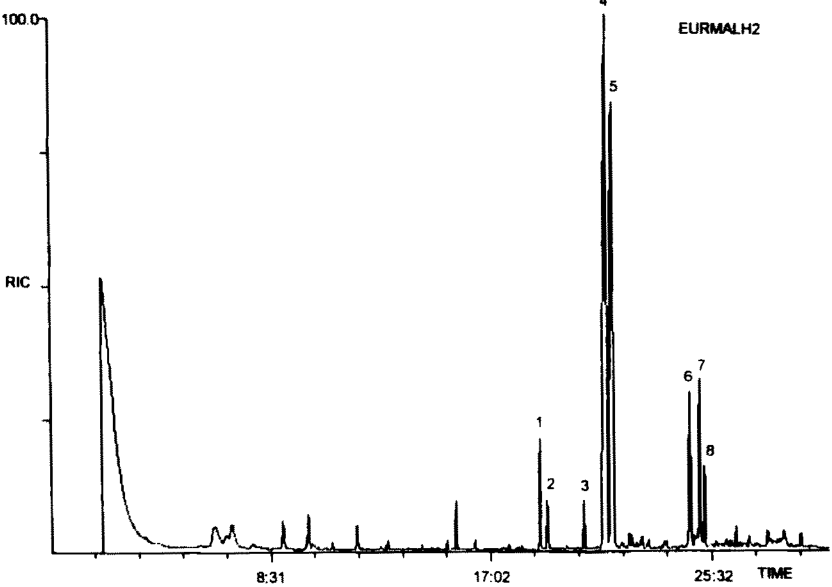

FIG. 2. Reconstituted total ion current trace, EURMALH2, *Eurygaster integriceps* hydrogenated male volatile extract; conditions as in Fig. 1.

Peak 3(H) MS: m/z 222 (M^+, 10%), 207 (<1), 137 (4), 124 (100), 109 (10), 95 (50), 81 (63), 67 (27), 55 (37), 41 (38). Possibly incompletely hydrogenated homosesquiterpene (MW 218 + 4H).

Peak 4(H) MS: m/z 224 (M^+, 18%), 209 (1), 167 (3), 125 (22), 111 (4), 97 (90), 81 (38), 69 (41), 55 (100), and 41 (48), suggesting that the homosesquiterpene (Figure 1, peak 17) contains three double bonds and one ring.

Peak 5(H) MS differed only slightly from that of peak 4, suggesting that peaks 4 and 5 are diastereoisomers.

Peak 6(H) MS: m/z 240 (M^+, 10%), 225 (12), 193 (3), 182 (9), 169 (3), 152 (3), 137 (9), 123 (12), 109 (9), 95 (23), 81 (21), 71 (100), 57 (41), 43 (60).

Peak 7(H) MS differed only slightly from peak 6, suggesting that peaks 6 and 7 are isomers.

Characterization of the Homosesquiterpenoid. The second airborne volatile extract obtained from male adults was found by GC-MS to contain one major peak (corresponding to peak 17 in Figure 1; EURMAL) and little else. On the basis of the MS data obtained, it was concluded that the major peak was almost completely homogenous. This sample was examined by NMR without further purification.

The sample, which was essentially pure according to the following spectra, showed the following data: δ_H 0.97 (3H, t, J 7.5), 1.69 (3H, br s), 1.65 (3H, br s), 1.59 (3H, br s), 1.96 (2H, q J, 7.5), 1.97–2.02 (2H, m), 2.03–2.07 (4H, m), 2.28–2.33 (2H, m), 2.72–2.76 (2H, br res.), 5.10 (1H, br t) and 5.33–5.36 (1H, br res.); δ_C 12.7 (CH_3), 16.0 (CH_3), 17.9 (CH_3), 23.5 (CH_3), 26.7 (CH_2), 27.0 (CH_2), 29.44 (CH_2), 31.7 (CH_2), 32.4 (CH_2), 34.5 (CH_2), 121.0 (CH), 122.7 (CH), 125.8 (C), 128.5 (C), 134.3 (C), and 137.0 (C). These data are consistent with a molecular formula of $C_{16}H_{26}$ (MW 218; four double-bond equivalents) determined by mass spectrometry.

Initial inspection of the ^1H NMR data clearly showed the presence of an isolated ethyl group, together with three methyl groups probably attached to double bonds (δ_H 1.6–1.7) and two olefinic protons (δ_H 5.10 and ~5.35), the former appearing as a broad triplet, characteristic of an acyclic isoprene group. The remaining methyl resonances were also indicative of a terpenoid structure. Especially diagnostic was the appearance of a broad singlet at δ_H ~ 2.74, characteristic of a double allylic methylene group in a γ-bisabolene **1** (Figure 3) (Wolinsky et al., 1976; Delay and Ohloff, 1979). ^1H–^1H COSY and ^1H–^{13}C correlation spectra, as well as the chemical shift data confirmed this assignment and also allowed the initial ^{13}C data to be assigned to the corresponding proton resonances. The initial assignment of structure was therefore a γ-bisabolene **1** having an additional methylene, present as part of an ethyl substituent, in place of one of the four methyl groups. The positioning of these substituents followed mainly from ^{13}C data. Firstly, ^1H–^1H COSY data indicated that the three-proton resonance at δ_H 1.65 was due to a methyl substituent on the cyclohexene ring. ^1H–^{13}C correlation spectra showed that this exhibited a resonance at δ_C 23.5 ppm, corresponding closely to previous literature assignments for similar structures (Delay and Ohloff, 1979; Pouchert and Behnke, 1993).

In addition, a well-established feature of ^{13}C spectra of acyclic terpenes **2** (Figure 4) is the appearance of resonances as shown for the two terminal methyl groups (Delay and Ohloff, 1979; Pouchert and Behnke, 1993). As the pheromone did not display a resonance at ~25.5 ppm in its ^{13}C data (the remaining three unassigned methyl resonances were all well below 20 ppm), it was deduced

1

FIG. 3. Structure of γ-bisabolene.

that the ethyl group was positioned at the terminal (*E*) methyl site in substructure **2**, resulting in a structural assignment shown in Figure 5.

Following this, it was then possible to assign the ^{13}C data as shown in Figure 6. These assignments are closely similar to those made for related homoterpenoid structures and fully support the stereochemistry shown (Freeman et al., 1988; Vaidya and Broger, 1984).

The stereochemistry was confirmed using NOE difference spectroscopy, the results of which are shown in Figure 7. These data also provide further support for the other structural features of this molecule, which we deduce to be (4*Z*,4′*E*)-4-(1,5′-dimethyl-4′-heptenylidene)-1-methylcyclohexene or, more simply, a homo-γ-bisabolene.

In the light of these data, the minor sesquiterpene (Figure 1, peak 14) is probably γ-bisabolene. Retention tests carried out with authentic, synthetic

~17.5

~25.5

2

FIG. 4. Structure of acyclic terpenes.

^{1}H NMR data

FIG. 5. ^{1}H NMR data.

^{13}C NMR data

[Quaternaries at δ_C 125.8; 128.5 (?); 134.3; 137.0]

FIG. 6. ^{13}C NMR data.

FIG. 7. NOE data.

standards ruled out α- and β-bisabolene, but at the time the work was carried out γ-bisabolene was not available for retention tests.

DISCUSSION

It is the conclusion of this work that monocyclic sesquiterpenoids are major components of the male scent of *E. integriceps*. Previously reported vanillin (Ubik et al., 1975) was also detected but only as a minor component of the male scent. Ethyl acrylate (Vrkoc et al., 1977) was not detected, although not specifically looked for in the male scent.

The sesquiterpenoids probably originate as components of the secretion from the abundant integumentary glands present in the sternites and laterotergites uniquely in male *E. integriceps* adults (Staddon and Edmunds, 1991). The major component of the male scent is a homosesquiterpenoid (MW 218). Minor components include other sesquiterpenoids, among them a bisabolene.

Sesquiterpenoids have been identified as major components of male-produced olfactory pheromones in several Heteroptera-Pentatomidae. Males of *Nezara* and *Acrosternum* spp. produce (*Z*)-α-bisabolene (MW 204) [(1′*Z*)-4-(1′,5′-dimethyl-1′,4′-hexadien-1-yl)-1-methylcyclohexene] and the related 1,2-epoxides (MW 220) in distinctive ratios (Baker et al., 1987; Marron and Nicolaou, 1989; Aldrich et al., 1987, 1989, 1993). In the case of *N. viridula*, the relative abundance of the *cis*-epoxide isomer released by males was found to vary according to geographic origin (Baker et al., 1987; Aldrich et al., 1989, 1993). Male pentatomid pheromones function in nature both as intraspecific attractants and as attractants for tachinid parasitoids (Aldrich et al., 1987).

From laboratory tests, Vrkoc et al. (1977) found that both vanillin and ethyl acrylate induce specific behavioral responses in sexually receptive females. Thus, both components may act as short distance attractants. They further showed that the stimulatory activity of a mixture (1 : 1) was greater than the activities of the compounds tested separately. Neither of the two compounds evoked

specific behavioral responses in the active males. It is now necessary to extend these tests to include the sesquiterpenoids and to examine the possibility that the male produced sesquiterpenoids function as long-range attractants.

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EFFECT OF TEMPERATURE ON BIOSYNTHESIS OF SEX PHEROMONE COMPONENTS IN POTATO TUBERWORM MOTH, *Phthorimaea operculella* (LEPIDOPTERA: GELECHIIDAE)

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Abstract—Rearing temperature modified the sex pheromone component ratio in the potato tuberworm moth, *Phthorimaea operculella*. This phenomenon seemed to be induced with ambient temperature by differences in timing and speed of biosynthesis between two pheromone components. (*E,Z,Z*)-4,7,10-Tridecatrienyl acetate (triene) was mainly synthesized during the pupal period, while most of (*E,Z*)-4,7-tridecadienyl acetate (diene) was synthesized during a short period just after emergence. Therefore, the ratio of triene in a newly emerging adult was relatively high at all temperatures although the amount of triene was relatively low at 35°C. On the other hand, the synthetic rate of accumulation of diene was clearly modified by ambient temperature. Biosynthesis of diene at 15°C was very low in the first two days and high in the third day. Consequently, a titer of the diene component at 15°C became approximately equivalent to that at 25°C one day later.

Key Words—Sex pheromone, pheromone components ratio, (*E,Z,Z*)-4,7,10-tridecatrienyl acetate, (*E,Z*)-4,7-tridecadienyl acetate, rearing temperature, Lepidoptera, Gelechiidae, potato tuberworm moth, *Phthorimaea operculella*.

INTRODUCTION

The pheromone emission of moths is influenced by many environmental conditions, especially temperature. McNeil (1991) has reviewed the factors affecting emission and reception of pheromone. Several studies have shown that temperature conditions affect calling periodicity (e.g., Cardé et al., 1975), gland extru-

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sion during calling (Conner et al., 1985), or the age at which calling is initiated (e.g., Delisle and McNeil, 1987).

The sex pheromone of *P. operculella* is a blend of (*E*,*Z*,*Z*)-4,7,10-tridecatrienyl acetate (triene) and (*E*,*Z*)-4,7-tridecadienyl acetate (diene) (Persoons et al., 1976). Ono (1993) showed that the ratio of the two pheromone components was modified by the rearing temperature, although this ratio also varies considerably among individuals (Ono et al., 1990).

In a previous paper, it was suggested that the critical period of the temperature sensitivity was in the pupal stage, because the ratio differed in females held at different temperatures after pupation (Ono, 1993). In that study, there was also a possibility that the temperature after adult emergence affected the pheromone component ratio. Ono (1993), however, considered that possibility, because even newly emerged adult females had considerable amounts (50–70% of maximal amount) of pheromone (Ono et al., 1990). A tentative conclusion was that females synthesize pheromone of the same component ratio during their pupal stage as well as after they emerge as adults (Ono, 1993), but this supposition has not yet been examined experimentally. It is possible that the component ratio in a moth sex pheromone may be affected by the timing or the speed of the biosynthesis of each component, because the enzymes involved may have different temperatures at which they function optimally.

The present study was conducted to clarify the details of the sensitive period during which biosynthesis of the two pheromone components is affected by temperature and to examine the effect of ambient temperature on the course of pheromone biosynthesis in *P. operculella*.

METHODS AND MATERIALS

Insect rearing procedures and methods for the GLC analysis have been described previously (Ono et al., 1990; Ono, 1993). The culture had been reared on potatoes in a rearing room (25°C and 14L:10D regime) over 90 generations. Rearing at different temperatures was conducted in a rearing chamber. There were five chambers set at different temperatures (at 5°C intervals between 15°C and 35°C with ±0.5°C fluctuation). The light and humidity conditions were not controlled. The light–dark cycle was the same as the laboratory condition (about 13–15 hr light). The pupae collected from each temperature were kept in the same chamber until gland extraction in the adult stage.

Glandular pheromone content of individual females was analyzed by GLC. The glands were extracted by 50 μl hexane, and 5 ng (*E*)-11-tetradecenyl acetate was added as an internal standard. Analyses were performed on Shimadzu GC-15A GLC with SP-2340 fused silica capillary column (30 m × 0.25 mm ID; 0.20-μm film thickness; Supelco Inc.). The initial column temperature was 80°C, then increased 15°C/min to a final temperature of 160°C with a 10-min hold.

Further details on the experimental procedure will be described in each section.

RESULTS

Females synthesize some of their sex pheromone components during the pupal stage (Ono, 1993), but it is unclear whether pheromone component ratios are affected by temperature after adult emergence. Figure 1 shows that temperature conditions during one day (24–40 hr) after adult emergence affect the pheromone component ratio, even though the temperature before emergence was the same (25°C) for each group of moths. The component ratios in each condition were very similar to those of females reared under the corresponding temperature conditions after pupation (Ono, 1993), although no statistical significance was shown between 15 and 25°C (Duncan's multiple-range test).

In contrast, temperature changes occurring only during the pupal period did not induce a difference in component ratio (Figure 2). All the female adults that were brought back to 25°C for one day after emergence contained similar pheromone component ratios (no significant difference, Duncan's multiple-range test), even though they had been reared under different temperature conditions during pupal stage.

These two results show that my previous supposition (Ono, 1993) is incor-

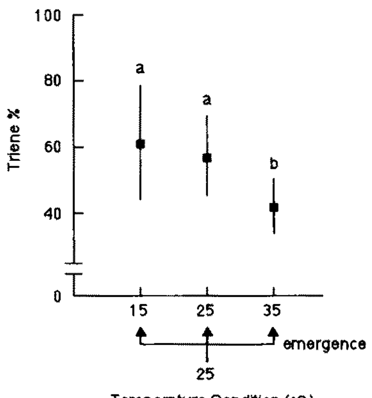

FIG. 1. Pheromone component ratio of females, when the ambient temperature was changed after emergence. See text for details of rearing conditions. The solid squares show mean values and bars show SD ($N = 28$ female glands in each condition). Bars with the same letters are not significantly different ($P < 0.05$, Duncan's multiple-range test).

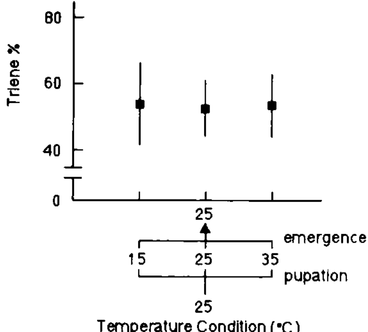

FIG. 2. Pheromone component ratio of females, when the rearing temperature was changed at pupation and brought back to 25°C after emergence. See text for details on rearing conditions. The solid squares show mean values and bars show SD ($N = 27$ in each condition). No significant differences were observed among each condition.

rect and that the critical sensitive period of pheromone biosynthesis to temperature conditions is not in the pupal stage, but in the adult stage. However, if so, how is it that newly emerged females can biosynthesize considerable amounts of pheromone as evidenced by gland extracts? To answer this question, the timing of biosynthesis of each component was examined in the next two experiments.

The insects were reared at 25°C until pupation. The pupae were separated into three groups and kept in three different temperature conditions, 15, 25, and 35°C. These pupae were allowed to emerge as adults under each temperature condition. The pheromone contents were examined from four groups of about 15 females of different age; 1, 15, 36, and 60 hr old, in each temperature condition. The 36- and 60-hr old females correspond to those classed as two and three days after emergence, respectively, in the earlier data. All the extractions were made during daytime (1000–1500 hr) to minimize the effect of daily rhythm in pheromone content (Ono et al., 1990), because *P. operculella* has no clear periodicity of emergence.

The results are summarized in Figure 3. Under all conditions, the percentage of diene was relatively low just after emergence, although the amounts of total pheromone, comprised mainly of the triene component, were different in each condition. The total amount of pheromone (or triene component) in the 25 and 15°C regimen, was higher than that in the 35°C regimen ($P < 0.01$ and $0.01 < P < 0.05$, respectively, t test). The amount of triene component did not change appreciably after adult emergence. However, the increase in the amount of diene was gradual in 35°C but rapid in 25°C up to 36 hr. In contrast, the diene component increased sharply during 36–60 hr at 15°C, and the total

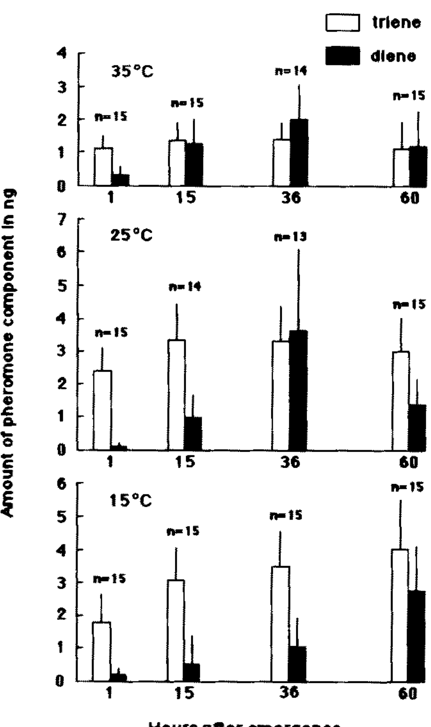

FIG. 3. The sequential changes in amount of pheromone from emergence to 60 hr after emergence under different temperature conditions. The females were kept at each temperature condition after pupation and also as adults. The histograms and bars show the mean amount ± SD.

amount of pheromone at 60 hr at 15°C was approximately equivalent to the total amount at 36 hr at 25°C ($P = 0.851$, t test).

In the experiment shown in Figure 3, the pupae were kept under different temperature conditions during the pupal period as well as during adulthood. There is a possibility that the temperature condition during the pupal period also modified pheromone biosynthesis after adult emergence. Actually, the quantities of pheromone just after emergence are different between the 35°C and 25°C conditions ($P < 0.01$, t test), which may suggest that the temperature condition during the pupal stage also affects the biosynthesis after emergence ($P = 0.057$ between 35°C and 15°C, and $P = 0.092$ between 25°C and 15°C, t test).

To further determine the effect of temperature on biosynthesis after adult emergence, the next experiments were conducted. All the females were kept at

25°C until adult emergence, when they were transferred to the different temperature conditions (15, 25, and 35°C). The amounts of each pheromone component were measured at 36 and 60 hr after emergence (Figure 4). The value of 1–3 hr is a control for all three temperature conditions, and the same data are therefore plotted three times in this figure. Again, the percentage of diene in total pheromone was relatively low at 1–3 hr. Similar to Figure 3, the diene component increased rapidly after emergence at 35 and 25°C, although the triene amount decreased gradually in this case. In addition, the diene component clearly increased during 36–60 hr after emergence at 15°C, and the total amount of pheromone at 60 hr at 15°C was approximately equivalent to the total amount at 36 hr at 25°C ($P = 0.964$, t test).

DISCUSSION

The progression of changes in pheromone titer in female gland extracts related to pheromone biosynthesis appears to differ among species. The pharate adults of *Holomelina lamae* have about the same amount of sex pheromone as adults (Schal et al., 1987), while in other species, such as *Chilo suppressalis*, pheromone titer increases after adult emergence, indicating more active synthesis during adulthood (Usui et al., 1988). A daily rhythm of pheromone biosynthesis is also known in some species [*Helicoverpa zea* (Raina et al., 1986); *Adoxophyes* sp. (Kou, 1992)]. These differences suggest that the timing of pheromone biosynthesis is influenced by calling periodicity.

In this study, females of *P. operculella* synthesized their sex pheromone before and after adult emergence. The biosynthesis of the triene component was mostly completed before adult emergence and was affected by temperature conditions, whereas the diene component accumulated mainly during 0–2 days after emergence. The diene synthesis was clearly affected by the ambient temperature. The speed of biosynthesis of this component was slow at the lower temperature (15°C) and the synthesis continued until three days after adult emergence. This is the main reason that the pheromone component ratio of triene at the lower temperature was very high (Ono, 1993). The titer of the diene component at the lower temperature, however, reached the same level as that of higher temperature conditions one day later, because the diene component was synthesized continuously until 60 hr after emergence.

Activity of triene synthesis was relatively low ($P < 0.01$ from 25°C, t test), and diene was synthesized faster at 35°C, while triene synthesis was rather high ($P = 0.050$ from 25°C, t test) but diene was synthesized very slowly at 15°C. There is not, therefore, a large difference in the total amount of pheromone at 36 hr between lower (15°C) and higher temperature (35°C) conditions (although the difference is significant; $P = 0.042$, t test), and this is why the

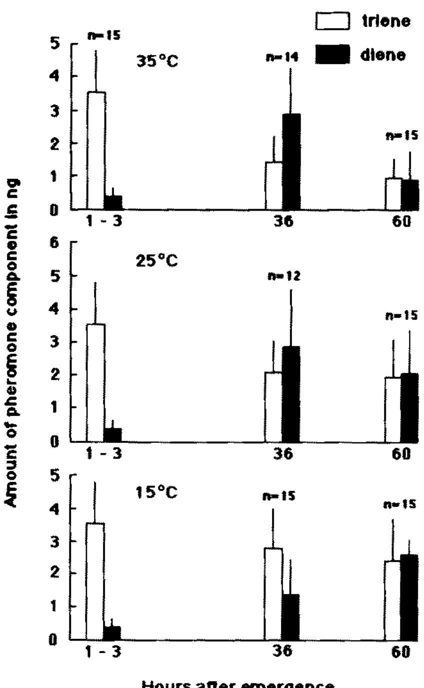

FIG. 4. The sequential changes in amount of pheromone from emergence to 60 hr after emergence under different temperature conditions. The females were moved to each temperature condition after emergence. The histograms and bars show the mean amount ± SD.

difference of pheromone amount between these two conditions was small in the previous paper (Ono, 1993). However, the total amount at 25°C was very high compared with the other two conditions due to higher triene synthesis during the pupal stage and quick synthesis of diene after emergence.

The amount of pheromone content in the gland may also be influenced by pheromone emission during calling. In the course of these experiments (Figures 3 and 4), females passed the scotophase at least twice in the 60-hr condition. Therefore, the decrease in the total amount for the 25 and 35°C conditions may be caused by the release of pheromone during calling. On the other hand, pheromone might not be released in the 15°C condition. Ono et al. (1972) have reported that the copulatory activities of *P. operculella* were quite low in cool temperatures and that no copulations were observed. Calling behavior was not checked in the course of these experiments. Furthermore, from the results shown here, it is not clear whether calling behavior influences pheromone biosynthesis.

The relationship between the process of pheromone synthesis and the timing of calling behavior also raises the possibility that the actual component ratio of the pheromone emitted may be almost the same in both higher and lower temperature conditions, if the female calls and releases the pheromone one day later at the lower temperature condition. Analysis of airborne sex pheromone emitted from females in each temperature condition is needed to clarify this.

The differences in the timing of pheromone synthesis seem to reflect the enzyme activity related to the biosynthesis of each component, because enzyme activity is usually sensitive to temperature conditions. This implication may agree with the idea that diene and triene components are synthesized from different precursors, as proposed by Roelofs and Bjostad (1984).

The temperature condition modifies the component ratio of the sex pheromone easily in this species, as shown above. These situations are quite different from tortricid species, in which the component ratio is rigidly controlled (Roelofs an Brown, 1982). Other lepidopterous species may show a similar controlled ratio, because the pheromone blends emitted by females usually have relatively small variability. Mechanisms controlling the pheromone component ratio that are independent of the ambient temperature may be involved in these species.

Then, why have similar mechanisms not evolved in *P. operculella*? Possible reasons include biosynthetic limitations, characteristics of pheromone component chemicals, the relationship with sympatric species, etc. (Ono et al., 1990).

There is a possibility that a genetically "unfixed" trait, such as pheromone component ratio in *P. operculella*, which is affected by some environmental conditions, may gradually be fixed genetically under selection pressure in the evolutionary process. The modification of pheromone component ratio by ambient temperature in *P. operculella* may change to a genetically fixed trait in the process of evolution. Male responsiveness to the sex pheromone is usually related genetically to the pheromone production mechanism (Klun and Maini, 1979). Therefore, if a change in sex pheromone blend is induced genetically in the females, the males' responsiveness to the blend will be tuned with the conspecific females' blend at the same time. These processes may, therefore, induce the evolutionary change of a pheromone's component ratio through the process of reproductive isolation by means of the pheromonal communication system.

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Note

INTEGUMENTAL SLIME AND WAX SECRETION: DEFENSIVE ADAPTATIONS OF SAWFLY LARVAE[1]

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Abstract—Evidence is presented from predation tests with ants (*Formica exsectoides*) that the slimy coating of *Caliroa cerasi* and the waxy investiture of *Eriocampa ovata* serve in defense.

Key Words—Hymenoptera, Symphyta, Tenthredinidae, predation, chemical defense, ants, Formicidae.

INTRODUCTION

Sawfly larvae (Hymenoptera: Symphyta) have evolved a multiplicity of chemical defenses. Cimbicid larvae (fam. Cimbicidae), when disturbed, eject a secretory spray from glands opening above the spiracles. Nematine larvae (fam. Tenthredinidae, subfam. Nematinae) derive protection from eversible glands, positioned midventrally along the abdominal segments. Other larvae, of the families Diprionidae and Pergidae, which live, respectively, on conifers and eucalypts, shunt the resins of their hosts into diverticulae of the foregut when feeding and

[1] Paper no. 125 of the series Defense Mechanisms of Arthropods. No. 124 is T. Eisner et al., *Experientia* 50:610–615 (1994).

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disgorge the fluids orally when attacked. A recent treatise on symphytan biology (Wagner and Raffa, 1993) includes a comprehensive review of sawfly defenses (Codella and Raffa, 1993).

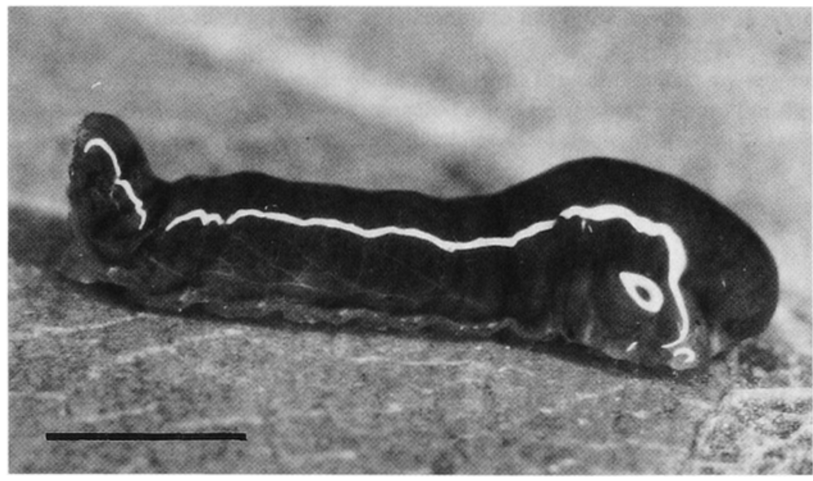
I here report some simple observations with ants that indicate that two other adaptations of sawfly larvae, integumental secretion of slime and of wax, are also protective. My observations were on two species, *Caliroa cerasi* (fam. Tenthredinidae, subfam. Heterarthrinae), a so-called "slug" sawfly, whose body is typically coated with slime (Figure 1, top), and *Eriocampa ovata* (fam. Tenthredinidae, subfam. Allantinae), which bears a fluffy covering of wax (Figure 1, bottom). It had been suggested that these investitures, which are also present in related species, might serve for defense (Codella and Raffa, 1993), but evidence to that effect was lacking.

METHODS AND RESULTS

Tests with C. cerasi.

The larvae (nearly full grown) were located on a cherry tree (*Prunus cerasus*) in Ithaca, New York, and transported on leaves to the laboratory, where they were gently coaxed into Petri dishes, within which had been placed a number (three–eight) of ants (*Formica exsectoides*) from a laboratory colony. Five separate dishes were prepared, each with a single larva. The results were consistent with four of the dishes. Individual ants, upon contacting a larva, turned away without either biting or ejecting their spray (they were not seen to bend their gaster forward, as they typically do when spraying), and then engaged in cleansing activities. The deterrence was prompt, and the preening behavior stereotyped: the ants wiped antennae with forelegs, and cleansed legs by mouthing them or by wiping them against one another (Figure 2A). The contamination with slime following such brief encounters was minimal, and none of the ants reacted by scurrying about erratically as if contacted by an irritant. All eventually recovered.

With the fifth dish, the ants were brought to a state of agitation by prodding them repeatedly with a metal probe, causing them to scurry about with their mandibles agape as they typically do when directly disturbed. Two such ants came upon the larva and clamped down with the mandibles to bite, but they too were quick to back away and engage in cleaning. One ant, visibly encumbered with slime, contaminated another that it touched, causing the latter to commence cleansing itself. The larva was injured, induced to bleed, and eventually killed by further assault. Inspection at close range revealed that it gave off a strong odor of formic acid, indicating that it had been sprayed.



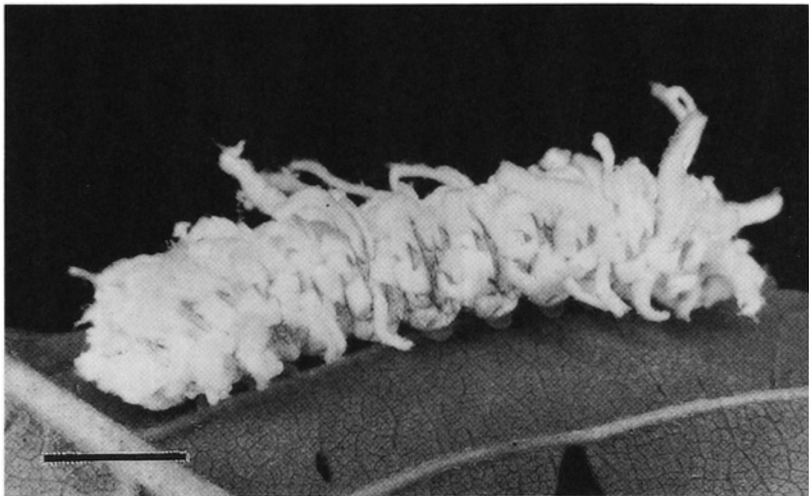


FIG. 1. Top: larva of *Caliroa cerasi*; bottom: larva of *Eriocampa ovata*. Bars = 5 mm.

Tests with E. ovata.

Larvae (nearly full grown) were located on a stand of alder (*Alnus rugosa*) by a pond near Rensselaerville, New York. They were taken on leaves and transported to a site beside a natural mound of *Formica exsectoides*. Ten larvae, each still clinging to its leaf, were presented to the ants, one at a time, on the

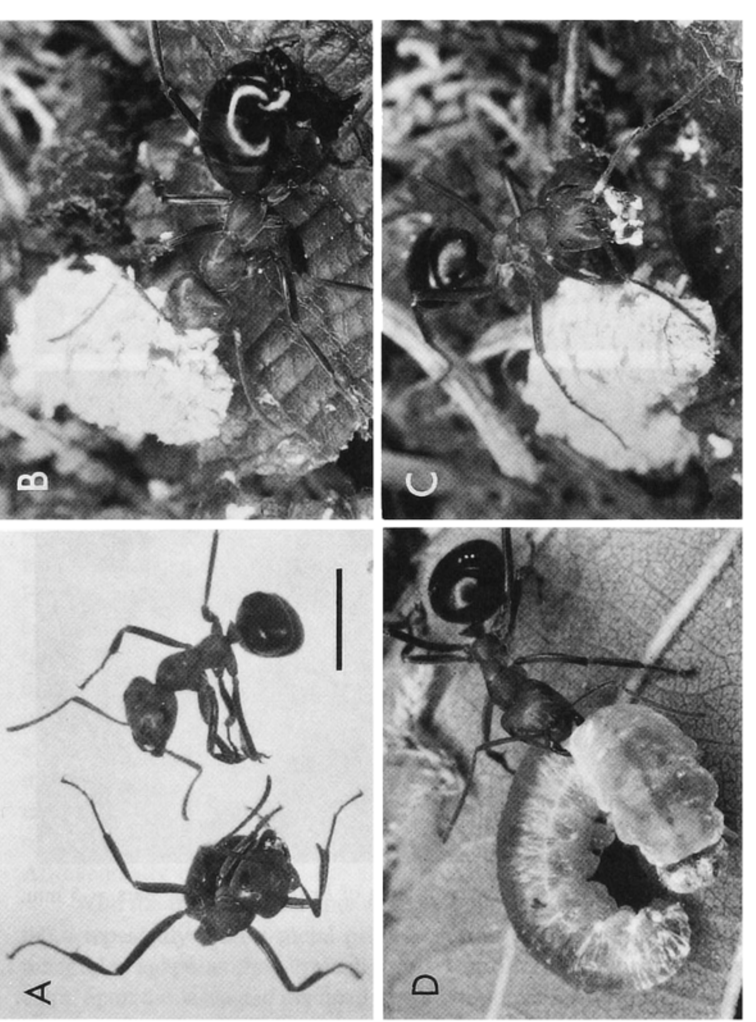


FIG. 2. (A) *Formica exsectoides*, preening, following contact with a *C. cerasi* larva; (B) *F. exsectoides*, biting into waxy investiture of *E. ovata* larva and, moments later (C), turning away from the larva, its mouthparts now contaminated with wax; (D) *F. exsectoides*, biting into an *E. ovata* larva that had been experimentally divested of its coating; the larva was eventually carried away by the ant. Bar = 5 mm.

ground next to the mound. Care was taken not to touch the larvae, so as not to impair their coating.

To induce attack, ants were caused to swarm from the colony by tapping the mound. Individual ants that encountered a larva attempted to bite, but they seemed in most cases to grasp wax only and appeared never to attempt to spray (they were not noted to flex the abdomen). They usually desisted promptly from the assault and, with their mouthparts visibly laden with wax (Figure 2B, C), sped away. In some cases, the larva curled its abdomen when attacked, in such fashion as to wipe its flank against the ant. In such instances, the ants became more thoroughly contaminated with wax.

The results were different with another five larvae that were wiped clean of wax with a brush before presentation to the ants. The ants grasped these larvae in the mandibles (Figure 2D) and carried them off, piercing some in the course of the attack, and causing them to bleed.

DISCUSSION

Both the slime of *Caliroa* and the wax of *Eriocampa* can evidently provide protection against ants. The results were clear-cut with *Eriocampa*, but convincing also with *Caliroa*. As I know from experience, *F. exsectoides*, when confined in small dishes with soft prey, usually bite and kill their offering. Their having spared *Caliroa* in most cases suggests that these larvae were unacceptable to the ants.

Both types of coating appear to act purely as mechanical deterrents. While there is no reason to suspect either coating to contain chemical irritants or repellents, presence of such components cannot be definitively ruled out for *Caliroa*, given the absence of analytical data. The coating of *Eriocampa*, however, has been examined chemically and appears to consist exclusively of long-chain (20–32 carbons) primary alcohols (Percy et al., 1983), substances likely to be virtually inert. There can be no question that ants are potentially important enemies of *Caliroa* and *Eriocampa*, as they are probably of sawfly larvae generally (Codella and Raffa, 1993).

Slime production for defense has evolved in Onychophora, as well as in a number of arthropods, including lithobiid and geophilid centipedes, glomerid millipedes, cockroaches, and larvae of the lepidopteran family Dalceridae (Epstein et al., 1994; Jones et al., 1976; Plattner et al., 1972; older references in Eisner, 1970). The slime in these animals may be produced as a topical coating or may be discharged as an ooze or spray from special glands, and it may or may not contain active chemical deterrents, in addition to the proteinaceous materials that form the basis of the slime. Predators, including ants, have been shown in some cases to be thwarted by these secretions, which may act as entangling agents.

Coatings of wax occur also in other arthropods, notably in Homoptera (Weber, 1930), where they may also serve primarily for defense. Wax may even be secondarily appropriated by insects that do not themselves produce it. The larva of the green lacewing, *Chrysopa slossonae*, feeds on woolly aphids, *Prociphilus tesselatus*, and covers itself with tufts of wax that it plucks from its prey. Ants that bite into this acquired investiture are deterred (Eisner et al., 1978), just as were those that attacked *Eriocampa*.

Waxy coatings can be classed as "detachable integumental outgrowths" (Eisner, 1970), a category of defensive materials that includes, among others, the hastisetae of dermestid beetles (Nutting and Spangler, 1969), and the scales of moths. Moths, as a consequence of the detachability of their scales, tend to elude orb-weaving spiders by not sticking to their webs (Eisner et al., 1964). Mosquitoes, which also have scales, and caddis flies, which are coated with detachable hairs, may similarly avoid capture. Coatings of wax could themselves shield against adherence to viscid threads, and one could easily envision the alates of certain aphids, coccids, and whiteflies, which bear such coatings, to be relatively invulnerable to entrapment in webs.

The defensive production of wax, and for that matter of slime, must entail metabolic costs. While such costs have not been experimentally assessed, evidence from one species indicates that it must be real: nymphs of the flatid *Ormenaria rufifascia*, which are coated with wax and surround themselves with a defensive waxy pad, cut down sharply on wax production when they are infested with ectoparasitic mites (LaMunyon and Eisner, 1990).

The entire topic of the arthropod integument, and of its adaptive modification in response to special defensive demands, is worthy of increased attention.

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ALARM RESPONSE BY A PLETHODONTID SALAMANDER (*Desmognathus ochrophaeus*): CONSPECIFIC AND HETEROSPECIFIC "SCHRECKSTOFF"

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Abstract—The detection of chemical alarm cues plays an important role for predator avoidance in many taxonomic groups, but little is known about the presence of such chemical cues in adult or caudate amphibians. We investigated the response (i.e., aversion or nonaversion) to chemical cues from damaged salamander skin and mealworms (*Tenebrio molitor*) in the plethodontid salamander, *Desmognathus ochrophaeus*. Avoidance responses were demonstrated to skin extracts of both conspecific and heterospecific salamanders. However, salamanders (*D. ochrophaeus*) did not avoid heated conspecific skin, fresh conspecific viscera, fresh mealworm, or fresh *Plethodon richmondi* skin extracts. These results indicate that chemical alarm cues are: (1) present in the skin of *Desmognathus* salamanders, (2) not present in mealworm or the viscera of *Desmognathus* salamanders, and (3) denatured or deactivated by heating. These results also suggest that an avoidance response to chemical cues from damaged conspecifics has adaptive value in predator avoidance in terrestrial as well as aquatic vertebrates.

Key Words—Alarm response, chemoreception, chemical cues, Schreckstoff, skin extract, predator avoidance, plethodontid salamanders, *Desmognathus ochrophaeus*, *Desmognathus brimleyorum*, *Plethodon richmondi*, *Notophthalmus viridescens*.

INTRODUCTION

The ability to detect predators by chemoreception is often important in successful predator avoidance (e.g., Cooper, 1990). Avoidance of predator odors may

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enhance the survivorship of prey by simply reducing the probability that they will occupy the foraging areas of potential predators (Brodie et al., 1991). Many researchers have investigated the use of chemical cues by animals in predator detection (e.g., Cooper, 1990; Keefe, 1992; Petranka et al., 1987; Rehnberg and Schreck, 1987; Sih and Kats, 1991; Thoen et al., 1986). The ability to detect predators with chemical cues appears to be taxonomically broad (Weldon, 1983, 1990).

Aquatic organisms (i.e., invertebrates, fishes, and larval anurans) may indirectly detect predators through chemical alarm signals, alarm pheromones, or Schreckstoff [a term originally used in reference to ostariophysan fishes (Smith, 1992)] released from injured conspecifics (e.g., Hews and Blaustein, 1985; Lawrence, 1991; Parker and Shulman, 1986; Sleeper et al., 1980; Smith, 1992; Wilson and Lefcort, 1993). Hews (1988) demonstrated an alarm response in tadpoles (*Bufo boreas*) to alarm substance released from injured conspecifics. Similar to chemical detection of predators, the detection and avoidance of alarm pheromones (released from damaged skin) may also increase survivorship through predator avoidance. The detection of chemical alarm cues in fathead minnows (*Pimephales promelas*) increases survival during encounters with predatory northern pike (*Esox lucius*) (Mathis and Smith, 1993).

Although alarm responses to damaged skin occur in aquatic vertebrates, such as some species of larval anurans (Kulzer, 1954; Pfeiffer, 1966), little is known about the possible presence of alarm responses in terrestrial vertebrates. Adult semiaquatic newts (*Cynops pyrrhogaster* and *Notophthalmus viridescens*) detect and avoid chemical cues released from damaged newt skin (Marvin and Hutchison, in press). Such findings suggest the presence and use of chemical alarm signals by some species of adult salamanders in predator avoidance.

We conducted experiments to investigate the possible presence and use of chemical alarm signals in a plethodontid salamander (*Desmognathus ochrophaeus*). This investigation provides the first evidence of a conspecific and heterospecific alarm response and chemical cue avoidance in a terrestrial plethodontid salamander.

METHODS AND MATERIALS

We collected adult *Desmognathus ochrophaeus*, *Plethodon richmondi*, and *Notophthalmus viridescens* during August 1993 in Harlan County, Kentucky and collected adult *Desmognathus brimleyorum* during April 1993 from LeFlore County, Oklahoma. Salamanders were maintained and fed a diet of *Drosophila* and mealworms (*Tenebrio molitor*). We did not feed salamanders for approximately 10 days prior to experimentation and maintained them at 15°C under a 14L:10D photoperiod.

We tested the response (aversion or nonaversion) of individual *D. ochrophaeus* to conspecific viscera extract, fresh skin extract, and a heated skin extract. We also tested the response of *D. ochrophaeus* to skin extracts of other plethodontid salamanders (*D. brimleyorum* and *P. richmondi*), the red-spotted newt (*N. viridescens*), and mealworms (*T. molitor*). Extracts (stimulus solutions) were prepared by homogenizing 1.0 g of macerated tissue in 10.0 ml of distilled water. Heated extracts were prepared by placing the beaker containing the extract in a boiling water bath for 15 min.

We conducted two-choice experiments by placing individual salamanders ($N = 20$) in glass Petri dishes (150 mm diam. × 20 mm deep). We lined one half of each Petri dish with filter paper saturated with 1.0 ml of distilled water (control, nonstimulus side) and the other half with filter paper saturated with 1.0 ml extract solution (stimulus side). The two filter paper substrates in each dish were placed approximately 3 mm apart to minimize diffusion of the extract to the nonstimulus side.

All experiments were conducted during February 1994 in the animals' acclimated scotophase, under constant temperature (20°C) and dim illumination (40-W incandescent white-light bulb, 2 m from dishes). We arbitrarily chose the order in which extracts were presented to test animals as listed in Table 1. Individuals were used in several experiments, but no animal was tested twice against the same extract and at least two days were allowed between experiments. We recorded the response to left or right position (in the Petri dishes) of each salamander every 5 min for 4 hr. If salamanders were positioned across

TABLE 1. RESPONSE BY *D. ochrophaeus* TO SALAMANDER TISSUE EXTRACTS AND MEALWORM EXTRACT

Extract[a]	N (N^1)[b]	Responses to extract (Mean ± SE)	Responses to control (Mean ± SE)	Wilcoxon T[c]
DO_s	20 (20)	5.80 ± 1.8	17.00 ± 1.5	27**
DO_v	20 (20)	10.45 ± 1.9	12.65 ± 1.5	87NS
DO_{hs}	20 (20)	11.95 ± 2.0	13.50 ± 1.7	102NS
DB_s	20 (19)	8.90 ± 1.8	16.15 ± 1.3	36*
MW	20 (20)	9.25 ± 1.8	14.90 ± 1.3	54NS
PR_s	20 (20)	9.30 ± 2.2	14.65 ± 2.0	73NS
NV_s	20 (20)	7.75 ± 2.2	16.85 ± 2.0	50*

[a] DO_s = fresh *D. ochrophaeus* skin, DO_v = fresh *D. ochrophaeus* viscera, DO_{hs} = heated *D. ochrophaeus* skin, DB_s = fresh *D. brimleyorum* skin, MW = fresh mealworm (whole), PR_s = fresh *P. richmondi* skin, NV_s = fresh *N. viridescens* skin.

[b] N = number of individuals tested, N^1 = sample size after deleting ties.

[c] $**P < 0.01$; $*P < 0.05$; NS, $P > 0.05$.

both the left and right side, the position of the salamanders' snouts was used to determine response to the control or extract side.

Aversion or nonaversion was determined by comparing the total number of responses that each animal made toward the nonstimulus (control) side during the first and third hour to the total number of responses toward the stimulus (extract) side during the second and fourth hour of the 4-hr observation period. We allowed animals to acclimate to the Petri dishes for approximately 30 min prior to recording observations. Petri dishes were stacked with cardboard dividers between them to prevent visual contact between individuals. To control for possible orientation to a left or right direction, we rotated the Petri dishes 180° every hour. Possible position biases were also investigated by comparing the total number of responses by animals toward the left side during the first and third hour to the total number of responses toward the right side during the second and fourth hour.

A two-tailed Wilcoxon signed-ranks test (Sokal and Rohlf, 1981) was used for the statistical comparisons. We also used a Friedman repeated-measures ANOVA on ranks (Sigmastat, Jandel Scientific Software Corporation) to test for possible changes in response to extract over time. The numbers of responses to extract-saturated substrates were compared for each hour over the 4-hr testing period.

RESULTS

Individual *D. ochrophaeus* demonstrated avoidance to substrates saturated with fresh conspecific skin extract ($P < 0.01$). However, there was no avoidance response to either heated skin extract or viscera extract of conspecifics ($P > 0.05$). Tests of *D. ochrophaeus* with fresh skin extracts of *D. brimleyorum* and *N. viridescens* showed a significant avoidance. However, individuals did not show a significant avoidance response to *P. richmondi* skin and mealworm (*T. molitor*) extracts (Table 1).

No significant position biases were found for either the left or right side of experimental containers during the 4-hr testing period ($P > 0.10$). A Friedman repeated-measures ANOVA on ranks indicated no significant changes in activity during the testing periods; these results show that there was no time bias (i.e., results do not merely reflect avoidance responses limited to the first hour and randomly thereafter). However, during the mealworm extract experiment, the number of responses to the extract side significantly differed in the second hour ($\bar{X} = 2.45$, SE $= 0.993$, $N = 20$) from the number of responses in the third hour ($\bar{X} = 5.25$, SE $= 0.978$, $N = 20$) ($P = 0.03$). Because the numbers of observations in the third and fourth hour do not significantly differ from the first, we can conclude that no habituation to the mealworm extract occurred.

DISCUSSION

The plethodontid salamander, *D. ochrophaeus*, demonstrates an avoidance response to chemical cues released from damaged skin of conspecifics and congenerics (Figure 1). This study is the second to implicate an alarm response in an adult amphibian (Marvin and Hutchison, in press) and the first to report such chemosensory and related avoidance behavior in a terrestrial plethodontid salamander. *D. ochrophaeus* avoids areas containing odors from the damaged skin of both conspecifics and heterospecific salamanders (*D. brimleyorum* and *N. viridescens*). The avoidance response to *N. viridescens* skin extract may represent avoidance of newt skin secretions that are toxic and distasteful to many animals (Brodie, 1968a; Hurlbert, 1970). However, no aversion was observed for *P. richmondi* and mealworms (*T. molitor*). These results suggest that there is no chemosensory response to damaged *P. richmondi* skin or mealworms. In addition, individual *D. ochrophaeus* did not avoid chemical cues from damaged visceral organs or heated skin extracts of conspecifics. Therefore, this avoidance is apparently a specific chemosensory response to damaged *Desmognathus* skin and not a general response to damaged tissues.

Similar avoidance responses have been observed in *Cynops pyrrhogaster* and *N. viridescens* (Marvin and Hutchison, in press). These authors suggested that tetrodotoxin (TTX), a neurotoxin found in all newt species in the family Salamandridae (Fuhrman, 1967; Mosher et al., 1964; Wakely et al., 1966; Yotsu et al., 1990), or an undescribed heat-sensitive skin toxin may function as

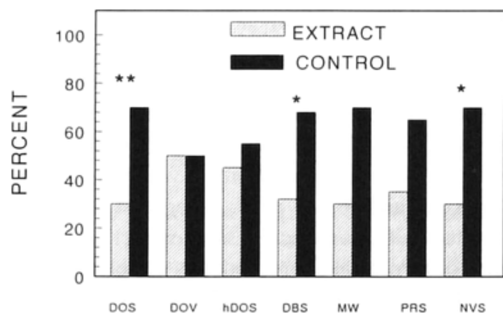

FIG. 1. Percentage of *Desmognathus ochrophaeus* spending the majority of time toward either extract or distilled water (control). Majority of time is based on the number of responses by individuals to extract and control substrates during the 4-hr test period. Significant differences ($*P < 0.05$, $**P < 0.01$) based on Wilcoxon signed-ranks test (Table 1). DO_s = fresh *D. ochrophaeus* skin, DO_v = fresh *D. ochrophaeus* viscera, DO_{hs} = heated *D. ochrophaeus* skin, DB_s = fresh *D. brimleyorum* skin, MW = fresh mealworm (whole), PR_s = fresh *P. richmondi* skin, NV_s = fresh *N. viridescens* skin. Sample sizes are given in Table 1.

a chemical alarm signal in addition to being a predator deterrent (Brodie, 1968a,b). Some alarm chemicals may have initially evolved to render prey toxic or unpalatable to predators, and the conspecifics' response to chemicals released during predation may have evolved secondarily (Weldon, 1983). Because the toxicity of newt (*Taricha*) skin extracts is reduced when heated (Brodie et al., 1974) and TTX is relatively heat stable (Budavari, 1989), the nonavoidance to heated skin extracts by salamanders provides evidence for an undescribed heat-sensitive skin toxin that may be the chemical cue for the observed avoidance response.

Additional research is needed to determine what chemical cues and/or toxins are used for the observed avoidance responses in plethodontid salamanders. Pseudotritontoxin (PTTX) in the salamander family Plethodontidae is comparable to TTX in toxicity (Brandon and Huheey, 1981). However, unlike TTX the toxicity of PTTX is destroyed by brief boiling (Goto et al., 1965, in Brandon and Huheey, 1981). Therefore, the aversion and nonaversion to freshly damaged skin extracts and heated skin extracts, respectively, are consistent with the hypothesis that PTTX serves as a chemical alarm cue in *Desmognathus*. Skin extracts of *Desmognathus* and *Plethodon* are not lethal and cause no severe hypothermia in mice (one of the symptoms of PTTX toxicosis) at concentrations equal to *Pseudotriton* skin extracts that are lethal (Brandon and Huheey, 1981). This indicates the possibility of only low PTTX concentration (i.e., which may still be detected by salamanders) and not necessarily the absence of PTTX in *Desmognathus* and *Plethodon* skin extracts. The avoidance to the *Desmognathus* skin extract and not the *Plethodon* skin extract may further suggest PTTX concentration differences (or other chemical differences) among different plethodontids.

Our observed results of an avoidance response may not be due to chemical alarm cues but simply to behaviors associated with territoriality or predator avoidance in desmognathids. Some species of *Desmognathus*, including *D. ochrophaeus*, exhibit aggressive defense of areas, which indicates territorial behavior (Jaeger, 1988; Keen and Reed, 1985; Keen and Sharp, 1984). Avoidance of substrates saturated with conspecific skin extract by *D. ochrophaeus* may represent avoidance of territorial pheromones as established for *Plethodon cinereus* (Jaeger, 1986; Jaeger and Gergits, 1979; Horne and Jaeger, 1988). However, we housed *D. ochrophaeus* together for six months prior to these experiments to lessen aggressive interactions between territorial neighbors due to "dear enemy" recognition (Jaeger, 1981). Avoidance response of *D. ochrophaeus* to *D. brimleyorum* skin extract may represent predator odor avoidance, as implicated for sympatric species of *Desmognathus* (Jacobs and Taylor, 1992; Hileman and Brodie, 1994; Roudebush and Taylor, 1987). However, *D. ochrophaeus* and *D. brimleyorum* are allopatric species and thus *D. ochrophaeus* may not exhibit avoidance behavior associated with predator–prey interactions.

Additional research is needed to conclusively determine if the avoidance behavior we observed is due to the presence of chemical alarm cues and not due to the presence of territorial pheromones (e.g., Jaeger, 1986) or predator odors (e.g., Elliott et al., 1993; Jacobs and Taylor, 1992).

Predator detection and avoidance by chemoreception of alarm cues may have significant adaptive value in enhancing survivorship in terrestrial salamanders. Predator avoidance by *D. ochrophaeus* has previously been explained as a response to predator odors (Hileman and Brodie, 1994); however, this predator avoidance may also represent an avoidance of alarm chemicals released from injured conspecifics.

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POTENTIAL ALLELOCHEMICALS FROM *Ruta graveolens* L. AND THEIR ACTION ON RADISH SEEDS

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Abstract—An aqueous extract of *Ruta graveolens* L. (250 g/liter) was tested for its allelopathic activity in vitro on radish germination and radicle growth in light and darkness. It caused a delay in the onset and a decrease in the rate of germination (40%) in the light. The photoinhibition of germination was accompanied by an inhibition of water uptake into the seed. Furthermore, the inhibition of radicle growth was slightly higher in the light than in darkness. Three potential allelochemicals, biologically active in the light, were isolated from the extract: 5-methoxypsoralen (5-MOP), 8-methoxypsoralen (8-MOP), and 4-hydroxy-coumarin at concentrations of 10^{-4} M, 2×10^{-4} M, and 0.4×10^{-5} M respectively. At a concentration of 2×10^{-4} M, 5-MOP was the most potent inhibitor, decreasing radish germination to 32% and radicle growth to 17% with respect to control. Microscopic observations of radish seeds treated with 5-MOP suggest that this substance changes the swelling of the seed coat and aleurone layer, which precedes radicle protrusion.

Key Words—Aleurone, 5-methoxypsoralen, 8-methoxypsoralen, 4-hydroxy-coumarin, coumarins, dormancy, light, radish, *Raphanus sativus*, root inhibition, *Ruta graveolens* rue, seed coat.

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INTRODUCTION

An important aspect of research in allelopathy is the identification of allelochemicals involved in plant–plant interactions and their possible mechanisms of action (Einhellig, 1986). Recently we have shown that coumarin, a well-known inhibitor of plant growth in vitro possesses significant properties required of an allelochemical (Aliotta et al., 1992, 1993). Coumarin confers a coat-imposed dormancy on radish seeds, which is influenced by light, and it inhibits the elongation of cells of the differentiating zone of the radicle. Some phenylpropanoid precursors of coumarin inhibit only radicle growth. In addition to the phenylpropanoids, coumarin is related to simple coumarins and furanocoumarins, which occur mainly in the Leguminosae, Moraceae, Rutaceae, and Umbelliferae. Coumarins are located on the leaf and seed surface, and they have a broad spectrum of biological activities that are often mediated by light. Thus, furanocoumarins are responsible for photophytodermatitis (Benezra et al., 1985) and function as attractants and repellents for insects (Murray et al., 1982; Zobel and Brown, 1991).

There are few studies concerning the allelopathic properties of plants rich in coumarins, and little is known about the functions of these substances in plant metabolism (Rice, 1984). This paper deals with the effects of potential allelochemicals isolated from rue, an ancient medicinal plant mentioned as an abortive by Pliny the Elder (23–79 AD) in his *Naturalis Historia* (XX. 143) (Rackam et al., 1938–1962). We have investigated differences in their action in light and darkness and also attempted to correlate the observed effects of rue extract with morphological aspects of germination.

METHODS AND MATERIALS

Plant Collection and Extraction. Fresh whole leaves of rue (*Ruta graveolens* L., Fam. Rutaceae) were collected from plants grown in the Naples botanic garden and extracted by a brief dipping in almost-boiling water (250 g/liter) according to Zobel and Brown (1988).

Bioassay. A bioassay based on radish germination and subsequent radicle growth was used to study inhibitory effects of the extract, the chromatographic fractions, and the isolated active coumarins of rue that possessed significant biological activity. Seeds of *Raphanus sativus* L. Saxa, collected during 1992, were purchased from Imperatore Co., Naples. The seeds were surface-sterilized in 95% ethanol for 15 sec and germinated on 30-ml layers of Bacto-Agar gel (10 g/liter) in covered 9-cm sterilized Petri dishes. Germination conditions were $20 \pm 1\,^{\circ}C$ with either a continuous light of 25 $\mu E/m^2/sec$ or in darkness.

The initial aqueous extract was prepared for bioassays as follows. Agar was added (10 g/liter extract) at the end of the plant extraction, when the water

was almost boiling. Then different volumes of this extract were mixed in Petri dishes with corresponding volumes of hot Bacto-Agar dissolved in H_2O in order to obtain final volumes of 30 ml containing different concentrations of rue extract. A control without rue extract was included in each treatment series. The radish seeds were placed in Petri dishes on agar containing the rue extract or chromatographic fractions and substances isolated (Aliotta et al., 1993). Germination and root growth were followed in light and darkness. Seeds were considered germinated when the protrusion of the radicle was evident. Effects on radicle elongation were determined by measuring to the nearest millimeter the radicle length five days after placing the seeds on the agar medium. Each determination was replicated three times, using Petri dishes containing 10 seeds each. Data are expressed as the mean $\pm$ standard deviation (SD).

Aqueous Extract Fractionation. The aqueous extract was lyophilized, and the residue (3.05 g) was extracted with methanol, to give 2.6 g of residue, then chromatographed, in 1.5-g lots, on a Sephadex LH-20 column (4 $\times$ 80 cm), eluting with MeOH. One hundred twenty-three fractions of 10 ml were recovered, analyzed by TLC on SiO_2 in *n*-BuOH—HOAc—H_2O (12:3:5) and in $CHCl_3$—MeOH—H_2O (70:30:3), and combined for chromatographic similarity, to give 17 main fractions.

Fractions VII and IX, biologically active, were purified. Purification of each fraction was achieved by RP-HPLC on a C_{18} μ-Bondapack column (30 cm $\times$ 7.8 mm). Fraction VII, eluted with MeOH—H_2O (3:1), flow rate 2.5 ml/min, resulted in the active compounds **1** (37 mg, elution time 14 min) and **2** (20 mg, elution time 16 min). From fraction IX, eluent MeOH—H_2O (1:1), flow rate 2.5 ml/min, the active compound **3** (7 mg, elution time 15 min) was recovered.

The structural determination of isolated active compounds was performed by spectroscopic methods (^{1}H NMR, ^{13}C NMR, ^{13}C DEPT NMR).

Compound 1 (8-Methoxypsoralen). ^{1}H NMR ($CDCl_3$ at 250 MHz) δ 6.24 (1H, d, J = 10 Hz) H-3; δ 6.72 (1H, d, J = 1.5 Hz) H-2′; δ 7.25 (1H, s) H-5; δ 7.61 (1H, d, J = 1.5 Hz) H-3′; δ 7.70 (1H, d, J = 10 Hz) H-4; δ 4.18 (OCH_3). ^{13}C NMR ($CDCl_3$ at 250 MHz) δ 160.31 (C-2); 114.40 (C-3); 144.28 (C-4); 116.27 (C-4a); 112.82 (C-5); 126.00 (C-6); 147.39 (C-7); 132.48 (C-8); 142.72 (C-8a); 146.49 (C-2′); 106.60 (C-3′); 61.07 (OCH_3). These data were in full agreement with those reported by Elgamal et al. (1979) and Lock de Ugaz (1988).

Compound 2 (5-Methoxypsoralen). ^{1}H NMR ($CDCl_3$ at 250 MHz) δ 6.18 (1H, d, J = 10 Hz) H-3; δ 6.95 (1H, d, J = 1.5 Hz) H-2′; δ 7.02 (1H, s) H-8; δ 7.55 (1H, d, J = 1.5 Hz) H-3′; δ 8.07 (1H, d, J = 10 Hz) H-4; δ 4.20 (OCH_3). ^{13}C NMR ($CDCl_3$ at 250 MHz) δ 161.22 (C-2); 112.45 (C-3); 139.26 (C-4); 106.33 (C-4a); 149.54 (C-5); 112.60 (C-6); 158.35 (C-7); 93.73 (C-8); 152.67 (C-8a); 144.76 (C-2′); 105.04 (C-3′); 60.04 (OCH_3). These data were

in full agreement with those reported by Elgamal et al. (1979) and Lock de Ugaz (1988).

Compound 3 (4-hydroxycoumarin). ^{1}H NMR (CD_3OD at 250 MHz) δ 7.23 (1H, d, J = 8 Hz) H-8; δ 7.25 (1H, s) H-3; δ 7.27 (1H, t, J = 8 Hz) H-7; δ 7.60 (1H, t, J = 8 Hz) H-6, δ 7.90 (1H, d, J = 8 Hz) H-5. ^{13}C NMR (CD_3OD at 250 MHz) δ 165.99 (C-2); 91.21 (C-3); 168.25 (C-4); 117.17 (C-4a); 124.48 (C-5); 125.17 (C-6); 133.87 (C-7); 117.46 (C-8); 155.06 (C-8a). The data were in agreement with literature references, reported by Chang and Floss (1977) and Lock de Ugaz (1988).

Stereo, Light, and Electron Microscopy. Eighteen hours after sowing in the presence of light, control and 5-MOP-treated radish seeds were differently moistened, having weight increases of 110 and 80%, respectively. The water uptake was evaluated as the difference in weight between moistened and unmoistened seeds as a percentage of the weight of unmoistened seeds (percent initial weight). Whole and excised moistened seeds were observed directly with a Stereomicroscope Wild M3Z. Moistened seeds were excised (cut in half along the two orthogonal planes of their major axis), the embryos were removed, and the seed coats and endosperms were fixed in 3% glutaraldehyde in 0.065 M phosphate buffer (pH 7.4) for 2 hr at room temperature. The specimens were then placed into 2% OsO_4 in 0.1 M phosphate buffer (pH 6.8) at 4°C before being dehydrated with ethanol and propylene oxide, and embedded in Epon 812 resin (Luft, 1961).

Thick sections (ca. 1 μm each) were stained with 0.1% toluidine blue and observed with a Zeiss light photomicroscope. Thin sections, obtained with a diamond knife on a Supernova microtome, were sequentially stained at room temperature with 2% uranyl acetate (aqueous) for 5 min and by lead citrate for 10 min (Reynolds, 1963). Ultrastructural studies were made using a Philips CM12 transmission electron microscope (TEM) operated at 80 kV.

Seed coats and endosperms of control and treated seeds, after fixation and ethanol dehydration, were critical-point dried and finally coated with carbon and gold in a sputter-coater. These specimens were observed at 20 kV with a Cambridge 250 Mark3 scanning electron microscope (SEM).

The observations were carried out at Centro Interdipartimentale di Ricerca sulle Ultrastrutture Biologiche (Faculty of Sciences, University of Naples).

Chemical Apparatus. The following instruments were used: for NMR, a Bruker MW 250 Spectrospin spectrometer; for HPLC, a Waters, model 6000 A pump equipped with a 6UK injector and a differential refractometer, model 401.

RESULTS AND DISCUSSION

Figure 1 reports the time-dependent germination patterns of radish seeds kept in continuous light (A) and darkness (B) in the presence of different amounts of rue extracts. Although the germination curves have the same general shape,

important differences between control and treated seeds are evident. In fact, the rue extract induces a delay in the onset and a decrease in the rate of germination, which are more pronounced in the light than in darkness. In the light, the proportion of seeds capable of completing germination in the presence of the higher concentration of rue extract was lower than in the control.

In order to identify the active constituents of rue extract, this latter was fractionated by means of CC and HPLC chromatography on the basis of bioassay results. Three active pure compounds were isolated and, on the basis of their physical features, were identified as coumarins: 5-methoxypsoralen (5-MOP), 8-methoxypsoralen (8-MOP), and 4-hydroxycoumarin. Their concentrations in the extract were 10^{-4} M, 2×10^{-4} M, and 0.4×10^{-5} M, respectively.

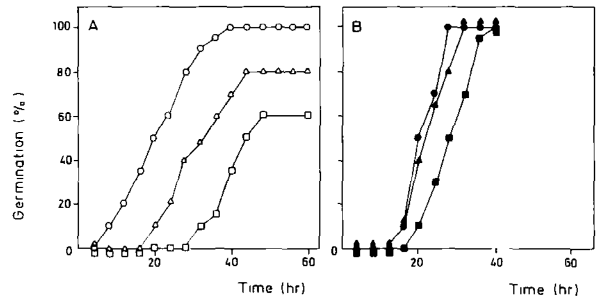


FIG. 1. Time course of germination of radish seeds in continuous light (A) and in darkness (B) in the presence of different concentrations of aqueous rue extract. ○ = control; △ = 15 ml extract/petri dish; □ = 30 ml extract/Petri dish. Germination is expressed as percentage of germinated seeds.

TABLE 1. INHIBITION BY AQUEOUS EXTRACT FROM RUE AND ITS ACTIVE ISOLATED COMPOUNDS (2×10^{-4} M) ON GERMINATION AND RADICLE GROWTH OF RADISH FIVE DAYS AFTER SOWING[a]

	GERMINATION		RADICLE LENGTH (mm)	
	Light	Dark	Light	Dark
Control	99 ± 1	99 ± 1	81 ± 5	72 ± 4
Rue aqueous extract	60 ± 3	99 ± 1	14 ± 6	35 ± 5
5-MOP	32 ± 4	99 ± 1	10 ± 3	23 ± 4
8-MOP	68 ± 3	99 ± 1	22 ± 4	23 ± 5
4-OH-coumarin	80 ± 2	99 ± 1	43 ± 6	50 ± 3

[a] Data are expressed as percentages of germination ± SD and root length ± SD.

Table 1 shows the effects of the crude extract, and of the active purified substances from rue, on the germination and radicle growth of radish five days after sowing, in both light and darkness. The crude extract and isolated coumarins, tested at 2×10^{-4} M, inhibited radish germination only in the light. They caused a delay in the onset of germination (not shown) and a dormancy that was released in darkness. Moreover, inhibition of radicle growth was slightly higher in the light than in darkness. 5-MOP was the most potent inhibitor. It is notable that 5-MOP and 8-MOP are psoralens responsible for the photophytodermatitis of rue that occurs when wet or damp skin is exposed to the sun after contact with the plant (Benezra et al., 1985).

To ascertain whether the effect of coumarins on radish germination was at the level of the embryo or if it was mediated by the seed coat, the coats were removed and embryos tested for their germination in light in the presence of each coumarin. It appeared that each coumarin inhibited the seeds with their coat, but did not significantly inhibit seeds without a coat (data not shown).

By means of stereo, light, and electron microscopy, we have focused our attention on the anatomical and ultrastructural aspects of the hilum–micropylar region of the radish seed, where the radicle protrudes. The investigations were carried out after 18 hr in the presence and absence of 5-MOP, when most of the seeds soaking in water were germinating, treated seeds were dormant, and different uptake of water into these seeds was evident (Figure 2).

According to Vaughan and Whitehouse (1971), radish seed is oval (3×2 mm) with a light brown to orange, reticulate surface. Three layers of cells may be recognized in the seed coat of the mature seed. They are the epidermis, formed by compressed cells; the palisade layer which presents cells more or less isodiametrical or radially elongated; and the inner parenchyma, which is a pigmented layer one cell thick. The endosperm persists as a well-formed aleu-

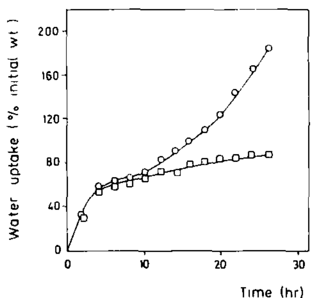


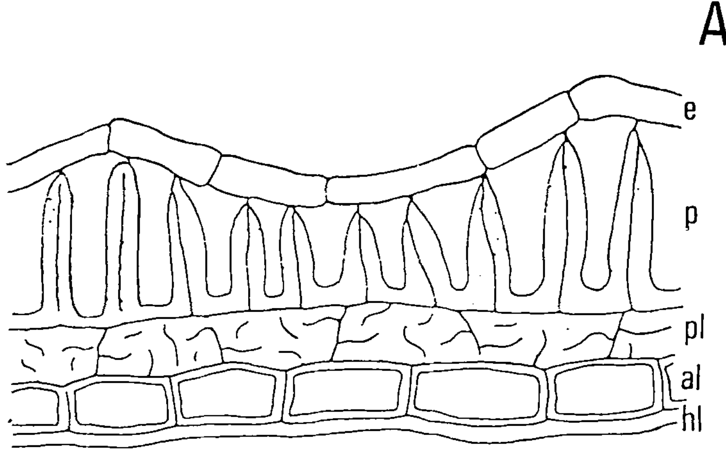
FIG. 2. Water uptake by radish seeds in presence (□) and absence (○) of 5-MOP 2×10^{-4} M (on a percent of initial weight of seeds).

rone layer intimately associated with the seed coat. The hyalin layer covers the embryo (Figure 3A). The embryo is folded with cotyledons against radicle.

The hilum–micropylar region of the seed is highly specialized and characterized by a flaking epidermis, thickened aleurone cells, and a hyalin layer. Moreover, this area represents a marker for comparison between seeds (Figure 3B). Stereo-microscopic observations in Figure 4 show that the section of hilum–micropylar region of control seed (A) presents two bands having different colors: the external band is black and the inner is gray. This latter does not appear in the treated seed (B). SEM and light microscope observations of the hilum–micropylar region show that the hilum is more evident in the control than in the treated seed (Figure 5).

This aspect is confirmed by the comparison of the hilum–micropylar semithin section of control and treated seeds under the light microscope (Figure 6 A and B). The hilum of the control seed is thicker and pigmented; the testa pigment layer is more evident, and there are more layers of aleurone cells that are filled by light-dense bodies. In this respect, it was interesting to compare the TEM ultrastructure of the seed coat and endosperm of control and 5-MOP-treated seeds (Figure 7). As can be seen, the observations made under the light microscope are confirmed. Moreover, the palisade layer of treated seed appears thicker than in the control. Figure 8, showing the TEM comparison between aleuronic cells of the control (Figure 8A) and treated cells (Figure 8B), reveals that the cells of the control are healthy with some evident organelles: nucleus, rough endoplasmic reticulum, plastid, plasmodesmata, conspicuous constrictions, protein bodies, and lipid droplets. By contrast, cells of the treated seed resemble those in the dried quiescent seed (Figure 8C). The differences observed between moistened and 5-MOP-treated radish seeds represent useful signals to establish whether the water uptake of the seed will culminate in radicle emergence.

Very little is known about the nature of the changes in seeds at the onset or cessation of dormancy (Bewley and Black, 1985). Moreland et al. (1974) reported that in radish seed cell elongation is sufficient for the radicle to rupture the seed coat, and cell division begins later. Schopfer and Plachy (1993) have shown that seed-coat constraint and the expansive force of the embryo interact additively on the level of the germination potential in radish seed. Moreover, photoinhibition of the germination by far-red light is accompanied by an inhibition of water uptake into the seed. These findings suggest that in radish seeds, 5-MOP in the presence of light provides, either directly or indirectly, a critical signal that inhibits water uptake into the seed, switches off the swelling of the seed coat epidermis, as well as of the cells of the endosperm aleurone layer and the related process of cell elongation in the embryo. Thus, it is possible that germination in the radish results from a combination of a lowered resistance of the swollen seed coat, endosperm, and of the related elongation of embryo cells.



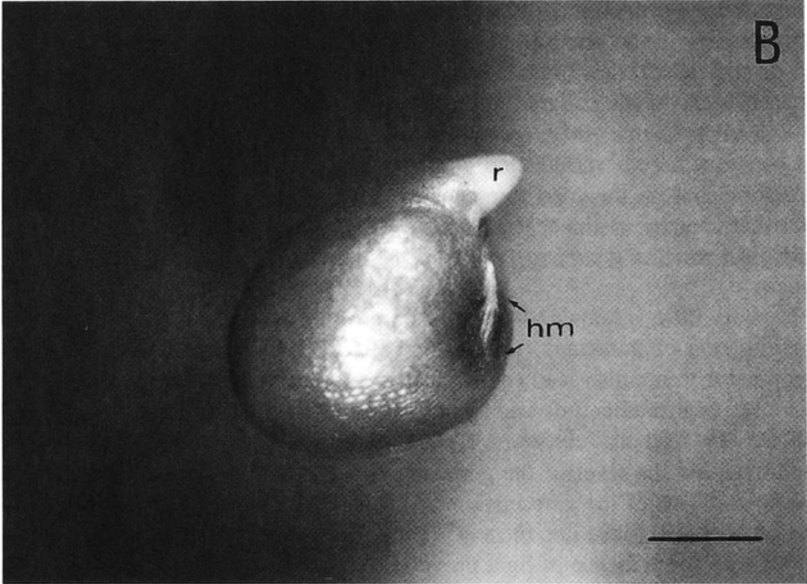
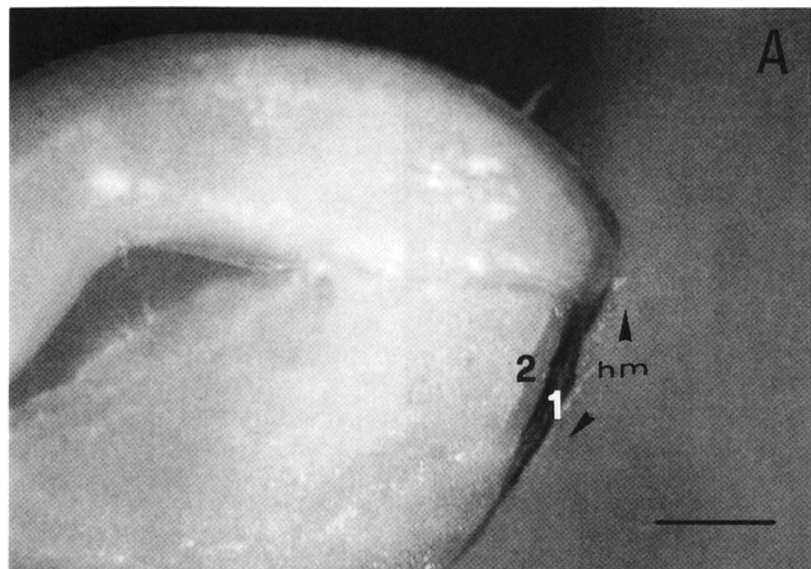


FIG. 3. Graphic showing the structural features of a seed coat and endosperm of radish seed, according to Vaughan and Whitehouse (1971) (A). Stereomicrograph of a germinating seed of radish showing the hilum micropylar region and the radicle (B). e, epidermis; p, palisade; pl, pigment layer; al, aleurone layer; hl, hyalin layer; r, radicle; hm, hilum-micropylar region. Bar = 1 mm.



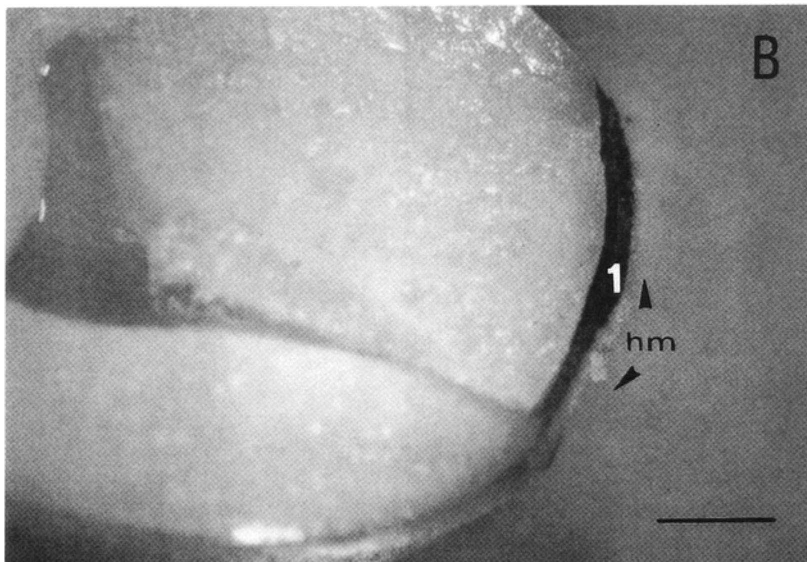


FIG. 4. Stereomicrographs of radish seeds, moistened (A) and 5-MOP-treated (B), 18 hr after sowing. Cut seeds were excised along the two orthogonal planes of their major axis. The hilum–micropylar region shows two bands in (A) and one in (B). Bar = 0.5 mm.

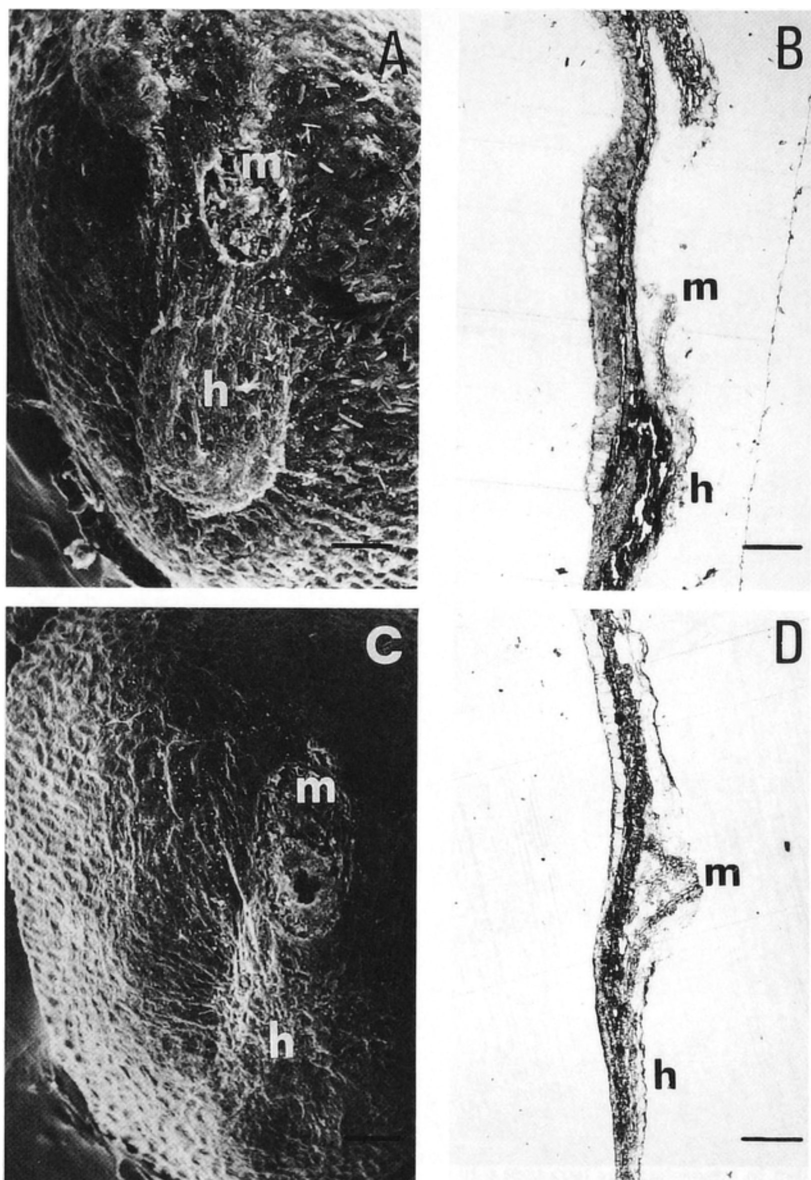


FIG. 5. SEM (A, C) and light microscope (B, D) micrographs of the hilum–micropylar region of radish; 18 hr after sowing the hilum is more evident in water-moistened seed, (A, B), than in 5-MOP-treated seed (C, D). Bar = 0.5 mm.

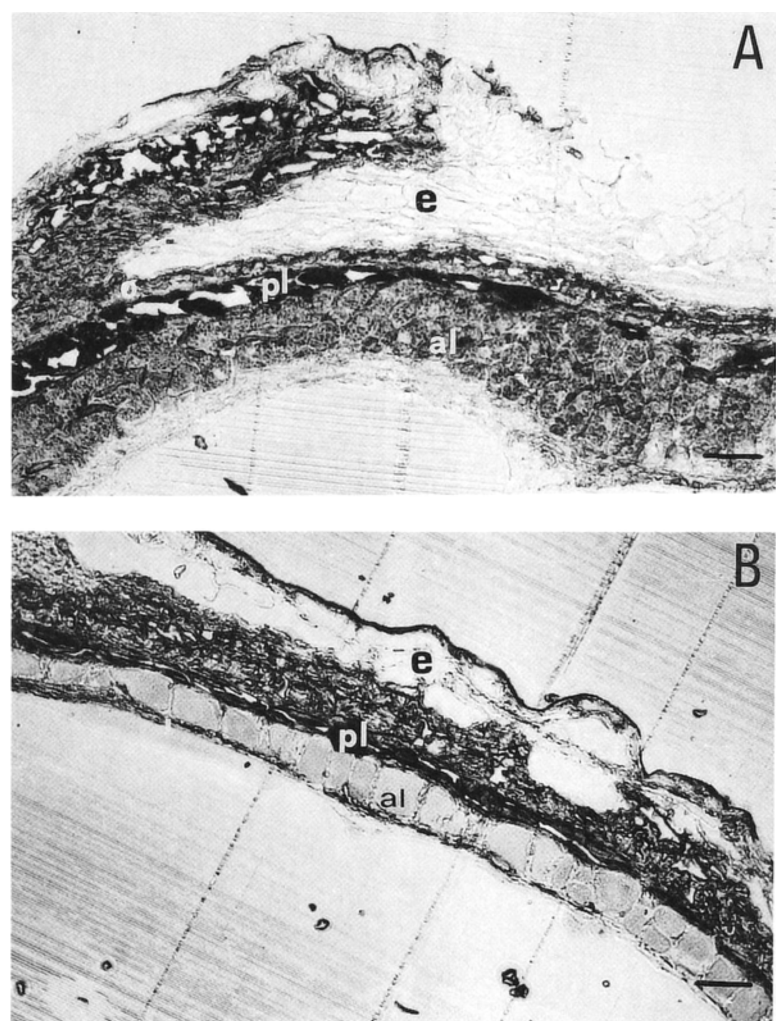


FIG. 6. Semithin light microscope section of hilum–micropylar region of water-moistened seed, 18 hr after sowing (A) and 5-MOP-treated radish seed (B). e, epidermis; pl, pigment layer; al, aleurone layer. Bar = 150 μm.

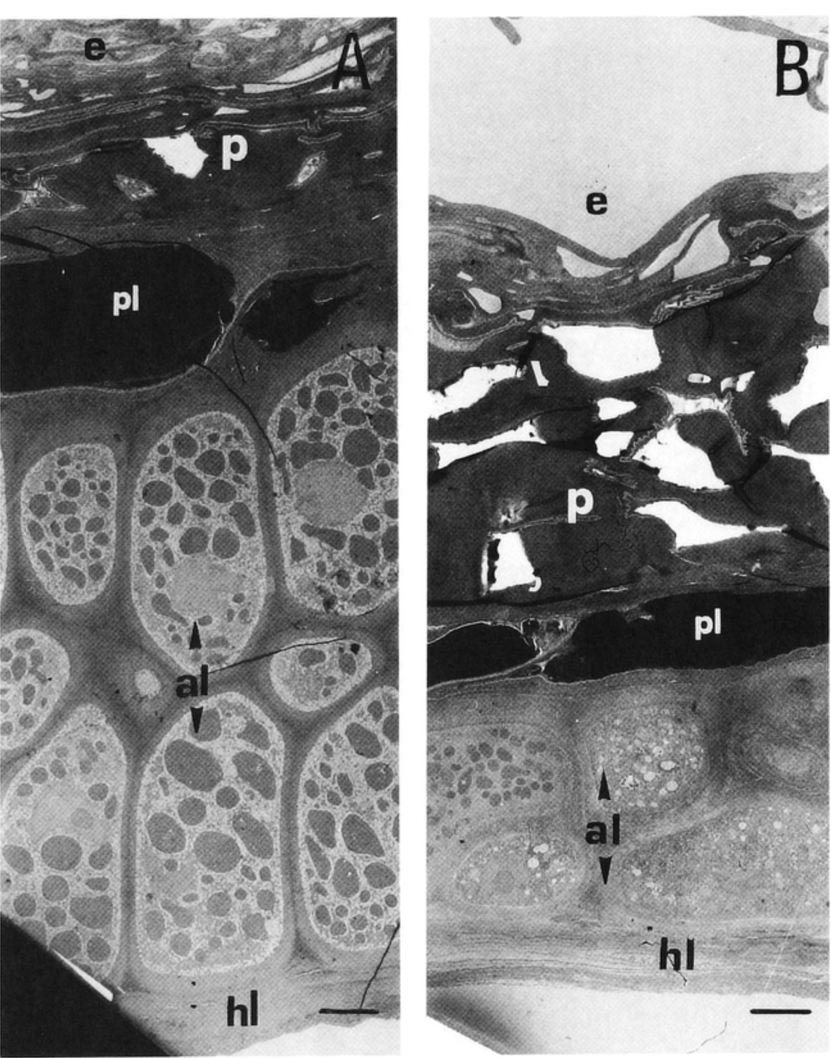


FIG. 7. TEM comparison between cells of seed coats and endosperm of radish 18 hr after sowing in water (A) and in presence the of 5-MOP (B). Control shows a thin layer of palisade cells, and more developed pigmented and aleuronic layers. Bar = 30 μm.

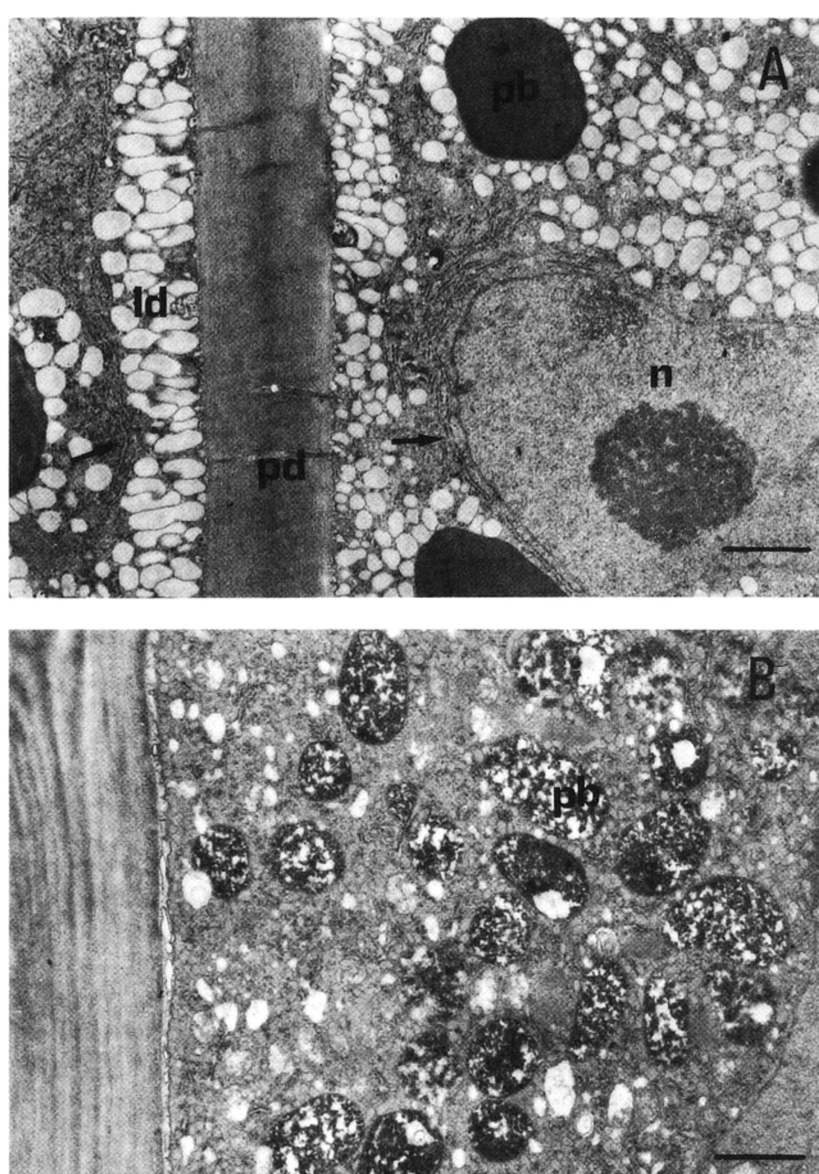


FIG. 8. TEM comparison of aleuronic cells of dried radish seed (C), water-moistened (A) and 5-MOP-treated seeds (B), 18 hr after sowing. Aleuronic cells of the control shows well-developed organelles: nucleus (n), rough endoplasmic reticulum (→), plasmodesmata (pd), protein bodies (pb) and lipid droplets (ld). Different profiles appear in the dried (C) and 5-MOP-treated seeds (B). Bar = 5 μm.

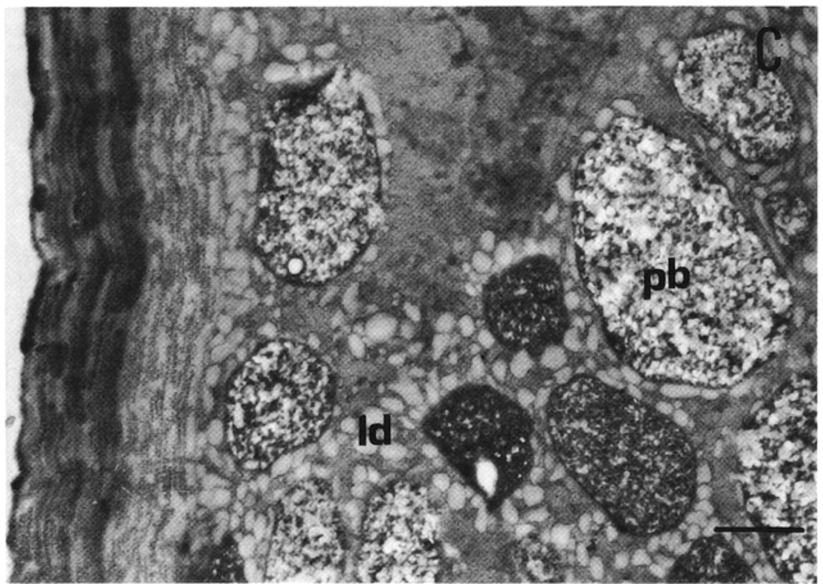


FIG. 8. Continued.

These effects due to the active coumarins are prevented by a preliminary removal of seed coats or by darkness. A similar pattern has been observed with coumarin by Aliotta et al. (1993).

To what extent the potential allelochemicals extracted from rue play an important role in plant-plant interaction remains to be seen. However, the biological properties of coumarins suggest that these substances may influence the growth of plants growing in the neighborhood of rue.

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ENHANCED MAIZE (*Zea mays* L.) PERICARP BROWNING: ASSOCIATIONS WITH INSECT RESISTANCE AND INVOLVEMENT OF OXIDIZING ENZYMES

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Abstract—The kernel pericarp of a maize (*Zea mays* L.) inbred, Mp313E, that browns rapidly at milk stage when damaged and that is resistant to *Aspergillus flavus* Link and the dusky sap beetle *Carpophilus lugubris* Murray compared to a susceptible inbred, SC212M, was examined for differing oxidizing enzymes (peroxidases) and their substrate specificity. Additional pericarp enzymes were constitutively produced by the Mp313E inbred compared to the SC212M inbred, as indicated by gel electrophoresis and isoelectric focusing. These enzymes oxidized relevent pericarp substrates such as ferulic acid. Similar results were seen with two varieties of maize containing the *Ch* mutant gene, which brown upon senescence in that enhanced oxidation of ferulic acid was seen in homogenates of browned pericarp compared to that which was cold-shocked and did not brown. Corn powder that was browned by mixing oxidizing enzymes with corn pericarp components ferulic acid and coumaric acid were typically less preferred/more toxic to caterpillars such as *Helicoverpa zea* (Boddie) and sap beetles such as *C. lugubris*. Thus, enhanced browning of maize pericarp can promote resistance to insects and is at least partly influenced by the presence of oxidizing enzymes. This mechanism may also promote resistance to maize pathogens, including those that produce mycotoxins.

Key Words—Maize, *zea mays* pericarp, insect resistance, peroxidase, *Helicoverpa zea*, *Carpophilus lugubris*, phenolic, flavonoid, hydroxamic lepidoptera, noctuidae, coleoptera, nitidulidae.

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INTRODUCTION

Resistant plant varieties can be an important component of integrates pest management programs. The mechanisms of host plant resistance are often due to the production of different levels or components of secondary chemicals. However, an increasing number of resistance mechanisms are being discovered that have protein or enzymatic components as well. These resistance factors include enzyme inhibitors such as trypsin inhibitor of soybeans (Hilder et al., 1987) or other proteins such as lectins (Gatehouse et al., 1991). Determining the components involved in resistance mechanisms can allow for more rapid and precise introduction into commercial varieties through conventional breeding. Protein-based mechanisms also have the potential for rapid incorporation into commercial varieties through genetic engineering.

The maize (*Zea mays* L.) inbred Mp313E is resistant to the aflatoxigenic fungus *Aspergillus flavus* Link (Scott and Zummo, 1988), and the sap beetles (Coleoptera: Nitidulidae) that vector this and other fungi (Dowd, 1994). Examination of kernels of this inbred in the milk stage revealed a rapid browning (a zone of 1–2 mm wide within 30 min) when the kernels were mechanically damaged as compared to other inbreds or commercial varieties that have been tested (Dowd, 1994). Plant enzymes involved in browning reactions, such as polyphenol oxidases or peroxidases, can oxidize appropriately hydroxylated phenolic compounds to quinones that are more toxic than their precursors to potentially pathogenic bacteria (Kojima, 1931; Urs and Dunleavy, 1975). This activity is indicated by rapid browning in, for example, apple, in varieties with demonstrated resistance to the brown rot fungus *Sclerotinia fructigena* where enhanced peroxidase activity is present (Byrde et al., 1960). These enzymes are also associated with maize resistance to different *Helminthosporium* strains (Macri et al., 1974; Cardena-Gomez and Nicholson, 1987). In addition, these enzymes are also involved in resistance to *Helicoverpa zea* (Boddie) in normal (Felton et al., 1989) and transgenic (Dowd and Lagrimini, 1994) tomato, to aphids in wild *Solanum bertheii* potato (Steffens and Waters, 1991), and to *H. zea* and the dusky sap beetle *Carpophilus lugubris* Murray in transgenic tobacco (*Nicotiana sylvestris* L.) (Dowd and Lagrimini, 1994).

Thus, it is likely that the rapid browning that occurs when the pericarp of Mp313E is damaged is at least part of a resistance mechanism. The potential for this reaction in the kernels to contribute to resistance to insects was determined by comparing responses of insects to brown and nonbrown corn material. Because initial studies indicated differing peroxidases were present in fungus- and insect-resistant vs. susceptible maize pericarps (Dowd, 1992), the potential involvement of enzymes in causing this reaction was further examined by comparing peroxidase isozymes of the Mp313E inbred with the *A. flavus*- (Scott and Zummo, 1988) and sap beetle- (Dowd, 1994) susceptible inbred SC212M,

and a hybrid of these two inbreds. Enzyme involvement in browning was also examined in varieties of corn containing the *Ch* gene, which produce a brown pericarp (Neuffer et al., 1969).

METHODS AND MATERIALS

Insects. The *H. zea* and *Spodoptera frugiperda* (J.E. Smith) larvae were reared on pinto bean diet at 27 ± 1°C, 40 ± 10% relative humidity, and 14L:10D photoperiod as described previously (Dowd, 1988). Neonate *Ostrinia nubilalis* Hübner were obtained from M.R. McGuire, also located at NCAUR. The dried-fruit beetle, *Carpophilus hemipterus* L., and *Carpophilus freemani* Dobson were reared under the same environmental conditions as described previously (Dowd and Weber, 1991). The *C. lugubris* were collected from oak wood–corn field interfaces using pheromone and host attractant baited traps as described previously (Dowd et al., 1992) and maintained on pinto bean diet until utilized. Maize weevils, *Sitophilus zeamaiz* (Motsch.) were reared on cracked corn under the same conditions as for the other insects except the relative humidity was 60 ± 10%.

Corn. Original seed of Mp313E and SC212M was obtained from G.E. Scott at Mississippi State University. Seed of the two maize lines homozygous for the *Ch* gene [89-726-1 (referred to hereafter as CH) and 90-1692-6 (referred to hereafter as CHH)] were provided by the Maize Genetics Stock Center at the University of Illinois, Urbana. The plants were field grown using standard soil and fertilizer treatments (Dowd, 1994). Inbreds were typically self-pollinated. Hybrid seed was obtained by hand cross pollination and was identified as hybrid seed when grown for experiments by self-pollination. Kernels of Mp313E are white, and SC212M are yellow. Self-pollinated hybrids were examined for a 1:3 production of white:yellow kernels following classical Mendelian genetics to confirm hybridization.

Chemicals. The chlorogenic acid, 4-chloronaphthol, *p*-coumaric acid, L-dihydroxyphenylalanine (DOPA), dimethyl-*p*-phenylenediamine (DMPD), gallic acid, kojic acid, mercuric chloride, 6-methoxybenzoxazolinone (MBOA), *o*-phenylenediamine, phenylthiourea, pyrogallol, quercetin, rutin, ultra-low-temperature gelling agarose, horseradish peroxidase (P8125), potato phenolase (P4788), and mushroom tyrosinase (T7755) were obtained from Sigma Chemical Co. Coniferyl alcohol and ferulic acid were obtained from Aldrich Chemical Co. Luteolin was obtained from Indofine Chemicals. Ascorbic acid and sorbic acid were obtained from U.S. Biochemical Corp. All other chemicals were of reagent grade.

Preparation of Browned Materials and Bioassays. For fresh kernel bioassays of *Ch* materials, mile-stage kernels were carefully pried from cobs using a

spatula with a pointed end. Assays were set up as described previously (Dowd, 1994). Briefly, individual kernels were placed in single wells of a 24-well tissue-culture plate, and individual *C. lugubris* adults were placed with a single kernel. Plates were incubated as described above (*Insects*) and examined after seven days to determine the number of kernels damaged.

Corn kernel powder assays were used in order to provide a simple simulation of conditions in the pericarp whereby addition of oxidizing enzymes would cause browned materials that could be compared with unbrowned ones for effects on insects. Preliminary assays with powdered corn pericarp indicated that *H. zea* larvae would only survive a few days, so whole corn kernels were used. Compounds such as ferulic acid and coumaric acid, that are naturally present in the pericarp (which makes up about 5% of the total weight of the kernel), were added to approximate concentrations potentially found in the pericarp (1000 ppm) (e.g., Classen et al., 1990). Air-dried milk-stage dent corn (Pioneer 3279) was ground to a powder that would pass a 0.5-mm screen using a Cyclotec 1093 sample mill. The corn powder was gelled using ultra-low-temperature gelling agarose at a rate of 250 mg/1.25 g of powdered corn. This allowed production of brown material that potentially could continue to brown (as the damaged or senescing pericarps did), since the added enzyme was not denatured by heating or solvent treatments. In order to obtain appropriate browning and proper gelling, materials had to be added and manipulated in a precise order. Sorbic acid (a mold inhibitor) was incorporated by adding 5 mg in 1.5 ml of acetone to 1.25 g of powder placed in a 5-cm-diameter Petri dish bottom (Falcon 1008). Acetone-soluble compounds were also added at this time so that the final concentration would be 1000 ppm once the diet was rehydrated by adding 3.75 ml water. The acetone was evaporated in a fume hood. Then, 2.5 ml of sterile, deionized water was added, and the material was held at 4°C overnight in order to rehydrate the powder. Enzyme stock solutions were prepared by adding 1 mg/1 ml water. Final enzyme solutions were made by adding 250 μl of the stock to 1 ml of water. When peroxidase was used, a stock solution of hydrogen peroxide, prepared as described earlier, replaced 250 μl of the water. The enzyme solutions and relevant controls were added to the room-temperature hydrated corn powder and allowed to react for 2 hr. The material was then chilled at least 2 hr at 4°C. The chilled reacted powder was mixed with the agarose (which was chilled to −20°C) and allowed to sit for 1 hr to allow gelling prior to use in bioassays. Degree of browning was rated using Munsell soil color charts (Kollmorgan Instruments Corp., 1992). In spite of the use of sorbic acid as a mold inhibitor, mold did occur in some wells of the longer-term assays. Insects in these wells were not included in the results.

For no-choice assays, treated diets were separated into approximately 20 equal parts, and each piece was placed in a well of a 24-well tissue-culture plate along with individual neonate caterpillars or sap beetle larvae as described pre-

viously (Dowd, 1988, 1990). Insects were examined for effects after 2, 4, 7, 10, and/or 14 days, depending on the insect and the rate the diet was consumed. Initial studies with *H. zea* indicated no or negligible browning when enzyme (and cofactor for peroxidase) alone or substrate alone was added to the powder and no significant effects on the insects compared to untreated diets (Table 1). Thus, in subsequent experiments the control consisted of the substrate alone, which was compared with the diets that had both substrate plus enzyme. Sap beetle choice assays were performed as previously described (Dowd, 1990) except that larger Petri plates (Falcon 1008), sealed with parafilm, were used. The gelled material was cut into eight sections of ca. 0.3 mg, and browned and nonbrowned sections were placed on opposite sides of the plates. Ten larvae or adults of *C. lugubris*, *Carpohilus freemani* Dobson, or *Carpophilus hemipterus* L. were added to each plate. The amount of diet consumed was rated on a 1–4 scale (Dowd, 1990) after 2–14 days (depending on size of insect and relative deterrency of the combinations). Each assay was typically replicated eight times. Larvae of sap beetles were also set up in no choice assays with ca. 0.6 mg of material and weighed after one series began to reach last instars (typically 2 to 5 days). Differences in mortality were examined for significance by Chi-square analysis, and feeding rates or weights were examined for significance by simple analysis of variance.

Some of the milk-stage kernels of the *Ch* varieties removed from the cobs were held at 25°C for two days to stimulate browning, while another set was held at 4°C for the same period to inhibit browning. Some of these kernels were used immediately in enzyme assays (see below), while others of each type were allowed to air dry and were used in maize weevil assays. Maize weevil adults were individually caged with a browned or unbrowned *Ch* variety kernel in the tissue culture plates, which were held under the same conditions used for maize weevil rearing. Numbers of surviving weevils were counted after seven days, and the degree of damage was determined by measuring the depth of penetration in millimeters. Each treatment was replicated with 30 insects. Differences in mortality were examined for significance by chi-square analysis, and feeding rates were examined for significance by simple analysis of variance.

In Vivo Corn Kernel Browning Assays. Milk-stage kernels were used for all assays. A series of 18 kernels was punctured through the pericarp with a 1-mm-wide stainless steel probe. Two microliters of each test solution was added to the hole with a pipet. Enzyme solutions were 0.1 mg/ml in 0.1 M pH 7.4 sodium phosphate buffer of peroxidase, phenolase, or tyrosinase and were used to indicate the presence of brownable substrates in the pericarp. When peroxidase was used, it was used alone and with 30 μl/10 ml solution of hydrogen peroxide. Hydrogen peroxide was also used by itself. Substrate solutions/suspensions, which were used as an indicator of the substrate specificity of the pericarp browning enzymes, were made up as 1 mg/ml water of ferulic acid

(5.1×10^{-3} M), gallic acid (5.9×10^{-3} M), pyrogallol (7.9×10^{-3} M), MBOA (6.1×10^{-3} M), and rutin (1.5×10^{-3} M). Potential inhibitor solutions (10^{-3} M) were mercuric chloride, potassium cyanide, phenylthiourea, kojic acid, and ascorbic acid. Two punctures were left untreated to serve as controls. Kernels were examined immediately after adding solutions and after 4 hr and 24 hr for browning reactions (typically the width of the browned area) or other changes. Treatments were replicated at least five times for each genotype. Differences in the degree of browning were examined for significance by simple analysis of variance.

Electrophoretic Separation of Peroxidase Isozymes. Pericarps from milk-stage kernels were removed by slicing the kernels longitudinally along the narrowest portion (sides) and pealing them apart. Typically eight kernels were used for each assay. Milk-stage kernels of the *Ch* varieties were removed from cobs by carefully prying them out with a pointed spatula in order to avoid damaging them. One set of kernels was held at 25°C for two days to stimulate browning, while another set was held at 4°C for the same period of time to inhibit browning. Some of these *Ch* kernels were used immediately in enzyme assays, while the rest were allowed to air dry and were used in subsequent insect bioassays. Tissues were homogenized in 2 ml of pH 7.4, 0.1 M sodium phosphate buffer with a ground glass homogenizer. The homogenates were centrifuged at 1200 *g* for 5 min, and the supernatants were used for the assays.

Electrophoretic separation on 7.5% polyacrylamide gels was performed as previously described (Dowd and Sparks, 1986). Ten microliters of homogenate was used per well, and bromophenol blue was used as a tracking dye. Tris glycine buffer (0.1 M, pH 8.6) was used as the mobile phase under 35 mA at 2000 V. Protein concentrations of the homogenates were determined using the Bio-Rad (1976) packaged assay.

Isoelectric focusing was performed using precast wide range (pH 3.5–9.0, Pharmacia-LKB) gels, as previously described (Dowd and Sparks, 1986). Homogenates (16 μl) were applied to filter paper application squares placed in the center of the gel lanes. Application squares were removed after 45 min of electrofocusing. Gels were electrofocused at 25 W until 1/2 hr after the Evan's blue tracking dye formed discrete bands (total run time was typically 1.5 hr). One-centimeter sections of gel were placed in 1 ml of deionized water at least overnight, and pH readings were taken. Assays were preformed at least in duplicate on two separate occasions.

Enzymes were detected on electrophoresed gels by a variety of methods. General peroxidase activity was determined by a method modified from a histochemical method (Conyers and Kidwell, 1991). Ten milligrams of 4-chloronaphthol and dimethylphenylenediamine (DMPD) were dissolved separately in 1.5 ml of ethanol. A detection solution consisted of 25 ml of pH 7.4, 0.1 M sodium phosphate buffer, 3 ml of 3% hydrogen peroxide (made from a

30% stock), and the ethanol solutions of 4-chloronaphthol and DMPD. DOPA oxidation was determined using the same buffer and peroxide solutions, plus 30 mg of DOPA, with or without hydrogen peroxide. Ferulic acid oxidation was determined directly using 30 mg of ferulic acid in the buffer–peroxide solution. MBOA oxidation was determined directly using 10 mg of MBOA dissolved in 1 ml ethanol in the standard buffer and peroxide mixture. It and ferulic acid oxidation were also measured in a coupled reaction where the gel was incubated under the conditions just described, then 4 ml of 1:30 solution of 4-chloronaphthol and DMPD allowed to react for 30 min were added. Oxidation of rutin, luteolin, and coumaric acid were also determined in this manner, except that 30 mg of substrate was used. Assays were performed at least in duplicate on two separate occasions.

Spectrophotometric Enzyme Assays. All assays were performed with the same total volume of 1 ml: typically with 100 μl of substrate solution, 100 μl of enzyme solution, 100 μl of a 30% hydrogen peroxide solution diluted 1:100, and 700 μl of pH 7.4, 0.1 M sodium phosphate buffer. Concentrations of substrates were typically 1 mg/ml dissolved in ethanol, buffer, or water. Controls contained relevent solvents. Assays were run for 10 min at 30°C on a Perkin-Elmer Lambda 4B high-performance spectrophotometer using the kinetics software package. Preliminary scans were made to determine peak absorbance values for quinone or other products generated to be used in subsequent assays and are as follows (in nm): ferulic acid, 420; DOPA, 480. Assays were performed at least in duplicate on two separate occasions. Values were examined for significance by simple analysis of variance.

Determination of Relative Phenolic Concentration of Pericarps. Relative phenolic content of brown and nonbrown CH and CHH pericarps was determined spectrophotometrically. Dried kernels were soaked for 48 hr in deionized water at 4°C to loosen pericarps. Kernels were split parallel to the widest portion of the crown in order to give an intact "side," and the pericarps were pealed off. The nongerm side pericarp was trimmed to 5 × 5 mm. The bottom was removed from a quartz cuvette, and the cuvette was separated diagonally along the long axis so that two pieces were obtained, each containing two sides, one of which was the transmission side. The pericarp squares were affixed to the inside of the transmission side of the cuvette with deionized water in the center of the light path. Preliminary scans with the same spectrophotometer described previously indicated two broad-crowned peaks with crowns that ranged in width from 240 to 290 nm and 410 to 450 nm, as well as a sharp peak that ranged in peak absorbance from 315 to 320 nm. Thus, absorbance was determined at three wavelengths: 280 nm (aromatic ring wavelength, and one at which the broad-crowned peak was typically at a maximum), 420 nm (browning wavelength as was monitored in spectrophotometric enzyme assays), and 315–320 nm (with peak absorbance determined by scanning each time for this narrower peak).

Absorbances were corrected for background with the trough value that typically occurred at 380 nm (typically about 0.5–0.6 absorbance units). Representative pericarps from four different kernels of each type were examined. Values were examined for significance by simple analysis of variance.

RESULTS

As reported previously (Dowd, 1994), the Mp313E inbred and, to a lesser extent, the hybrid exhibited greater browning when damaged compared to the SC212M inbred (see below). Although the zone of browning was larger for the Mp313E, the depth of color was approximately the same, but difficult to determine due to the small area browned on the SC212M. CH and CHH held at room temperature turned nearly as brown as original mature seed (Munsell ratings of 10YR3/3 = dark brown and 10YR3/6 = dark yellowish brown, respectively), while the corresponding refrigerated CH and CHH pericarps did not brown or browned only slightly (Munsell ratings of 10YR8/2 = very pale yellow and 5Y8/2 = pale yellow, respectively).

As described previously, the Mp313E had a mean of about one kernel damaged by *C. lugubris* in on-ear assays compared to several for the SC212M (Dowd, 1994). In the present study, only 5% (1/20) of the CH kernels and 7.7% (1/19) of the CHH kernels were damaged by *C. lugubris*, compared to over 25% of kernels damaged for some commercial hybrids in similarly conducted assays (Dowd, unpublished data). The CH and CHH mutants brown only during senescence, so on-plant assays with sap beetles (which generally feed on milk-stage kernels) were not relevant. However, they did brown to some extent during the laboratory assays.

Adding aryl oxidase enzyme to corn powder containing ferulic or coumaric acid darkened the powder. For the ferulic acid corn powder alone, the Munsell rating was 5Y8/2 = pale yellow, and when the peroxidase + hydrogen peroxide was added it was 2.5Y7/4 = pale yellow (but darker than the material treated with ferulic acid alone). For the coumaric acid corn powder alone, the Munsell rating was 2.5Y7/4 = pale yellow, and when tyrosinase was added the rating was 5Y6/4 = pale olive. Reduced feeding and/or increased mortality were typically noted for insects fed brown vs. nonbrown corn powder amended with corn-associated phenolics and plant enzymes from other sources, although development of sap beetle larvae in no-choice assays was typically reduced only slightly (Tables 1–3). An especially dramatic response was noted for the coumaric acid oxidized by tyrosinase and fed to neonate *H. zea* and *S. frugiperda*, both exhibiting increased mortality and decreased weights of survivors. This material was closest in darkness to the Mp313E inbred and hybrid pericarps, but less dark that the fully browned CH and CHH mutants. Maize weevils

TABLE 1. EFFECT OF BROWN VS. NONBROWN CORN KERNEL POWDER ON NEONATE CATERPILLARS[a]

Material	N	Mortality (%)	Weight (mg)
H. zea			
Solvent	33	36.4a	2.8 ± 0.3a
Coumaric acid	32	43.8a	2.4 ± 0.4a
Tyrosinase	37	45.9a	3.1 ± 0.5a
Coumaric acid + tyrosinase	32	75.0b	0.1 ± 0.1b
Solvent	34	29.4a	4.6 ± 0.7a
Ferulic acid	40	16.7a	4.4 ± 0.5a
H_2O_2 + peroxidase	36	27.5a	4.6 ± 0.6a
Ferulic acid + H_2O_2 + peroxidase	38	18.4a	3.1 ± 0.4a (P < 0.06)
S. frugiperda			
Coumaric acid	17	0.0a	2.4 ± 0.3a
Coumaric acid + tyrosinase	18	66.7b	0.3 ± 0.1b
Ferulic acid	18	5.6a	1.8 ± 0.2a
Ferulic acid + H_2O_2 + peroxidase	20	25.0a	0.2 ± 0.1b
O. nubilalis			
Coumaric acid	20	0.0a	1.1 ± 0.1a
Coumaric acid + tyrosinase	19	26.3b	0.8 ± 0.2a
Ferulic acid	20	5.0a	1.4 ± 0.2a
Ferulic acid + H_2O_2 + peroxidase	17	0.0a	1.4 ± 0.2a

[a]Weight values are means ± standard errors. Values in columns of like studies followed by the same letter are not significantly different at $P < 0.05$ by chi-square analysis (mortality) or simple analysis of variance (weights).

damaged nonbrown CH and CHH kernels to a greater extent than the browned forms and survived less effectively on the browned forms (Table 4).

Puncture assays on intact ears demonstrated a generally greater browning response by the Mp313E inbred pericarps than those of the Mp313E × SC212M hybrid, which was generally greater than that of the SC212M inbred both at 4 and 24 hr (Table 5). Browning of all pericarp sources was especially enhanced over controls by adding tyrosinase, chlorogenic acid, gallic acid, or pyrogallol. Browning of Mp313E pericarps was also significantly increased when all other potential substrates were added, except for rutin, after 24 hr. Inhibitors had no significant effect on the browning of SC212E pericarp, but all zero values prevented statistical analysis by analysis of variance at the 24-hr period. Kojic acid

TABLE 2. EFFECT OF BROWN VS. NONBROWN CORN KERNEL POWDER ON SAP BEETLE ADULTS AND LARVAE IN CHOICE ASSAYS[a]

Material	Feeding rating
C. lugubris adults	
Coumaric acid	3.9 ± 0.1a
Coumaric acid + tyrosinase	2.5 ± 0.3b
Ferulic acid	2.9 ± 0.3a
Ferulic acid + H_2O_2 + peroxidase	2.0 ± 0.2a
C. lugubris larvae	
Coumaric acid	3.8 ± 0.2a
Coumaric acid + tyrosinase	1.2 ± 0.2b
Ferulic acid	3.5 ± 0.3a
Ferulic acid + H_2O_2 + peroxidase	1.8 ± 0.2b
C. hemipterus adults	
Coumaric acid	3.0 ± 0.3a
Coumaric acid + tyrosinase	2.5 ± 0.2a
Ferulic acid	3.4 ± 0.3a
Ferulic acid + H_2O_2 + peroxidase	3.0 ± 0.4a
C. hemipterus larvae	
Coumaric acid	3.9 ± 0.1a
Coumaric acid + tyrosinase	2.0 ± 0.3b
Ferulic acid	3.6 ± 0.3a
Ferulic acid + H_2O_2 + peroxidase	2.4 ± 0.2b
C. freemani adults	
Coumaric acid	3.4 ± 0.3a
Coumaric acid + tyrosinase	2.4 ± 0.3b
Ferulic acid	3.2 ± 0.2a
Ferulic acid + H_2O_2 + peroxidase	1.9 ± 0.2b
C. freemani larvae	
Coumaric acid	3.5 ± 0.2a
Coumaric acid + tyrosinase	2.7 ± 0.3b
Ferulic acid	3.9 ± 0.1a
Ferulic acid + H_2O_2 + peroxidase	2.9 ± 0.2b

[a] Feeding rating values are means ± standard errors. Values in columns of like studies followed by the same letter are not significantly different at $P < 0.05$ by simple analysis of variance. All assays were run with eight groups of insects except for coumaric acid assays with *C. lugubris* larvae, which were run with four groups.

TABLE 3. EFFECT OF BROWN VS. NONBROWN CORN KERNEL POWDER ON SAP BEETLE LARVAE IN NO-CHOICE ASSAYS[a]

Material	N	Weight (mg)
C. lugubris		
Coumaric acid	20	2.5 ± 0.2a
Coumaric acid + tyrosinase	13	1.9 ± 0.2a ($P < 0.054$)
Ferulic acid	12	2.6 ± 0.1a
Ferulic acid + H_2O_2 + peroxidase	11	2.1 ± 0.1b
C. hemipterus		
Coumaric acid	20	2.9 ± 0.1a
Coumaric acid + tyrosinase	17	2.7 ± 0.2a
Ferulic acid	40	2.4 ± 0.1a
Ferulic acid + H_2O_2 + peroxidase	39	2.1 ± 0.1b
C. freemani		
Coumaric acid	14	1.2 ± 0.1a
Coumaric acid + tyrosinase	18	1.1 ± 0.1a
Ferulic acid	18	1.4 ± 0.1a
Ferulic acid + H_2O_2 + peroxidase	20	1.3 ± 0.1a

[a]Weight values are means ± standard errors. Values in columns of like studies followed by the same letter are not significantly different at $P < 0.05$ by simple analysis of variance.

TABLE 4. EFFECT OF BROWN VS. NONBROWN *Ch* KERNELS ON *S. zeamais* ADULTS.

Material	N	Mortality (%)	Damage (mm)
CH	30	16.7a	3.2 ± 0.3a
CH-brown	29	51.7b	3.9 ± 0.5a
CHH	30	56.7a	3.5 ± 0.3a
CHH-brown	30	76.7a ($P < 0.10$)	2.4 ± 0.5a

[a]Weight values are means ± standard errors. Values in columns of like studies followed by the same letter are not significantly different at $P < 0.05$ by chi-square analysis (mortality) or simple analysis of variance (damage).

TABLE 5. BROWNING RESPONSES OF DAMAGED KERNELS

	Browning of pericarp (mm)[b]					
	Mp313E		Hybrid		SC212M	
Treatment[a]	4 hr	24 hr	4 hr	24 hr	4 hr	24 hr
C	0.5 ± 0.2x	1.2 ± 0.5x	0.1 ± 0.3	0.7 ± 0.3y	0.0 ± 0.0	0.4 ± 0.2z
PH	0.5 ± 0.3	1.1 ± 0.6	0.0 ± 0.0	0.8 ± 0.3	0.0 ± 0.0	0.1 ± 0.3
TY	1.5 ± 0.6cx	2.3 ± 0.5cx	0.4 ± 0.7y	1.3 ± 0.8cy	0.6 ± 0.2y	0.8 ± 0.5y
H	0.4 ± 0.2	1.2 ± 0.5x	0.0 ± 0.0	0.8 ± 0.4y	0.0 ± 0.0	0.0 ± 0.0
PX	0.5 ± 0.3	1.2 ± 0.5x	0.0 ± 0.0	0.8 ± 0.4y	0.0 ± 0.0	0.0 ± 0.0
HPX	0.7 ± 0.3	1.2 ± 0.6x	0.0 ± 0.0	0.8 ± 0.5xy	0.0 ± 0.0	0.2 ± 0.3z
DP	1.4 ± 0.6cx	2.2 ± 0.8cx	0.8 ± 0.6cy	2.0 ± 0.7cx	0.1 ± 0.2x	0.5 ± 0.0
CA	1.0 ± 0.4cx	1.9 ± 1.2cx	0.1 ± 0.3y	1.1 ± 0.6cy	0.0 ± 0.0	0.1 ± 0.2z
FA	0.9 ± 0.6cx	1.8 ± 0.8cx	0.1 ± 0.3	0.9 ± 0.7y	0.0 ± 0.0	0.1 ± 0.2z
GA	1.5 ± 0.7cx	2.9 ± 0.7cx	1.2 ± 0.7cx	1.8 ± 0.9cy	0.2 ± 0.3y	0.6 ± 0.2z
RT	0.8 ± 0.4cx	1.6 ± 0.7x	0.0 ± 0.1y	0.9 ± 0.2y	0.0 ± 0.0	0.0 ± 0.3z
PY	1.6 ± 0.5cx	2.4 ± 0.7cx	1.0 ± 0.8cyz	2.4 ± 0.5cx	1.2 ± 0.5z	1.8 ± 0.6cy
PT	0.4 ± 0.3	1.1 ± 0.5x	0.0 ± 0.0	0.7 ± 0.3y	0.0 ± 0.0	0.5 ± 0.0
CN	0.3 ± 0.3	0.8 ± 0.2cx	0.0 ± 0.0	0.6 ± 0.4xy	0.0 ± 0.0	0.2 ± 0.3z
KA	0.3 ± 0.3c	1.1 ± 0.5x	0.0 ± 0.0	0.3 ± 0.4cy	0.0 ± 0.0	0.3 ± 0.3z
HG	0.3 ± 0.3	0.6 ± 1.0	0.0 ± 0.0	0.8 ± 0.8	0.0 ± 0.0	0.0 ± 0.0
AA	0.3 ± 0.3	1.0 ± 0.6x	0.2 ± 0.3	0.6 ± 0.2xy	0.0 ± 0.0	0.3 ± 0.3y

[a] C = control, PH = phenolase, TY = tyrosinase, H = hydrogen peroxide, PX = peroxidase, DP = DOPA, CA = chlorogenic acid, FA = ferulic acid, GA = gallic acid, RT = rutin, PY = pyrogallol, PT = phenylthiourea, CN = cyanide, KA = kojic acid, HG = mercury, AA = ascorbic acid.
[b] Treatments in columns followed by a "c" are significantly different from controls at $P < 0.05$ by simple analysis of variance. Treatments in rows of like time intervals followed by the same letter are not significantly different from each other at $P < 0.05$ by simple analysis of variance.

significantly inhibited browning of the hybrid pericarp, while CN^- and Hg^{+2} ion had a clear inhibitory effect on the Mp313E pericarp browning.

Spectrophotometric assays of pericarp homogenates indicated a greater rate of oxidation of ferulic acid and DOPA by the Mp313E compared to the hybrid and SC212M (Table 6). The same trend was noted for ferulic oxidation by brown vs. nonbrown pericarp homogenates from CH and CHH. However, oxidation of DOPA by the *Ch* preparations was much lower than that by the inbreds and hybrid. Due to the large volume of enzyme preparation required to see oxidation for luteolin, rutin, coumaric acid, and MBOA, these assays were not run.

More peroxidase isozymes visualized by the general CN-DMPD assay were typically present from pericarp homogenates of varieties that browned (Mp313E vs. SC212M, brown CH and CHH vs. nonbrown CH and CHH) compared to

TABLE 6. RELATIVE RATES OF OXIDATION OF PHENOLICS BY PERICARP HOMOGENATES DETERMINED SPECTROPHOTOMETRICALLY[a]

	Change in absorbance over 10 min/mg protein	
Homogenate	Ferulic acid	DOPA
MP313E	1.25 ± 0.06a	0.32 ± 0.01a
Hybrid	0.66 ± 0.02b	0.26 ± 0.01b
SC212M	0.61 ± 0.04b	0.16 ± 0.01c
CH-nonbrown	0.36 ± 0.01a	0.02 ± 0.01a
CH-brown	1.23 ± 0.04b	−0.01 ± 0.02a
CHH-nonbrown	0.29 ± 0.02a	0.03 ± 0.01a
CHH-brown	1.01 ± 0.04b	0.03 ± 0.01a

[a]Values are means ± standard errors. Values in columns for like material followed by the same letter are not significantly different by simple analysis of variance.

corresponding materials (Figures 1 and 2). The best comparison is for the CH and CHH, since these were identical germplasms that were treated with different temperatures to induce/retard browning. A similar trend was noted when isoelectric focusing was used (Figure 3 and 4). In both separation methods, some of the enzymes produced appeared to be the result of a dominant gene, since they were present at the same spot in Mp313E and the hybrid, but not in SC212M. Some cathodic forms were also more prominant in the brown(ing) vs. nonbrown(ing) forms. Identity with relevant corn-associated substrates was generally similar to that noted with the general CN-DMPD assay, although many substrates tested produced fewer bands. Although faint, banding with ferulic acid and MBOA alone was the same as when they were used in coupled assays. Ferulic acid bands often coincided with "extra" bands found in Mp313 and the hybrid and the brown CH and CHH when the CN-DMPD assay was used. Flavonoids were not oxidized by the fast anodic bands in conventional polyacrylamide gel electrophoretic separations of the Mp313 series, while basic isozymes acted against the most substrates when separated by isoelectric focusing in this series. The majority of activity induced in the CH series and separated by conventional polyacrylamide gel electrophoresis was cathodic and had wide substrate specificity, while the CHH-induced activity was primarily anodic and reacted with few substrates. Isozymes in the CH and CHH series that were induced and separated by isoelectric focusing and had the widest substrate range were primarily basic, although some acidic isozymes also reacted with a number of substrates.

Absorbances at 420 nm were typically higher for brown compared to non-

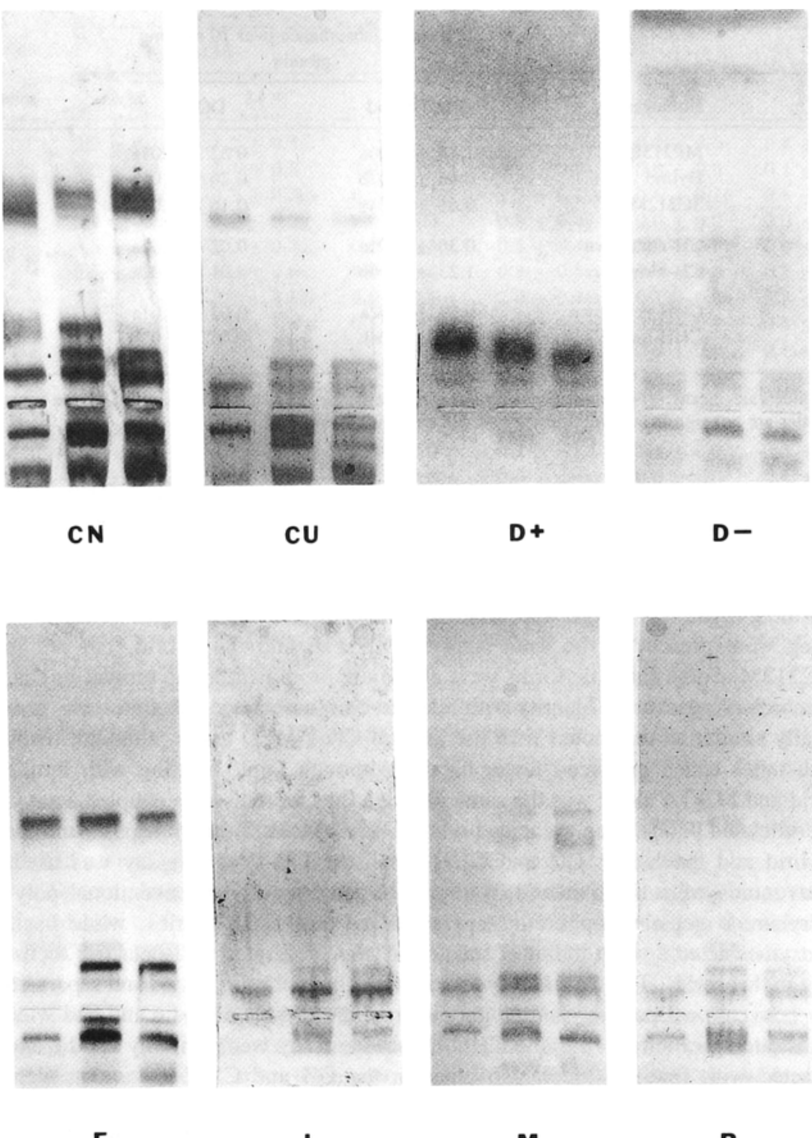

FIG. 1. Oxidation of substrates by pericarp peroxidase isozymes separated by conventional polyacrylamide gel electrophoresis. CN = 4-chloronaphthol, CU = coumaric acid, D+ = DOPA + hydrogen peroxide, D− = DOPA without hydrogen peroxide, F = ferulic acid, L = luteolin, M = MBOA, R = rutin. For each substrate, isozymes are located in lanes as follows: Mp313E, right; SC212M, left; and hybrid, center. See text for assay conditions.

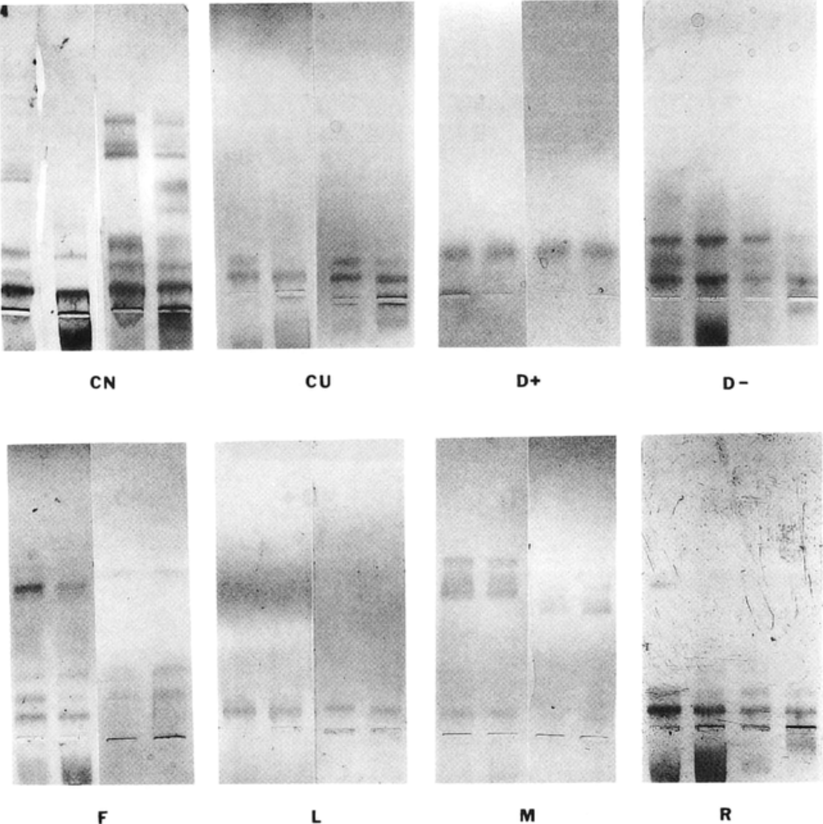

FIG. 2. Oxidation of substrates by pericarp peroxidase isozymes separated by conventional polyacrylamide gel electrophoresis. CN = 4-chloronaphthol, CU = coumaric acid, D+ = DOPA + hydrogen peroxide, D− = DOPA without hydrogen peroxide, F = ferulic acid, L = luteolin, M = MBOA, R = rutin. For each substrate, isozymes are located in lanes as follows: CH nonbrown, far left; CH brown, near left; CHH nonbrown, near right; and CHH brown, far right. See text for assay conditions.

brown *Ch* pericarps, as were absorbances at the 320-nm region [scans varied in peak location from 320 to 315 nm (Table 7)]. Absorbances at 280 nm were less for the brown compared to the nonbrown *Ch* pericarps. The differences in absorbances at 320 and 280 nm were greater for the brown compared to the nonbrown material. Scans of peroxidase browning of ferulic acid solutions indicated little change in absorbance of a peak at 285 nm, but the reduction of a peak at 305 nm and the appearance of the peak in the 420-nm region.

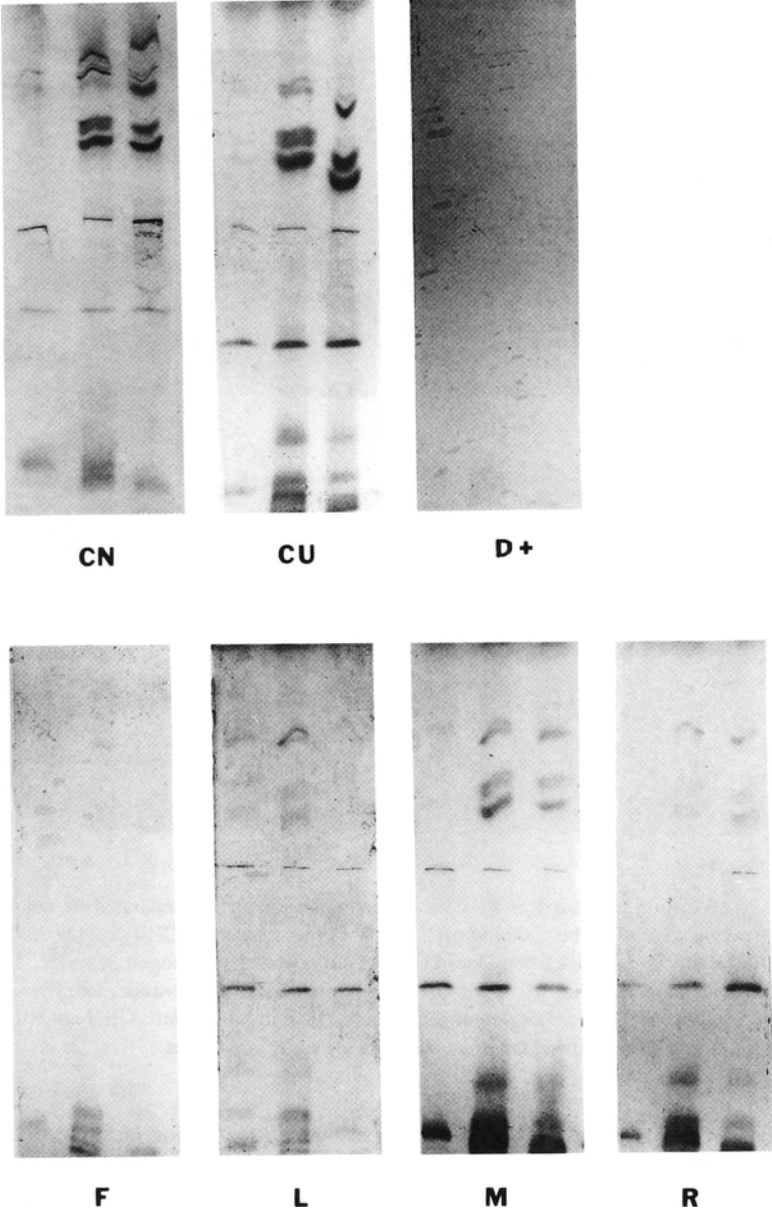

FIG. 3. Oxidation of substrates by pericarp peroxidase isozymes separated by isoelectric focusing. CN = 4-chloronaphthol, CU = coumaric acid, D+ = DOPA + hydrogen peroxide, F = ferulic acid, L = luteolin, M = MBOA, R = rutin. For each substrate, isozymes are located in lanes as follows: Mp313E, right; SC212M, left; and hybrid center. Top of gel is pH 3.5, bottom of gel is pH 9.0. The pH range along the gel is uniformly distributed within 0.1 pH units. See text for assay conditions.

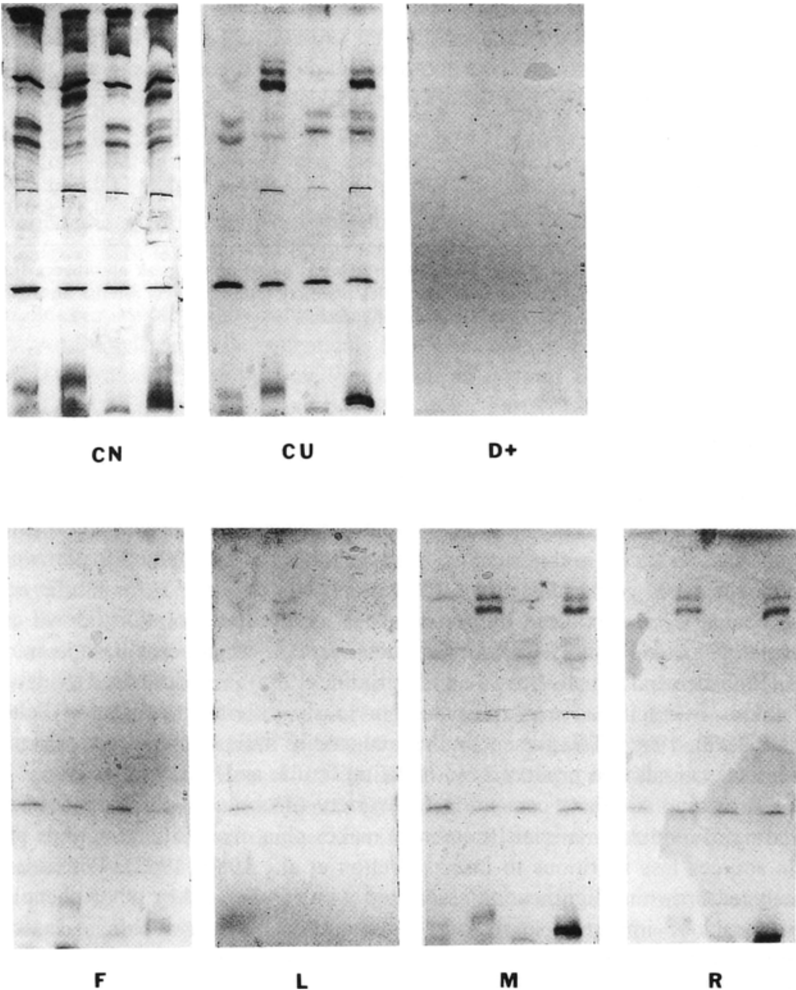

FIG. 4. Oxidation of substrates by pericarp peroxidase isozymes separated by isoelectric focusing. CN = 4-chloronaphthol, CU = coumaric acid, D+ = DOPA + hydrogen peroxide, F = ferulic acid, L = luteolin, M = MBOA, R = rutin. For each substrate, isozymes are located in lanes as follows: CH nonbrown, far left; CH brown, near left; CHH nonbrown, near right; and CHH brown, far right. Top of gel is pH 3.5, bottom of gel is pH 9.0. The pH range along the gel is uniformly distributed within 0.1 pH units. See text for assay conditions.

TABLE 7. ABSORBANCES OF BROWN AND NONBROWN PERICARPS AT DISCRETE WAVELENGTHS[a]

Pericarp	420 nm	320 nm	280 nm	320–280 nm
CH-nonbrown	0.09 ± 0.02	0.60 ± 0.09	0.26 ± 0.03	0.34 ± 0.08
CH-brown	0.11 ± 0.03	0.70 ± 0.08	0.23 ± 0.02	0.47 ± 0.06
CHH-nonbrown	0.17 ± 0.01	0.79 ± 0.05	0.55 ± 0.06	0.24 ± 0.03*
CHH-brown	0.19 ± 0.01	0.86 ± 0.02	0.41 ± 0.04	0.45 ± 0.02

[a] Values are means ± standard errors. The 320-nm value is based on the peak absorbance from scans in this region. Values in columns of like pericarp variety followed by an asterisk are significantly different at $P < 0.05$ by simple analysis of variance.

DISCUSSION

Browning Reactions and Insect Responses. Compared to nonbrowned material, browned material, whether naturally generated in the pericarp or generated by combining purified enzymes and substrates, was generally resistant to feeding by the insects tested in this study. The same trend has been noted for browned vs. nonbrowned corn callus tissue (Dowd and Norton, 1995), for ferulic acid and coumaric acid oxidized by peroxidase in protein-free solution (Dowd and Vega, 1995), and rapidly browning tissues overexpressing peroxidase in transgenic tobacco and tomato (Dowd and Lagrimini, 1994). Tomato-based oxidation of chlorogenic acid has been associated previously with insect resistance (Felton et al., 1989, 1992). The present and related studies indicate enhanced resistance to insects can also be produced by oxidizing ferulic and coumaric acids.

Browning reactions can produce a variety of obstacles to insect feeding. Binding of oxidized materials (quinones) makes plant material and various protein sources less nutritious to insects (Felton et al., 1989, 1992). Peroxidase-catalyzed browning/lignification associated with ferulic acid or other phenolics also could be important in making other nutrients less available to insects. Ferulic acid can be dimerized by peroxidases and cross-linked to other compounds (Markwalder and Newkom, 1976; Fry, 1986). Ferulic acid often exists as sugar esters bound to cell walls (Harris and Hartley, 1970; Kato and Nevins, 1985; Ohashi et al., 1987), and steroid esters (Evershed et al., 1988; Seitz, 1989; Norton, 1994). Thus, dimerization or complexing with proteins should decrease the availability of all of these complexed nutrients to insects and increase cross-linking of structural components.

The pericarp of corn is also high in hydroxyproline-rich proteins (Hood et al., 1991). Quinones are known to react with these proteins in melon and bean to form inpenetrable barriers to pathogens through cross-linking (O'Connel et

al., 1990). These proteins may also form a framework for lignin deposition (Vance et al., 1980). Cell wall rigidity of spinach cell cultures is increased by peroxidase activity that cross-links ferulic acid sugars bound to cell walls (Fry, 1979). Tomato fruit skin, which is analogous to corn kernel pericarp, was more highly lignified in transgenic plants overexpressing tobacco anionic peroxidase than wild-type plants (Lagrimini et al., 1993); these transgenic fruits were also more resistant to neonate *H. zea* and were penetrated less frequently by third-instar *H. zea* (Dowd and Lagrimini, 1994), suggesting lignification reduced or deterred the insect's ability to penetrate the skin. Tougher plant material is associated with resistance to insect feeding (Coley, 1983; Raupp, 1985). Thus, if the pericarps are more highly lignified and otherwise cross-linked, the increased toughness may physically limit insect penetration.

Additional corn allelochemicals that were oxidized by pericarp enzymes, such as peroxidases, as indicated by on-ear assays and assays of enzymes separated by electrophoresis, may occur in other parts of the corn plant and be oxidized by some of the same isozymes that occur in the pericarp and are widely distributed in different corn tissues (Brewbaker and Hasegawa, 1975). These oxidation products may also adversely affect insects. DOPA is a major component of brown rice koji (Ohba et al., 1971) and has been reported from some plants (Reese, 1978). Cross-linking of tyrosine residues attached to the hydroxyproline-rich glycoproteins (Fry, 1982) in a manner analogous to that described for ferulic acid esters can also occur. The structural similarity of tyrosine and DOPA suggests isozymes visualized with DOPA may be the ones involved in cross-linking the tyrosine esters. A peroxidase product of MBOA adversely affects leafhopper survival (Dowd and Vega, 1995). Flavonoids may be acted on by peroxidases to ultimately form potentially toxic benzoquinone derivatives (Barz and Hoesel, 1978). Rutin oxidized by peroxidases can significantly deter feeding by leafhoppers (Dowd and Vega, 1995). Thus, peroxidases may also interact with a number of other plant allelochemicals found in maize to deter insect feeding.

The work with pericarps reported here and past work with other plant tissues have demonstrated the involvement of oxidative, browning type reactions in insect resistance. However, there are other examples that suggest browning enzymes may reduce the toxicity of their substrates to insects. Browning of corn silks is often inversely correlated with *H. zea* resistance (Byrne et al., 1989). An exception is the variety Zapalote Chico, which browned rapidly but was also resistant to *H. zea* (Byrne et al., 1989). Recent work by Wiseman and Isenhour (1993) demonstrated that adding antioxidants to diets containing silks from Zapalote Chico, for which the resistance compound is maysin, a luteolin derivative (Waiss et al., 1979), inhibits toxic effects compared to diets that contain no antioxidants. This suggests that maysin needs to be oxidized to be effective and that silk-derived peroxidases could be the normal source of oxidation. In

other varieties, the silk components may polymerize to less harmful compounds, as has been suggested by Miles and Oertli (1993) for catechin and potentially other compounds in assays with rose aphids and the aphid-produced peroxidase. However, the peroxidases of bird cherry oat-aphids (*Rhopalosiphon padi* L.), are inhibited by several phenolic compounds, including ferulic and coumaric acids (Urbanska and Leszczynski, 1992). It appears that appropriate enzymes and appropriate substrates need to be present (or absent) to obtain a browning response that results in insect resistance. In the case of the pericarp, additional reactions other than simple quinone formation and protein binding are also involved, which would produce additional mechanistic effects not likely to be present in silks.

Peroxidase Properties. The activity of peroxidases is characteristically enhanced by hydrogen peroxide. Although polyphenol/catechol oxidase may be present in plants, as indicated by use of differing substrates for peroxidase vs. polyphenol oxidase (van Loon, 1971), in the present study the same bands were detected using a general substrate such as 4-chloronaphthol with hydrogen peroxide (a peroxidase assay) in a short period of time (10–15 min) as were detected using DOPA without hydrogen peroxide (a polyphenol/catechol oxidase assay) over a long period of time (4 hr) for isozymes separated by conventional polyacrylamide electrophoresis. This enhancement of activity in the presence of hydrogen peroxide suggests it would be more relevant to consider the isozymes detected to be peroxidases, as was found for the enzymes tested spectrophotometrically and electrophoretically in the present study.

The activity of peroxidases is often inhibited by cyanide, PTU, ascorbic acid, and/or mercury ions (Gaspar et al., 1982), as was found for the activity in the present study during in vivo assays. Kojic acid is produced by several species of kernel-invading *Aspergillus* and *Penicillium* (Turner and Aldrich, 1983). Kojic acid inhibits metal-ion containing oxidative enzymes, such as amino acid oxidase (Turner and Aldrich, 1983), unspecific monooxygenase (Dowd, 1988), and polyphenol oxidase (Chen et al., 1991). In cases where oxidative enzymes, such as peroxidase or polyphenol oxidase, are involved in fungus resistance, kojic acid could interfere with the response. The ferulic acid oxidation monitored spectrophotometrically was not significantly inhibited by kojic acid, but oxidation of DOPA was in both Mp313E and SC212M extracts (60.2 $\pm$ 3.6% and 56.4 $\pm$ 1.2%, respectively). Thus, kojic acid could interfere with cross-linking of tyrosine residues. However, because both fungus-resistant and -susceptible materials responded similarly to kojic acid, the importance of this resistance to the potential effects of kojic acid remains unclear.

Although the relevant substrate/enzyme relationship was not always clear, the present study indicates that enhanced browning of pericarp in corn is associated with additional peroxidase isozymes. This was true in the case of representative inbreds, hybrids, and most notably and clear-cut for varieties of corn

containing the mutant *Ch* gene in browning vs. nonbrowning tissue. As would be expected, these additional enzyme types are often associated with corresponding increases in the ability to oxidize materials that either directly produce brown materials (quinones), such as ferulic acid, or are known to bind to protein (or other polymers) and produce browned material. However, the in vitro browning simulation assays may not include all possible effects, especially that of cross-linked, hardened, and/or lignified cell walls, which would produce a barrier to insect feeding and eventual breaching of the pericarp so that the inside of the seed can be fed upon. Inconsistencies may be due to additional substrates or quantities of substrate present in the pericarp and not tested to or complexing with additional compounds present in the pericarp. In the case of the wider substrate specificity of the Mp313E pericarp vs. the SC212M and the hybrid seen in on-kernel assays and assays with enzymes separated electrophoretically, it is possible that synergistic interactions between oxidized substrates enhance browning. This synergism of quinones appears to be involved in enhanced browning of damaged fruit tissue above what is seen in reactions with individual compounds (Lee, 1992). The strong reaction seen with tyrosine in the on-ear assays by all three varieties tested indicated phenolics occur in the pericarp of all of them. However, the significant difference in the degree of the reaction seen with the three varieties suggests the quantity and/or composition may differ, although tyrosinase increased the browning zone over that of the control by approximately twofold in each variety after 24 hr. Interestingly, compared to the SC212M, the Mp313E activity (additional bands) appears to be constitutive, while that of the *Ch* mutants is induced. This may explain the rapid browning response of the Mp313 inbred when damaged, since this would promote intermixing of compartmentalized peroxidases and substrates.

Different roles have been attributed to anionic/anodic and cationic/cathodic peroxidases but the role of a particular isozyme defined in this way may vary in different plant species. In tobacco (Schloss et al., 1987) anionic peroxidases are known to be associated with cell walls and involved in the lignification process and can rapidly oxidize phenolic acids such as caffeic or ferulic acid (Pickering et al., 1973). These isozymes are also associated with wound healing (Espelie et al., 1986), which may involve binding of ferulic and coumaric acids to cutins and suberins (Riley and Kolattukudy, 1975). Anionic isozymes were constitutive in Mp313E compared to SC212M and induced in browned CH and CHH materials. The isozymes present in Mp313E pericarp had a wider substrate range than those from SC212M that included ferulic acid, as indicated by on-ear assays. Assays involving enzymes separated by electrophoresis indicated the anionic forms of Mp313E had a wider substrate range than those from SC212M. However, the anionic enzymes from the CH and CHH did not have such a wide substrate range.

Cathodic (basic) peroxidases, associated with lignification and disease

resistance in reed canary grass, *Phalaris arundinacea* L. (Vance et al., 1976), may generate hydrogen peroxide from NAD(P)H and thus provide it for other peroxidase isozymes (Campa, 1991). Some cathodic forms may oxidize ferulic acid and be involved in cell wall stiffening (van Huystee and Zheng, 1993). In the present study, the activity of similar cathodic forms, which oxidized ferulic acid, were also enhanced in Mp313E vs. SC212M and in brown vs. nonbrown CH and CHH. In general, cathodic forms of peroxidases are thought to be involved in IAA regulation (Gaspar et al., 1982), although work with corn tissues and caffeic vs. IAA specificity indicates a less clear-cut trend (Brewbaker and Hasegawa, 1975). Isozyme 5, which is a slow cathodic, has a 3 : 1 ratio of band-rating intensity for caffeic acid to indole acetic acid oxidation (Brewbaker and Hasegawa, 1975). What is apparently this same isozyme was found at enhanced levels for some of the Mp313E material tested in the present study. Higher activity by this isozyme correlates well with prior studies (Fry, 1979) that indicate cross-linking of ferulates to increase cell wall rigidity is also associated with IAA degradation, which prevents wall expansion and also contributes to rigidity.

Although some of the isozymes produced by multiple alleles of the same peroxidase genes may have overlapping R_f values with those produced by other peroxidase genes when separated by electrophoresis (Brewbaker et al., 1985), the bands visualized in the present study appear to correspond with the standard groupings. The results of the present study indicate that both anionic, peroxidases and cathodic peroxidases are more likely involved in browning reactions, such as lignification, and can preferentially oxidize lignin precursors and their relatives, such as ferulic acid, and the corresponding lignin precursor, coniferyl alcohol. However, in the pericarp, oxidation of ferulic acid appears to be more relevant in promoting resistance. The anodic forms seen in the Mp313E, but not in the SC212M, appear to have a different substrate specificity than those from the browned vs. nonbrowned *Ch* mutants because the Mp313E isozymes were able to oxidize ferulic acid. Because the same isozymes occur in other corn plant tissues, with differing allelochemical content (e.g., flavonoids in leaves and silks), the presence of additional isozymes in tissues other than the pericarp may enhance resistance to insects in these tissues as well.

Implications. The present study indicates that brown pericarp, probably associated with oxidation of ferulic and coumaric acids and/or their derivatives, is an insect resistance factor. Differences in rates of browning are associated with increased rates of oxidation of appropriate substrates, such as ferulic and coumaric acids. However, other mechanisms may also be involved in pericarp-based resistance to insects in these and other varieties, such as pericarp architecture (Gomez et al., 1983), thickness (Tracey and Schmidt, 1987), and secondary chemicals reported in whole corn kernels (Serratos et al., 1987; Classen

et al., 1990). The secondary compound chemistry of the varieties used in this study is presently being determined.

Varieties of maize in which enhanced levels of ferulic acid and coumaric acid are associated with insect resistance (e.g., Serratos et al., 1987, Classen et al., 1990) and fungal resistance (Assabgui et al., 1993) when mature may also be more resistant at milk stage due to the activity of the peroxidases on ferulic acid. Enhanced peroxidase activity may be responsible for increased levels of ferulic acid seen in mature corn kernels. Binding of peroxidase-generated ferulyl quinones with more materials would take them out of the free pool. Because some of the enzymes responsible for ferulic acid biosynthesis are subject to feedback inhibition by ferulic acid and its relatives (Kahl, 1975; Ralph and Helm, 1993), continually taking ferulic acid out of the free pool through generation of reactive quinones by peroxidases would ultimately result in a higher ferulic acid content (in bound and unbound form). Work with developing wheat has indicated the free pool of ferulic acid in the pericarp decreases and the bound forms increase as the kernel matures, with total ferulic acid content also increasing (McCallum and Walker, 1991). Spectrophotometric scans of brown and nonbrown *Ch* pericarps indicated a reduction in absorbance in the aromatic region in the brown vs. nonbrown pericarps that was also noted in the enzymatic assays with ferulic acid. This suggests that the total phenolic content has not increased in the brown pericarps because peak absorbance in the aromatic region (260–280 nm) did not increase. The significance of the increase in the peak at 320 nm is unknown but could relate to transformation or binding of ferulic or coumaric acid.

Breeding programs that produce varieties with both high phenolic acid levels and high appropriate peroxidase isozyme activity may also produce material that is cross-resistant to insects and kernel-invading fungi. Enhanced peroxidase activity, which is inducible, occurs in maize hybrids and inbreds that are resistant to *Fusarium moniliforme* compared to susceptible ones (Brad et al., 1974). An additional advantage to general insect and fungal resistance would be that ferulic acid is known to inhibit aflatoxin production by *A. flavus* (Bilgrami et al., 1981). The ease of following the browning reaction directly or with reactive substrates through kernel (or other tissue) damage suggests breeding for this trait should be relatively straightforward. A simple visual indicator could be used to follow transference of the trait. Provided suitable concentrations of substrates are available, incorporating the ability to more rapidly brown as a response to wounding through genetic engineering should be a way to increase plant resistance to relevant insects and plant pathogens. Work with transgenic tobacco and tomato overexpressing tobacco anionic peroxidase has already provided a practical example of this in insect resistance (Dowd and Lagrimini, 1994).

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ATTRACTION OF *Carpophilus* spp. (COLEOPTERA: NITIDULIDAE) TO SYNTHETIC AGGREGATION PHEROMONES AND HOST-RELATED COATTRACTANTS IN AUSTRALIAN STONE FRUIT ORCHARDS: BEETLE PHENOLOGY AND PHEROMONE DOSE STUDIES

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Abstract—Synthetic aggregation pheromones of *Carpophilus hemipterus* (L.) and *Carpophilus mutilatus* Erichson were field tested during a 10-month period in southern New South Wales stone fruit orchards to determine *Carpophilus* spp. phenology and the effect of two pheromone doses on attraction. Aggregation pheromones synergize the attraction of host volatiles to *Carpophilus* spp. Four major species, *C. hemipterus*, *C. mutilatus*, *C. davidsoni* Dobson and *C. (Urophorus) humeralis* (F.), were trapped, with greater numbers of each species in *C. hemipterus* pheromone/fermenting whole-wheat bread-dough-baited traps, than in dough-only-traps. In *C. mutilatus* pheromone/fermenting-dough-baited traps, only *C. mutilatus* and *C. davidsoni* responded in greater numbers than to dough-only traps. Beetles first appeared in traps in late September (early spring) when daily maximum temperatures averaged 17.5°C. Trappings reached a peak during October and declined to very low levels in November–December (late spring–early summer). Numbers trapped of all species increased during February–March (late summer–early autumn), presumably due to the presence of abundant host resources (ripening and fallen fruit), and continued at high levels until May (late autumn). An 18-week study demonstrated significantly greater responses by *Carpophilus* spp. to 5000-μg than to 500-μg doses of *C. hemipterus* and *C. mutilatus* pheromones.

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Greatest responses to 5000 μg were recorded for *C. hemipterus* and *C. mutilatus* responding to their own pheromones (increased attraction over dough alone of 259× and 21.2×, respectively). Implications of the study and the potential for using synthetic aggregation pheromones for managing *Carpophilus* spp. populations in Australian stone fruit are discussed.

Key Words—*Carpophilus hemipterus*, *C. mutilatus*, *C. davidsoni*, *C. humeralis*, Coleoptera, Nitidulidae, aggregation pheromones, stone fruit, phenology, dose.

INTRODUCTION

Nitidulid beetles (primarily *Carpophilus* spp.) are worldwide pests of fruits and grains, both before and after harvest (Hinton, 1945). In southern Australia these beetles are serious pests of ripening stone fruit, particularly apricots, nectarines, and peaches (Gaven, 1964; Hely et al., 1982). Fruit loss is caused primarily by adult beetles penetrating ripening fruit and causing rapid breakdown. Beetles can enter fruit by chewing through the skin, usually around the stem end or in sutures, although they often enter at sites of mechanical damage. *Carpophilus* spp. also serve as mechanical carriers of brown rot disease (Kable, 1969). The importance of *Carpophilus* spp. in Australian stone fruit production has increased in recent years, following a decline in insecticide use for key pests such as the Oriental fruit moth, *Grapholita molesta* Busck. The development and widespread utilization of a management strategy for *G. molesta* based on pheromonal disruption of mating (Vickers et al., 1985) has made stone fruit production in some areas of southern Australia largely insecticide free.

Current control of *Carpophilus* spp. in stone fruit is based on the use of broad-spectrum insecticides applied near harvest. Control is often unsatisfactory, requiring multiple applications, which can result in insecticide residues on harvested fruit. In addition, the use of broad-spectrum materials can precipitate outbreaks of other pests such as mites. Management of *Carpophilus* spp. using effective, nondisruptive techniques is essential if stability of stone fruit ecosystems and associated integrated and biological control systems is to be preserved.

Male-produced aggregation pheromones to which both sexes respond have been identified for a number of *Carpophilus* spp. including *C. mutilatus* Erichson (Bartelt et al., 1993) and *C. hemipterus* (L.) (Bartelt et al., 1990a). Both of these pheromones combined with food volatiles were active under field conditions in the United States (Bartelt et al., 1992, 1993, 1994), Israel (Blumberg et al., 1993), and Australia (James et al., 1993). Cross-attraction of various *Carpophilus* spp. to *C. hemipterus* and *C. mutilatus* pheromones has also been reported (Bartelt et al., 1992; Blumberg et al., 1993; James et al., 1993). Bartelt et al. (1994) showed the response of *C. mutilatus* and *C. hemipterus* to their

respective pheromones in a date garden in southern California increased with dose.

Synthetic pheromones appear to offer considerable potential for monitoring and control of *Carpophilus* spp. populations in stone fruit orchards. However, effective practical use of these infochemicals requires a better understanding of *Carpophilus* biology/ecology in orchard ecosystems, together with more information on optimal pheromone doses. Here we report the results of pheromone-trapping studies on *Carpophilus* spp. in two stone fruit orchards in the Murrumbidgee Irrigation Area of southern New South Wales. The studies provide data on the phenology of *Carpophilus* spp. responses to the synthetic pheromones of *C. hemipterus* and *C. mutilatus* during a 10-month period. They also provide information on responses to two doses of these pheromones.

METHODS AND MATERIALS

Synthetic Pheromones. The pheromone for *C. mutilatus* consisted of a 100:7 blend of (3*E*,5*E*,7*E*)-5-ethyl-7-methyl-3,5,7-undecatriene and (3*E*,5*E*,7*E*)-6-ethyl-4-methyl-3,5,7-decatriene (Bartelt et al., 1993). The pheromone for *C. hemipterus* consisted of a 100:31:11:8 blend of (2*E*,4*E*,6*E*,8*E*)-3,5,7-trimethyl-2,4,6,8-decatetraene, (2*E*,4*E*,6*E*,8*E*)-3,5,7-trimethyl-2,4,6,8-undecatetraene, (2*E*,4*E*,6*E*,8*E*)-7-ethyl-3,5-dimethyl-2,4,6,8-decatetraene, and (2*E*,4*E*,6*E*,8*E*)-7-ethyl-3,5-dimethyl-2,4,6,8-undecatetraene, respectively (Bartelt et al., 1992). The synthetic pheromones were purified only by distillation and open-column chromatography on silica gel. These procedures did not remove the small amounts of *Z* isomers produced in the syntheses (Bartelt et al., 1990b), but there is no evidence that these (presently unavoidable) impurities are detrimental to pheromonal activity (Bartelt et al., 1992).

Pheromones were appropriately diluted with hexane and stored in a freezer until needed. Concentrations of components were determined by gas chromatography on diluted aliquots [instrumentation as described by Bartelt et al. (1990a)]. Pheromone solutions (between 3 and 100 μl, depending on concentration and desired dose) were applied to rubber septa (11 $\times$ 20 mm, red rubber, Aldrich Chemical Co., Milwaukee, Wisconsin), followed by 300 μl of methylene chloride. Once the liquid had soaked into the septa, they were aired in a fume hood for 1 hr and stored in a freezer in tightly closed bottles until needed. For the dose studies, septa were prepared with 500 or 5000 μg of all *E* isomers per septum (proportions as noted above). For the phenological study, only the 500-μg dose was used.

Traps and Coattractant Baits. Wind-oriented pipe traps based on the design of Dowd et al. (1992) were used. Beetles entered the trap through a cone-shaped piece of screen and were finally trapped in a plastic 140-ml bottle attached to

the bottom of the trap. No killing agent was used, and a screen partition prevented beetles from contacting the bait. Fermenting whole-wheat bread dough was used as the pheromone synergist (approximately 10 ml/trap) held in a 20-ml glass tube. Pheromone septa were pinned to the screens of the bait compartments of the traps. Traps were suspended approximately 1.5 m above the ground from a wire attached to a tree branch. They were oriented to the wind by a fin so that the trap opening was always accessible to beetles approaching the traps from downwind.

Phenological Study. The response of *Carpophilus* spp. to *C. hemipterus* and *C. mutilatus* synthetic pheromones was monitored over a 10-month period in two small (approximately 50 × 50-m) stone fruit orchards near Leeton in southern New South Wales. *C. hemipterus* pheromone was used in an apricot orchard, adjacent to approximately 8 ha of mixed stone fruit (peaches, nectarines, plums). *C. mutilatus* pheromone was used in a canning peach orchard situated in a peach-growing district. Neither orchard had been exposed to broad-spectrum insecticides for 5–10 years.

At each site, 16 traps were used and incorporated four replicates of four treatments (pheromone only, fermenting dough only, pheromone plus dough, unbaited). Treatments were randomized in a 4 × 4 grid of trees spaced at 6-m intervals. Traps were examined weekly, beetles collected, and dough replaced. Pheromone septa were replaced fortnightly. Both orchards were flood irrigated with the apricots harvested in December and peaches in February.

Dose Study. The response of *Carpophilus* spp. to two doses (500 and 5000 μg) of *C. hemipterus* and *C. mutilatus* synthetic pheromone was monitored over an 18-week period (December 1992–April 1993) in a small peach orchard (25 × 25 m) at Yanco Agricultural Institute in southern New South Wales. Twenty-four traps incorporated four replicates of six treatments (unbaited, fermenting dough only, 500 μg *C. hemipterus* pheromone plus dough, 5000 μg *C. hemipterus* pheromone plus dough, 500 μg *C. mutilatus* pheromone plus dough, 5000 μg *C. mutilatus* pheromone plus dough). Treatments were randomized, although missing trees prevented the use of a uniform grid. Trees were spaced at 5-m intervals, and the orchard was flood-irrigated. Beetle collection and replacement of dough and septa were conducted as described above. Fruit was harvested in February.

Meteorological data were obtained from a recording station at the Yanco Agricultural Institute. All trapping data were subjected to analysis of variance and least significant difference procedures.

RESULTS

Phenological Study. Six species of *Carpophilus* were trapped at both sites: *C. hemipterus*, *C. mutilatus*, *C. davidsoni* Dobson, *C. (Urophorus) humeralis* (F.), *C. gaveni* Dobson, and an unidentified species. However, the latter two

species were trapped infrequently (<1 individual/trap/week) and are not considered further.

In the apricot orchard, significantly greater numbers of *C. hemipterus*, *C. mutilatus*, *C. davidsoni*, and *C. humeralis* were trapped in the *C. hemipterus* pheromone/dough-baited traps than in the other traps (Table 1). Pheromone increased the attraction of dough-baited traps to *C. hemipterus* by 46.2× over the whole period. The corresponding values for *C. mutilatus*, *C. davidsoni*, and *C. humeralis* were 3.7×, 3.7×, and 7.2×, respectively. Attraction of pheromone/dough-baited traps to *C. hemipterus* and *C. mutilatus* was greatest during February–June (late summer–early winter) (Table 1).

Numbers of beetles trapped, for all species, were relatively low (<5 beetles/trap/week) during August–January but increased substantially during February–May (Figures 1 and 2). No beetles were trapped during July, August, and most of September (mid-winter–early spring). Beetles were first trapped during September 22–29, when daily maximum temperatures exceeded 20°C for the first time and the mean maximum was 17.5°C (Figure 3). Thereafter, mean daily maxima remained above 17.5°C and beetles continued to be trapped for the next four to five weeks. *C. mutilatus* was the dominant species during this early period (even though *C. hemipterus* pheromone was used), and data for this species are therefore used in Figure 3. Spring flight activity of all species was largely confined to October, with very few beetles trapped during September and November (Figures 1 and 2). Trappings of all species increased substantially during February–March (late summer–early autumn) and generally continued at high levels until May (late autumn) (Figures 1 and 2).

In the peach orchard, significantly greater numbers of *C. mutilatus* and *C. davidsoni* were trapped in the *C. mutilatus* pheromone/dough-baited traps than in the other traps (Table 2). Very small numbers of *C. hemipterus* and *C. humeralis* were trapped. Over the whole trapping period, pheromone increased the attractiveness of dough-baited traps to *C. mutilatus* and *C. davidsoni* by 4.6× and 8.1×, respectively. The increase in attraction of *C. davidsoni* was greatest during August–January.

Numbers of beetles trapped in the peach orchard were substantially lower than in the apricot orchard. However, the same seasonal pattern of trapping, with a spring peak, summer lull, and greatest activity in autumn, also occurred. Greatest numbers of *C. mutilatus* (mean: 8–10 beetles/trap) were trapped in October and May.

Dose Study. *C. hemipterus* and *C. mutilatus* responded to both doses of their respective pheromones at well above the level recorded for dough only (Table 3). In addition, responses to the higher dose were significantly greater than responses to the lower dose ($P < 0.05$). The 5000-μg dose of *C. hemipterus* pheromone increased the attraction of *C. hemipterus* by 4.9× compared to the 500-μg dose. Attraction to the high dose was 259× greater than to dough alone

TABLE 1. MEAN NUMBER (± SE) OF *Carpophilus* spp. CAUGHT PER WEEK IN *C. hemipterus* PHEROMONE/DOUGH-BAITED TRAPS DURING AUGUST 1992–JUNE 1993 IN A SOUTHERN NEW SOUTH WALES APRICOT ORCHARD[a]

	C. hemipterus			*C. mutilatus*			*C. davidsoni*			*C. humeralis*		
Treatment[b]	Aug–Jun	Aug–Jan	Feb–Jun	Aug–Jun	Aug–Jan	Feb–Jun	Aug–Jun	Aug–Jan	Feb–Jun	Aug–Jun	Aug–Jan	Feb–Jun
Pheromone +	25.43	3.92	53.74	10.98	4.0	20.16	24.18	3.8	51.0	2.45	1.32	3.95
dough	±5.91[c]	±0.81[c]	±10.58[c]	±2.66[c]	±1.41[c]	±5.15[c]	±5.50[c]	±1.31[c]	±9.61[c]	±0.72[c]	±0.86[c]	±1.12[c]
	(46.2×)	(10.9×)	(68×)	(3.7×)	(2.3×)	(4.4×)	(3.7×)	(4.1×)	(3.6×)	(7.2×)	(11×)	(6.3×)
Pheromone	6.36	1.16	13.21	0.25	0.04	0.053	0.34	0	0.79	0	0	0
alone	±1.88	±0.62	±3.37	±0.13	±0.04	±0.29	±0.23		±0.51			
Dough alone	0.55	0.36	0.79	2.98	1.76	4.58	6.59	0.92	14.05	0.34	0.12	0.63
	+0.33	0.19	±0.72	±0.69	±0.61	±1.29	±2.68	±0.37	±5.76	±0.16	±0.09	±0.33
Control	0.5	0	1.16	0.02	0.04	0	0	0	0	0	0	0
	±0.34		±0.76	±0.02	±0.04							

[a] Values in parentheses represent the increase in attraction with pheromone and dough compared to dough alone.
[b] Pheromone dose = 500 μg.
[c] Significantly greater than other treatments ($P < 0.05$).

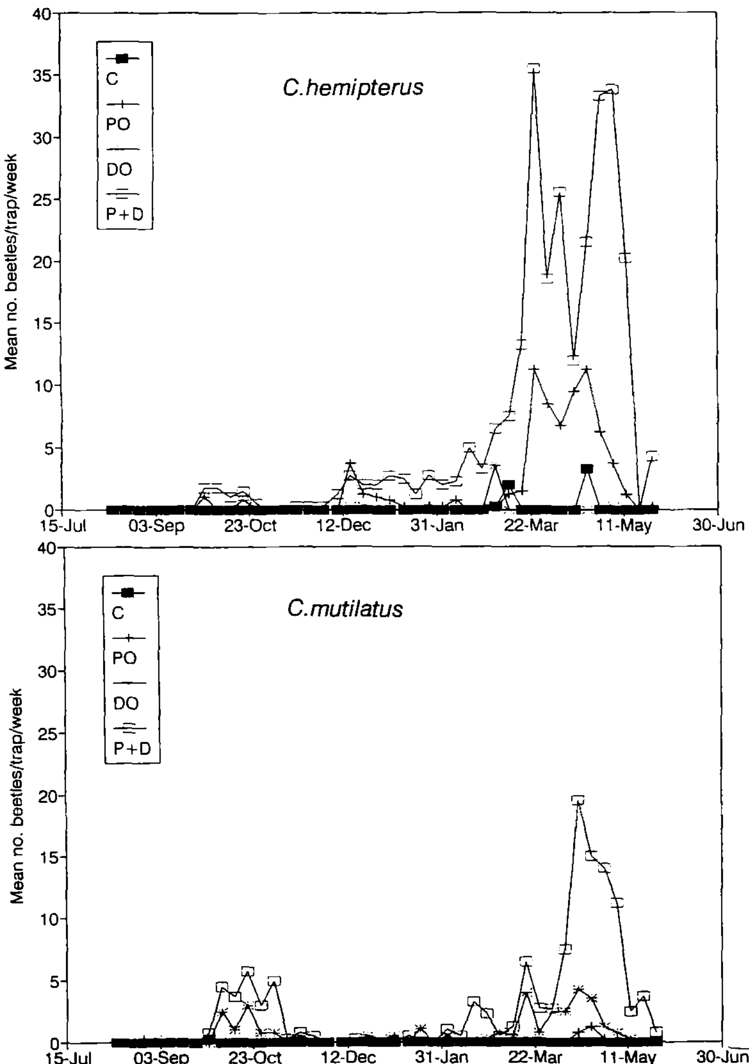

FIG. 1. Mean number of *C. hemipterus* and *C. mutilatus* trapped weekly in *C. hemipterus* pheromone- (500 μg) and fermenting-dough-baited traps in an apricot orchard near Leeton in southern New South Wales during 1992–1993 (C = unbaited, PO = pheromone only, DO = dough only, P+D = pheromone + dough).

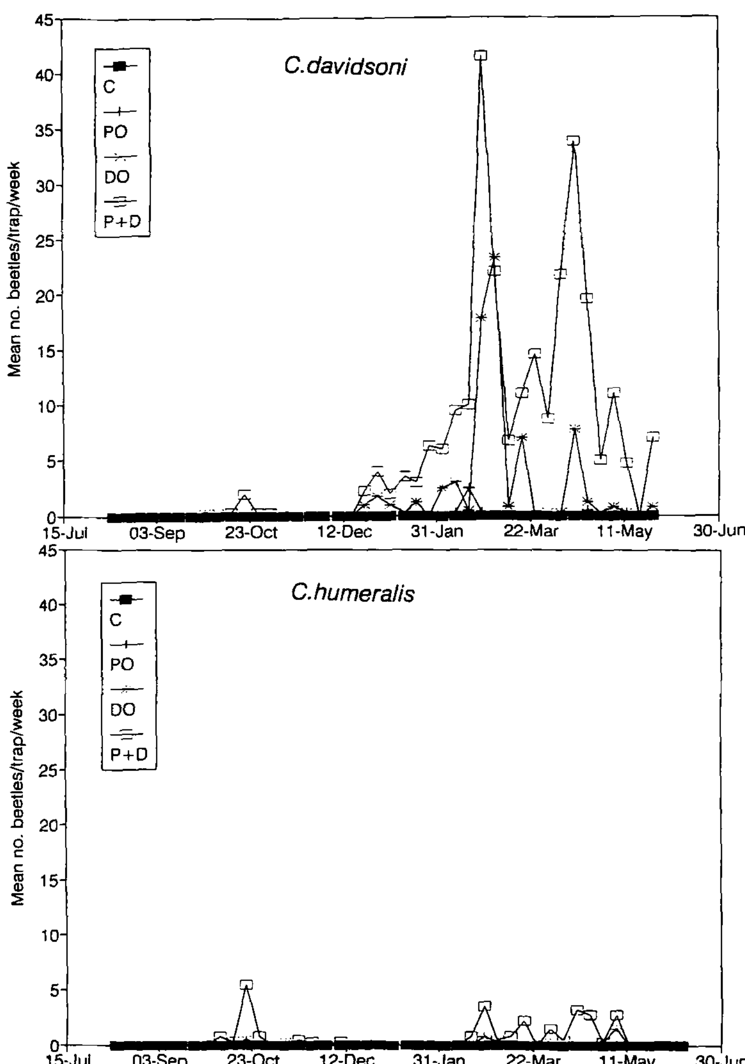

FIG. 2. Mean number of *C. davidsoni* and *C. humeralis* trapped weekly in *C. hemipterus* pheromone- (500 μg) and fermenting-dough-baited traps in an apricot orchard near Leeton in southern New South Wales during 1992–1993 (C = unbaited, PO = pheromone only, DO = dough only and P+D = pheromone + dough).

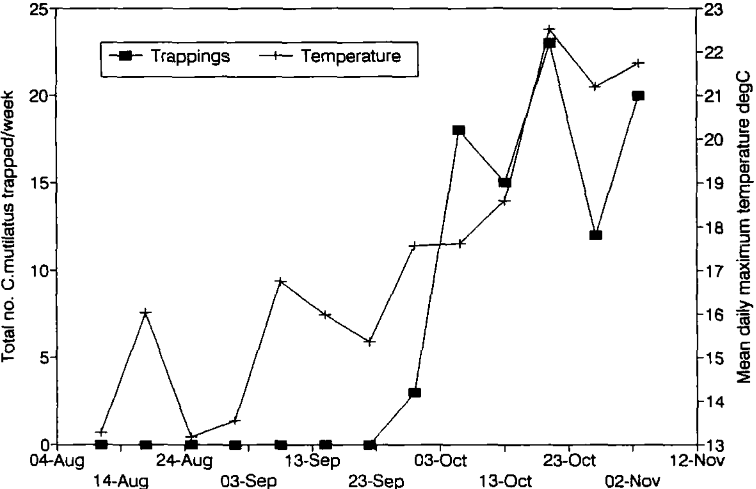

FIG. 3. Total number of *C. mutilatus* trapped weekly in four *C. hemipterus* pheromone-(500 μg) and fermenting-dough-baited traps during late winter and early spring 1992 in an apricot orchard near Leeton in southern New South Wales. Mean daily maximum temperatures calculated weekly.

(Table 3). The high dose of both pheromones also increased attraction of the other three species, although this was not significant ($P > 0.05$), except for *C. davidsoni* responding to *C. mutilatus* pheromone ($P < 0.05$), (Table 3).

Greatest numbers of *C. hemipterus* were trapped during December–February, declining in March before a late-season increase in April (Figure 4). Numbers of *C. mutilatus* were lower than for *C. hemipterus* but followed a similar trend.

DISCUSSION

This study further demonstrated the powerful attraction of synthetic aggregation pheromone/host volatile combinations to *Carpophilus* spp. This response was previously shown for *Carpophilus* populations in the United States (Bartelt et al., 1992, 1994) and Israel (Blumberg et al., 1993) as well as in Australia (James et al., 1993). Furthermore, a pheromone dose 10× the standard dose used to date increased attraction of *C. hemipterus* by almost fivefold and doubled the attraction of *C. mutilatus*.

Carpophilus hemipterus in a Californian date garden responded significantly to its pheromone at doses ranging from 15 to 15,000 μg (Bartelt et al.,

TABLE 2. MEAN NUMBER (±SE) OF *Carpophilus* spp. CAUGHT PER WEEK IN *C. mutilatus* PHEROMONE-DOUGH-BAITED TRAPS DURING AUGUST 1992–JUNE 1993 IN A SOUTHERN NEW SOUTH WALES PEACH ORCHARD.[a]

	C. mutilatus			*C. hemipterus*			*C. davidsoni*			*C. humeralis*		
Treatment[b]	Aug–Jun	Aug–Jan	Feb–Jun	*Aug–Jun*	*Aug–Jan*	*Feb–Jun*	Aug–Jun	Aug–Jan	Feb–Jun	Aug–Jun	Aug–Jan	Feb–Jun
Pheromone +	7.80	6.20	9.89	0.57	0.08	1.21	3.18	2.36	4.26	0.27	0.36	0.16
dough	±1.64[c]	±2.10[c]	±2.52[c]	±0.50	±0.08	±1.13	±1.03[c]	±1.26[c]	±1.68[c]	±0.10	±0.16	±0.08
	(4.6×)	(3.5×)	(6.1×)				(8.1×)	(29.5×)	(5.4×)	(1.2×)	(2.2×)	
Pheromone	0.93	1.52	0.16	0.05	0	0.11	0.14	0.16	0.11	0	0	0
alone	±0.83	±1.45	±0.08	±0.04		±0.10	±0.08	±0.12	±0.07			
Dough alone	1.70	1.76	1.63	0.02	0.04	0	0.39	0.08	0.79	0.23	0.16	0.32
	±0.83	±1.31	±0.84	+0.02	±0.04		±0.27	±0.05	±0.62	±0.15	±0.12	±0.31
Control	0	0	0	0	0	0	0	0	0	0	0	0

[a] Values in parentheses represent the increase in attraction with pheromone and dough compared to dough alone.
[b] Pheromone dose = 500 μg.
[c] Significantly greater than other treatments ($P < 0.05$).

TABLE 3. MEAN NUMBER (±SE) OF *Carpophilus* spp. CAUGHT PER WEEK IN *C. hemipterus* AND *C. mutilatus* PHEROMONE/DOUGH-BAITED TRAPS DECEMBER 16, 1992–APRIL 16, 1993, IN A PEACH ORCHARD AT YANCO AGRICULTURAL INSTITUTE[a]

Treatment	*C. hemipterus*	*C. davidsoni*	*C. mutilatus*	*C. humeralis*
C. hemipterus pheromone (500 μg) + dough	10.08 ±1.81[b] (53×)	3.15 ±0.73[b] (9.5×)	1.33 ±0.30 (2.3×)	1.57 ±0.60[b] (39.2×)
C. hemipterus pheromone (5000 μg) + dough	49.21 ±6.3[c] (259×)	5.32 ±0.86[b] (16×)	2.73 ±0.59[b] (4.7×)	1.56 ±0.39[b] (39×)
C. mutilatus pheromone (500 μg) + dough	0.33 ±0.10 (1.7×)	2.38 ±0.44[b] (7.2×)	5.68 ±0.79[b] (9.8×)	0.41 ±0.13[b] (10.2×)
C. mutilatus pheromone (5000 μg) + dough	0.50 ±0.21 (2.6×)	6.85 ±1.19[c] (20.8×)	12.32 ±1.99[c] (21.2×)	0.54 ±0.15[b] (13.5×)
Dough alone	0.19 ±0.05	0.33 ±0.11	0.58 ±0.18	0.04 ±0.03
Control	0	0.07 ±0.05	0.03 ±0.03	0.03 ±0.02

[a] Values in parentheses represent the increase in attraction with pheromone and dough compared to dough alone.
[b] Significantly greater than dough alone ($P < 0.05$).
[c] Significantly greater than 500 μg rate of same pheromone ($P < 0.05$).

1994). However, little difference in response to 500- and 5000-μg doses was observed, with only a doubling in attraction when doses were increased from 500 to 15,000 μg. Californian *C. mutilatus* doubled their response to *C. hemipterus* pheromone when it was increased from 500 to 5000 μg; a similar response for this species was also recorded in the current study.

In the same Californian study, *C. mutilatus* demonstrated a 5.3–8.6× increase in response when the dose of its pheromone was increased from 500 to 5000 μg (Bartelt et al., 1994). Calculation of response to different pheromone doses is likely to be influenced by the total number of beetles trapped in the experiment. The Californian study was characterized by extremely large populations of *C. mutilatus* and small populations of *C. hemipterus*. Pheromone trapping data for the former species might therefore be expected to be more robust than for the latter. Similarly, the larger number of *C. hemipterus* trapped in the current study probably makes these data more reliable than those of *C. mutilatus*. Nevertheless, the results from this study and those of Bartelt et al. (1992, 1994) and James et al. (1993) clearly demonstrate the generally substantial synergistic effect on attraction of *Carpophilus* spp. when synthetic aggre-

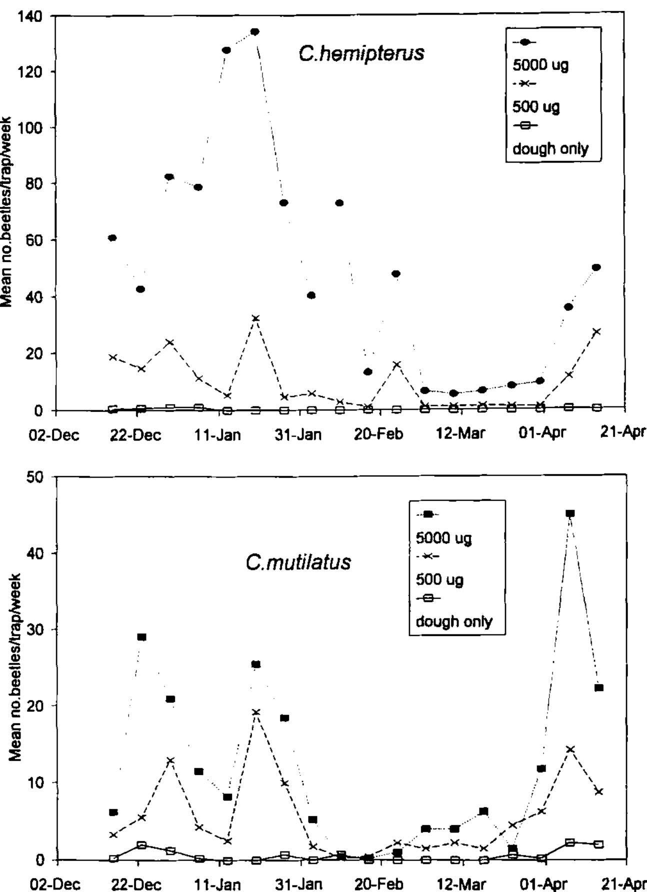

FIG. 4. Mean number of *C. hemipterus* and *C. mutilatus* trapped weekly responding to two doses of their respective pheromones in combination with fermenting dough in a peach orchard at the Yanco Agricultural Institute during 1992–1993.

gation pheromones are used in combination with host-related volatiles. This is particularly true when responses of *Carpophilus* spp. to their own pheromones are measured. Cross-attraction responses of *Carpophilus* beetles responding to other *Carpophilus* sp. pheromones, are generally much lower and in some cases (e.g., *C. hemipterus* responding to *C. mutilatus* pheromone) are very slight (Bartelt et al., 1992; James et al., 1993). *C. davidsoni* responded reasonably well to both *C. mutilatus* and *C. hemipterus* pheromone in this study (approximately 10× increase in attraction over dough alone using 500-μg doses). The

aggregation pheromone of this species has recently been identified, synthesized and field tested and is far more efficient in trapping this species (Bartelt and James, 1994).

The ecology of *Carpophilus* spp. in Australian stone fruit orchards has not been studied, and such data will clearly be fundamental to the effective use of synthetic pheromones in monitoring or managing *Carpophilus* populations. This study provides the first information on *Carpophilus* spp. incidence, abundance, and flight activity in southern New South Wales stone fruit orchards during an entire season. In combination with the results of James et al. (1993) and other studies underway, the current data will help establish a basic picture of *Carpophilus* beetle ecology in stone fruit. Observations in earlier years and the data on *Carpophilus* spp. abundance in Kable (1969) strongly indicate a spring flight activity peak in October or November. James et al. (1993), working in the same apricot orchard used in this study, recorded high numbers of *C. hemipterus* at the commencement of trapping in late November 1992. Numbers declined rapidly during December. Although numbers of *Carpophilus* spp. trapped during spring in the current study were small, a peak of activity in October was evident. Early season peaks in flight activity were also reported for *C. hemipterus* and *C. mutilatus* in a California date garden (Bartelt et al., 1992).

Preliminary studies indicate *Carpophilus* spp. overwinter as adults in southern New South Wales (James, unpublished). Temperature and probably rainfall are likely to be the main factors influencing timing of initial spring flight activity. This study showed flight activity commenced when daily maximum temperatures reached 17°C. Spring/summer 1992 in southern New South Wales was characterized by temperatures 2–4°C below long-term averages, which may have contributed to the relatively low number of beetles trapped at this time. The spring flight activity peak was followed by four to six weeks (*C. hemipterus*, *C. davidsoni*) or 10–12 weeks (*C. mutilatus*, *C. humeralis*) of minimal flight activity. Increased trapping of beetles in late summer–autumn indicated the development of larger populations, presumably associated with the presence of abundant host resources, i.e., ripening and fallen fruit.

These phenological data indicate a possible vulnerability of *Carpophilus* spp. populations during spring. Host resources (i.e., fermenting materials for nutrition and reproduction) are very limited during this period, and it is likely that *Carpophilus* spp. populations suffer a natural decline at this time. The use of synthetic pheromones as the basis of a mass-trapping program is likely to be most effective during this period.

Considerable potential exists for using synthetic aggregation pheromones in the management of *Carpophilus* spp. populations in stone fruit production. Options for their use include population monitoring, and possible disruption of egg-laying and mating behaviors. Synthetic pheromones of *Carpophilus* spp. are clearly a sensitive population monitoring tool and will greatly improve cur-

rent insecticide-based control in stone fruit orchards. *Carpophilus* spp. are rarely seen in stone fruit orchards during spring, even though pheromone-trapping shows that considerable populations can actually be present. Consequently, growers are often unaware of the problem until fruit starts to ripen. Chemical control at this time is usually unsatisfactory and risks producing insecticide-contaminated fruit. Early-season pheromone monitoring of *Carpophilus* will enable more timely and better targeted applications of insecticide, which should prevent the occurrence of damaging populations at harvest.

The substantial attraction of *Carpophilus* beetles to pheromone/host-volatile-baited traps suggests mass trapping could provide within-orchard management of these pests. Warner (1960, 1961) was the first to suggest mass-trapping as a management option for *Carpophilus* following studies with fermenting baits in Californian fig orchards. The value of mass-trapping *Carpophilus* spp. in stone fruit orchards will be largely dependent on the currently unknown population threshold at which damage to ripening fruit occurs. It is possible that only very large populations cause damage to fruit ripening on trees; smaller populations may be restricted to fallen fruit. Mass trapping may enable populations to be reduced to below the economic threshold, as has been demonstrated for the sweet potato weevil *Cylas formicarius* (F.) in Taiwan (Hwang and Hung, 1991). Studies investigating the mass trapping option for *Carpophilus* spp. are in progress. Mating disruption through saturation of orchard atmospheres with synthetic pheromone has been demonstrated for a number of pest Lepidoptera (e.g., Vickers et al., 1985). There is little information on the potential of this technique for other insect orders, although Mason and Janson (1990) demonstrated its potential for management of *C. formicarius*.

A considerable amount of work remains to be done before synthetic aggregation pheromones can be used effectively in reducing the impact of *Carpophilus* spp. on Australian stone fruit production. More information is needed, particularly on pest biology and ecology, so that optimal use can be made of the pheromones. Other studies in progress indicate that species composition of the *Carpophilus* fauna affecting stone fruit differs between fruit-growing regions in eastern Australia (James, unpublished). There may also be significant changes in faunal composition between seasons. More information is also needed on the effective trapping radius of synthetic pheromones and on optimal doses for monitoring, mass trapping or mating disruption. *C. hemipterus*, *C. mutilatus*, and *C. davidsoni* are likely to be the major economic *Carpophilus* spp. in Australian stone fruit production. The availability of effective synthetic aggregation pheromones for these species should ensure future management of these pests is more sustainable and environmentally acceptable than the application of broad-spectrum insecticides.

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EFFECTS OF BENZOIC AND CINNAMIC ACIDS ON GROWTH, MINERAL COMPOSITION, AND CHLOROPHYLL CONTENT OF SOYBEAN[1]

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Abstract—Organic acids are major water-soluble allelochemicals found in soil infested with quackgrass and are involved in several processes that are important in plant growth and development. This study was carried out to gain more information on the effects of benzoic acid (BEN) and *trans*-cinnamic acid (CIN) on growth, mineral composition, and chlorophyll content of soybean [*Glycine max* (L.) Merr. cv. Maple Bell] grown in nutrient solution. The two allelochemicals reduced root and shoot dry biomass of soybean. Treated plants had fewer lateral roots and tended to grow more horizontally compared to the untreated plants. Lateral roots were stunted and less flexible. The amounts of P, K, Mg, Mn, Cl^-, and SO_4^{2-} were lower, and Zn and Fe contents were higher in roots of plants grown with BEN or CIN as compared to untreated plants. Shoots of plants grown with the allelochemical showed greater accumulation of Ca, Mg, and Zn, whereas P and Fe contents were reduced. The BEN and CIN also caused reductions in leaf chlorophyll content. The BEN and CIN may be responsible for negative allelopathic effects of quackgrass on soybean by inhibiting root growth, by altering ion uptake and transport, and by reducing chlorophyll content.

Key Words—Allelochemical benzoic acid, *trans*-cinnamic acid, mineral uptake, quackgrass, *Elytrigia repens*, root morphology, soybean, *Glycine max*.

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INTRODUCTION

Quackgrass [*Elytrigia repens* (L.) Nevski] is a widespread weed that causes significant crop yield reductions in the temperate zone. The detrimental effects of quackgrass have been associated with production of toxic substances by living or decaying tissues (Rice, 1984). Root and shoot residues of quackgrass were shown to be phytotoxic to several crops including soybean [*Glycine max.* (L.) Merr.], navybean (*Phaseolus vulgaris* L.) and alfalfa (*Medicago sativa* L.) (Weston and Putnam, 1986).

Phenolic compounds are the major allelochemicals found in soil infested with quackgrass (Whitehead et al., 1982; Bobnick and Hagin, 1985). More recently, high concentrations of benzoic acid (BEN) and *trans*-cinnamic acid (CIN) (Figure 1) were identified in the rhizosphere of quackgrass, ranging from 50 to 250 μM (Baziramakenga et al., 1994). These organic acids inhibit germination and plant growth when applied exogenously in millimolar concentrations (Chou and Patrick, 1976; Lynch, 1980; Ramirez and Garraway, 1982; Ueda, 1989). However, the physiological and biochemical aspects of how these compounds affect growth remains obscure. Glass (1974a) found that the absorption of K by excised roots of barley (*Hordeum vulgar* L. cv. Karlsberg) was inhibited by the two compounds. Benzoic acid also inhibited P uptake by excised roots of barley (Glass, 1973, Glass and Dunlop, 1974). Tillberg (1970) observed that CIN has no effect on P uptake by *Scenedesmus obtusiusculus*. None of the above studies was conducted on intact root systems. The BEN and CIN were also reported to reduce chlorophyll content in wheat (*Triticum aestivum*) (Mattagajasingh and Kar, 1989) and soybean (Patterson, 1981).

The objective of this research was to describe the effects of BEN and CIN on growth and chlorophyll and mineral contents of intact seedlings of soybean [*Glycine max* (L.) Merr.] grown hydroponically in order to contribute to the understanding of the mechanisms by which quackgrass affects soybean growth.

METHODS AND MATERIALS

Plant Material. Soybean [*Glycine max.* (L.) Merr. cv Maple Bell] seeds were sterilized for 30 min with 5% hypochlorite, rinsed with distilled water, and placed on sterilized vermiculite saturated with 10 mM $CaSO_4$. The plants

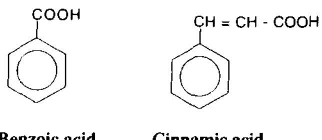

Fig. 1. Structure of benzoic and cinnamic acids.

were maintained in a growth chamber with a 28:20°C day–night temperature, a light–dark regime of 16:8 hr, an irradiance of 280 μmol/m^2/sec and a 70% relative humidity.

Mineral Uptake. Two-week-old seedlings were transferred into an Erlenmeyer containing 100 ml of nutrient solution of the following composition (μM): 750 KNO_3, 325 $Mg(NO_3)_2$, 10 KH_2PO_4, 1000 $CaSO_4$, 8 H_3BO_3, 0.2 $MnSO_4$, 0.2 $ZnSO_4$, 0.2 $CuSO_4$, and 0.2 $(NH_4)_6Mo_7O_{24}$ (Cakmak and Horst, 1991). After three days of acclimatization, the seedlings were transferred into nutrient solutions containing BEN or CIN at concentrations ranging from 0 to 250 μM and adjusted to pH 5.8 with 0.01 M NaOH or 0.01 N HCl. The nutrient solution was replaced every two days because more than 70% of the acids disappeared within three days by plant uptake or microbial degradation (Blum and Dalton, 1985) and to avoid a fall in pH of the nutrient solution.

Seedlings were harvested after four weeks and separated into roots and shoots. Dry weights were obtained after drying the plant parts for 24 hr at 50°C in a forced-air oven. Dried samples were heated in a glass beaker for 3 hr at 450°C in a muffle furnace, and then ground into a fine powder with a Willey mill (20-mesh screen). Ash residue was dissolved in 5 ml of 0.5 N HCl. The solution was filtered through Whatman No. 42 filter paper, and the filtrate was adjusted to 25 ml with distilled water. The K, Ca, Mg, Cu, Fe, Mn, and Zn contents were determined by atomic absorption spectroscopy on a Perkin-Elmer atomic absorption spectrometer 3300. Phosphorus content was measured colorimetrically using a phosphovanadomolybdate complex color on a Hitachi U-1000 Spectrophotometer (Murphy and Riley, 1962).

Anions were extracted according to Cataldo et al. (1975). Briefly, plant tissues (100 mg) were incubated with 10 ml of distilled water for 1 hr at 45°C. The suspensions were filtered through Whatman No. 2 filter paper. The Cl^-, NO_3^-, and SO_4^{2-} contents were measured by ion chromatography (Dionex 4000i, Dionex Corp., Sunnyvale, California) using an anion exchange column (Dionex IonPac AS4A) isocratically eluted with 3 mM $NaHCO_3$ and 2.4 mM Na_2CO_3. Anions were detected by suppressed conductivity (Dionex Micromembrane Suppressor using 0.025 N H_2SO_4 at 5 ml/min as conductivity suppressor).

Determination of Chlorophyll Contents. Fully expanded leaves were taken randomly from seedlings treated with BEN or CIN at 0, 50, 100, and 200 μM. Their petioles were removed, and fresh weights determined. The chlorophyll pigments were extracted by 95% methanol for 16 hr in the dark. Absorbance of the extracts was read at 649 and 665 nm on a Hitachi U-1000 Spectrophotometer. Chlorophyll *a* and *b* contents were calculated using formulas of Knudson et al. (1977):

$$\frac{\text{Chlorophyll } a\,(\mu g)}{\text{Solution (ml)}} = [(13.70)(A_{665\,nm})] - [(5.76)(A_{649\,nm})]$$

$$\frac{\text{Chlorophyll } b\,(\mu g)}{\text{Solution (ml)}} = [(25.80)(A_{649\,nm})] - [(7.60)(A_{665\,nm})]$$

Each experiment was carried out according to a completely randomized design with three replicates and was repeated twice. The data of root and shoot biomass, chlorophyll *a* and *b*, and elemental concentration were submitted to standard analysis of variance after testing for homogeneity of variance. For each compound, the selected concentration treatments were compared to the untreated control using a *t* test.

Root Morphology. In an additional experiment, root morphology was examined. Both aromatic acids were used at 0, 50, 100, 200, and 250 μM. Three days after germination, soybean seedlings were transferred into an Erlenmeyer containing 40 ml of the nutrient solution for 24 hr and then the allelochemicals were added. The Erlenmeyer flasks were kept as previously described for 14 days. At the end of the experiment, root morphology was evaluated visually.

RESULTS

Seedling Growth and Root Morphology. Both BEN and CIN reduced soybean root and shoot dry biomass (Figure 2). A significant reduction of shoot and root biomass occurred for plants subjected to 150 μM of CIN, whereas significant reduction was observed at 200 μM for BEN. When BEN was used at 250 μM, root biomass was reduced by 40% and shoot biomass by 35% compared to the control. The corresponding reductions were respectively 25 and 32% for CIN. Visual differences in soybean root systems were also apparent (Figures 3 and 4). Primary root elongation and the number and length of secondary roots were inhibited by the two allelochemicals. Lateral roots were stunted and less flexible.

Mineral Absorption and Translocation. Each of the two allelochemicals consistently affected the soybean mineral content, the effect being dependent on the concentration of allelochemicals and on the mineral element. Root absorption of P, K, and Mg was severely inhibited by BEN (Table 1). The Ca and Cu absorption was found to be unaffected by BEN. The BEN at concentrations higher than 150 μM strongly enhanced the Fe absorption compared to the control. Low concentrations of BEN (50 and 100 μM) slightly stimulated the uptake of Mn, but larger concentrations were inhibitory. The BEN (250 μM) significantly enhanced Zn root content.

Absorptions of P, K, Mg, and Mn were severely depressed by CIN (Table 1). For example, at 250 μM of CIN, K uptake was inhibited by 64%, P uptake by 42%, and Mg and Mn uptake by 74%. The CIN significantly promoted the absorption of Zn and Fe in soybean roots. The Cu content of roots increased in the presence of low concentrations of CIN. Root absorption of Ca was not affected by CIN.

The BEN treatments did not result in statistically significant changes in

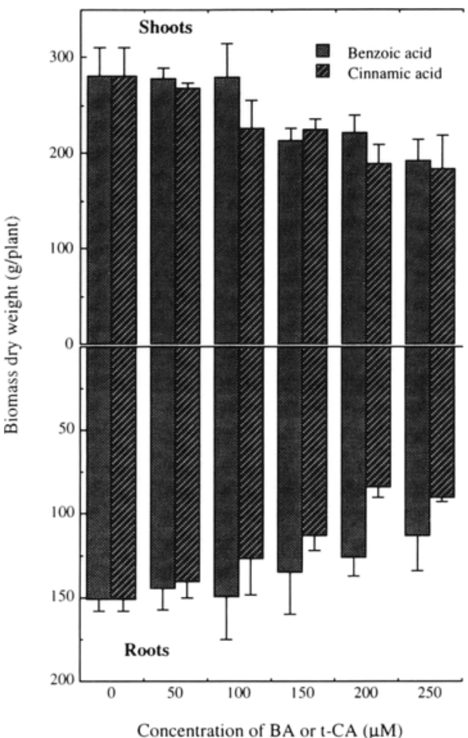

Fig. 2. Effects of benzoic and cinnamic acids on root and shoot dry biomass of soybean.

shoot K and Cu contents. The highest concentrations of BEN significantly promoted the shoot Ca, Mg, and Zn contents while the opposite effect was observed for P and Fe (Table 2). The Mn content of shoots was raised at 100 μM of BEN, as compared with untreated plants.

The amounts of K, Ca, Zn, and Mn measured in the shoots were not changed by the treatments with CIN (Table 2). The shoot Mg content was significantly enhanced by CIN. A significant reduction in shoot P and Fe content was observed in seedlings treated with CIN. Higher concentrations of CIN raised the amount of Cu in soybean shoots.

The shoot Cl^- content was reduced by CIN or BEN (Figure 5). Only CIN depressed the root Cl^- concentration (Figure 6). The contents of SO_4^{2-} in root decreased with increasing rates of the two allelochemicals. Low concentrations of BEN or CIN stimulated the translocation of SO_4^{2-} to the shoots, while the

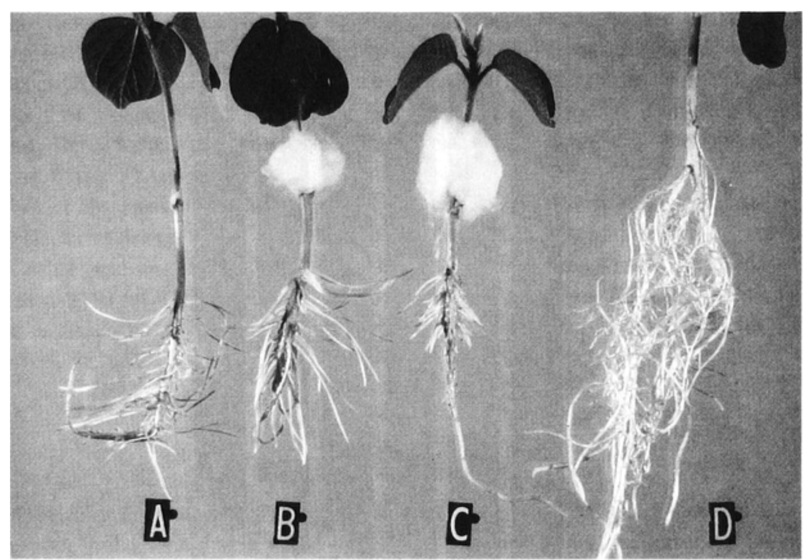

Fig. 3. Soybean root system as affected by benzoic acid: (A) 50 μM, (B) 100 μM, (C) 200 μM, and (D) control.

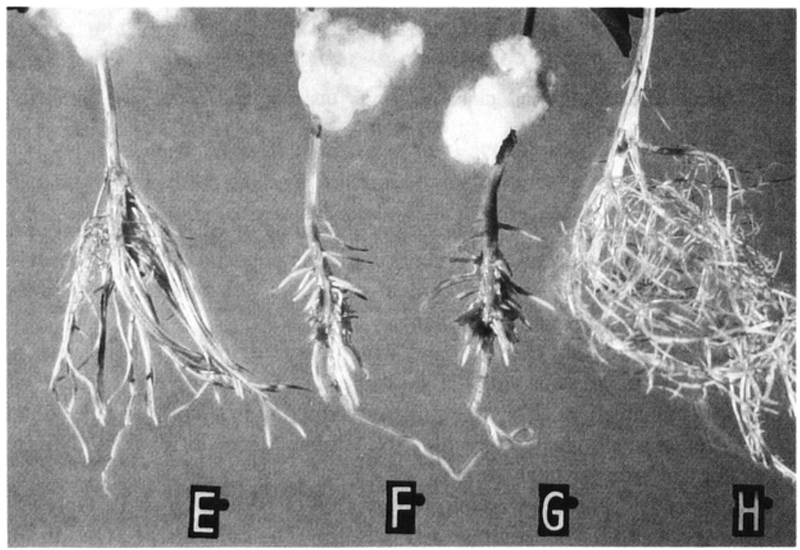

Fig. 4. Soybean root system as affected by cinnamic acid: (E) 50 μM, (F) 100 μM, (G) 200 μM. Control at right.

TABLE 1. EFFECTS OF BENZOIC ACID (BEN) AND *trans*-CINNAMIC ACID (CIN) ON NUTRIENT CONTENT OF ROOTS OF SOYBEAN SEEDLINGS[a]

	Nutrient concentration							
	μmol/g dry wt				nmol/g dry wt			
Treatment	P	K	Ca	Mg	Cu	Zn	Mn	Fe
Control	123	949	94	179	376	354	1412	2435
BEN								
50 μM	120	877	79	155*	265	326	1605	2307
100 μM	102**	823	75	133*	254	313	1464	2596
150 μM	92**	804	77	128**	308	407	1249	3486
200 μM	78**	731*	75	110**	434	465	1071*	4593**
250 μM	72**	721**	80	104**	470	531*	884**	9092**
CIN								
50 μM	117	817	75	151*	409	409	1331	2305
100 μM	107*	739*	79	118**	532*	539*	1281	2842
150 μM	98**	608**	87	96**	489	514*	962**	3745*
200 μM	71**	451**	96	66**	460	674**	593**	6302**
250 μM	71**	343**	111	48**	476	731**	367**	6876**

[a]Levels of significance (compared to the untreated control): *$0.01 < P < 0.05$; **$P < 0.01$.

highest concentrations produced the inverse effect. Concentrations larger than 150 μM promoted the accumulation of NO_3^-; this effect was much more noticeable in roots than in shoots.

Chlorophyll Content. Seedlings grown in the presence of BEN or CIN showed chlorosis, especially at concentrations higher than 100 μM; this was reflected in the chlorophyll content (Figures 7 and 8). At lower concentrations, there was a slight increase in chlorophyll content. Chlorophyll *a* content was affected to a greater extent than chlorophyll *b*. At 200 μM, chlorophyll *a* declined by 37% with BEN and by 27% with CIN. Only BEN at a concentration greater than 100 μM decreased chlorophyll *b*.

DISCUSSION

The growth of soybean seedlings and the formation of lateral roots were inhibited by BEN and CIN, and the extent of inhibition was related to the concentration of the allelochemicals. Vaughan and Ord (1990) also observed that BEN and CIN derivatives altered root morphology in pea (*Pisum sativum*). Our data could explain the results of Weston and Putnam (1986) who reported quackgrass inhibition of legume root hair formation. The allelopathic substance

TABLE 2. EFFECTS OF BENZOIC ACID (BEN) AND *trans*-CINNAMIC ACID (CIN) ON NUTRIENT CONTENT OF SHOOTS OF SOYBEAN SEEDLINGS

	Nutrient concentration							
	μmol/g dry wt				nmol/g dry wt			
Treatment	P	K	Ca	Mg	Cu	Zn	Mn	Fe
Control	91	437	213	73	101	480	441	1125
BEN								
50 μM	89	449	218	87*	97	503	516*	1080
100 μM	82	462	217	87*	92	525	490	1028
150 μM	75**	466	238	105**	135	573	484	982
200 μM	67**	431	253*	106**	138	629*	417	827*
250 μM	66**	422	250*	109**	136	700**	382	784*
CIN								
50 μM	88	485	214	92*	126	511	446	934
100 μM	81*	465	239	96**	131	577	487	850*
150 μM	73**	500	215	97**	154**	575	431	817*
200 μM	73**	398	202	101**	156**	570	416	812*
250 μM	65**	381	216	104**	196**	581	401	833*

[a]Levels of significance (compared to the untreated control): *$0.01 < P < 0.05$; **$P < 0.01$.

may interfere with the root meristematic processes, resulting in impaired cell division (Vaughan and Ord, 1990). Alternation of hormonal balance, such as that of indoleacetic acid (IAA), is one likely mechanism by which allelopathic compounds affect plant growth (Rice, 1984). The BEN and CIN derivatives, depending on the extent and pattern of substitution, influence IAA concentrations in plant tissues by stimulating or inhibiting enzymes involved in IAA synthesis and destruction (Vaughan and Ord, 1991). This mechanism could also be operative for the two allelochemicals used in the present study. Several BEN and CIN derivatives were reported to interfere with metabolic processes such as cell-wall extension, protein synthesis, enzymatic activities, mitochondrial respiration, and plant–water relations (Rice, 1984; Tan et al., 1992; Sato et al., 1982; Dube et al., 1992).

Results from the present work indicate that BEN and CIN affect mineral uptake and their translocation from roots to shoots in soybean. This direct effect on mineral composition constitutes another possible explanation for the inhibition of soybean growth by BEN or CIN. Inhibitions of the same order of magnitude have been reported for soybean and sorghum (*Sorghum bicolor* L. Moench) exposed to ferulic acid (McClure et al., 1978; Kobza and Einhellig, 1987).

The mechanisms of inhibition of ion uptake are still unclear. Glass (1974b)

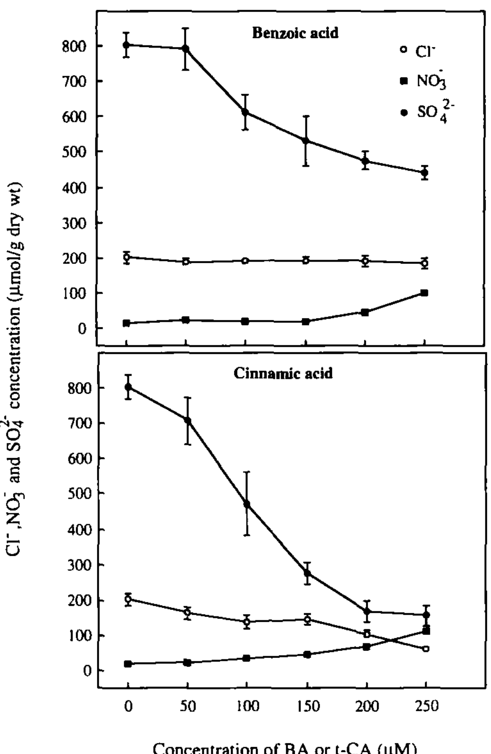

Fig. 5. Effects of benzoic and cinnamic acids on the concentration of Cl^-, NO_3^-, and SO_4^{2-} in shoots of 14-day-old soybean seedlings.

indicated that the inhibitory effects of benzoic and cinnamic derivatives are strongly correlated with their lipid solubilities as reflected by their octanol–water partition coefficients. BEN and CIN can depolarize the root cell membrane, causing a generalized increase in membrane permeability and dysfunction. BEN depolarized the electrical potential of oat coleoptiles (Bates and Goldsmith, 1983) and membranes of epidermal cells of barley roots (Glass and Dunlop, 1974). In contrast, Macri et al. (1986) concluded that BEN had no effect on depolarization of pea stem mitochondria. Rice (1984) noted that phenolic compounds could affect respiration and mitochondrial metabolism, thus limiting ATP synthesis; however, BEN has no effect on mitochondrial ATPase (Macri et al., 1986) and a small inhibitory effect on the respiration of tomato slices (Baker et al., 1978). A recent study with *Saccharomyces cerivisiae* suggests that BEN lowers ATP content as a result of high ATP utilization rather than

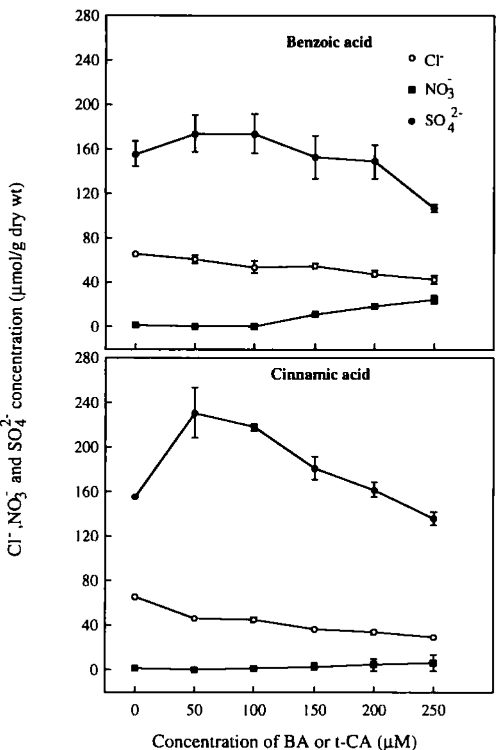

Fig. 6. Effects of benzoic and cinnamic acids on the concentration of Cl^-, NO_3^-, and SO_4^{2-} in roots of 14-day-old soybean seedlings.

from inhibition of its synthesis (Warth, 1991). Tillberg (1970) noted an increase in ATP content for cells of *Scenedesmus* treated with CIN.

The current study showed that NO_3^- accumulated in roots treated with BEN or CIN. It is difficult to speculate on the reason for this accumulation. It could be related to inhibition of enzymes involved in nitrate metabolism, lack of reducing power, or antagonism between nitrates and other anions. The limited availability of information in this area indicates that some BEN and CIN derivatives inhibited nitrate reductase (Pospisil and Sindearova, 1981).

Our results indicate that low concentrations of BEN and CIN can promote chlorophyll content, whereas larger concentrations produced the inverse effect. These results are in accordance with Mattagajasingh and Kar (1989), who concluded that BEN (100 μM) raised the chlorophyll content in wheat. Patterson (1981) reported that CIN (1 mM) reduced the chlorophyll content of soybean.

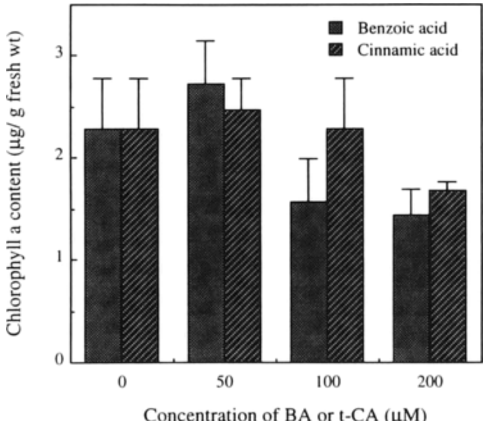

Fig. 7. Effects of benzoic and cinnamic acids on chlorophyll *a* content of 14-day-old soybean seedlings.

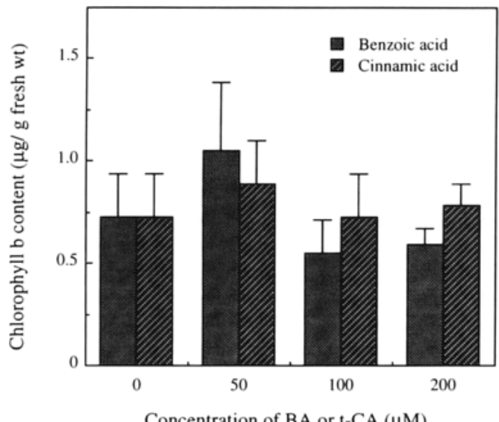

Fig. 8. Effects of benzoic and cinnamic acids on chlorophyll *b* content of 14-day-old soybean seedlings.

Since the chlorophyll content is closely related to plant dry matter production (Buttery and Buzzell, 1977), any reduction in leaf chlorophyll content would limit net photosynthesis and thus diminish total plant growth. The present study does not establish whether reduction in the chlorophyll content is due to a reduction in synthesis or to an allelochemical-induced degradation. The involvement of minerals in chlorophyll synthesis suggests that allelochemicals could

influence its synthesis by limiting the supply of these ions. For instance, our data show that Fe was reduced in shoots treated with BEN.

Results from the present work indicate that BEN and CIN affect plant growth, particularly root growth and morphology, alter the uptake and transport of ions, and reduce the chlorophyll content. BEN and CIN may be responsible for the allelopathic effects of quackgrass on crops and weeds under field conditions. As suggested by Einhellig (1986), inhibition of seedling growth might better be regarded as a tertiary effect, with perturbation of mineral nutrition as a secondary effect and the cause of such inhibitions the true primary effect. Cell membranes are the prime targets for alleochemicals and further investigations could clarify the mechanisms by which inhibition of growth is related to changes in membrane properties.

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INFLUENCE OF ANTIFUNGAL COMPOUNDS FROM A SOIL-BORNE ACTINOMYCETE ON *Fusarium* spp. IN ASPARAGUS

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Abstract—Asparagus decline syndrome is caused by fungal infection of asparagus roots and crowns by *Fusarium oxysporum* f.sp. *asparagi* (FOA) and *F. moniliforme* (FM). Several soil-borne microorganisms have been found inhibitory to *Fusarium* pathogens in other crops. A novel *Streptomyces* spp. (ME2-27-19A) was isolated from asparagus field soil and found to be inhibitory to FOA and FM in vitro. Solvent extraction of ME2-27-19A and chromatographic purification of the extract yielded compound(s) that were inhibitory to FOA and FM at 40 μg/ml. ME2-27-19A extract produced variable control of FOA and FM in vitro, and was phytotoxic at 1000 μg/ml. In soil, ME2-27-19A extract reduced the *Fusarium* population at 100 μg/ml, but also reduced the asparagus shoot length.

Key Words—Antifungal, *Asparagus officinalis*, biological control, *Fusarium oxysporum*, *Fusarium moniliforme*, *Streptomyces*.

INTRODUCTION

Fusarium crown and root rot of asparagus (*Asparagus officinalis* L.) are caused by *Fusarium oxysporum* (Schlecht.) emend Snyd. & Hans. f.sp. *asparagi* Cohen (FOA) and *Fusarium moniliforme* (Sheld.) emend Snyd. & Hans. (FM). Effective methods of controlling these diseases of asparagus are not currently available. Resistant varieties, which have proven effective in other vegetable crops (Mace et al., 1981), have not been found in asparagus (Stephens et al., 1989). Chemical treatments with benomyl [methyl 1-(butylcarbamoyl)-1H-benzimid-

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azol-2-ylcarbamate] and captan [*N*-[(trichloromethythio)thio]-4-cyclohexene-1,2-dicarboximide] produced no positive effects on crown survival or yields over the controls (Lacy, 1979).

However, Smith et al. (1990) found that faeriefungin, a polyene macrolide produced by *Streptomyces griseus* var. *autotrophicus*, inhibited *F. oxysporum* f.sp. *asparagi* in vitro and in the greenhouse. Kemira Oy, a European chemical company, recently has begun marketing a biofungicide named Mycostop. It is based on *S. griseovirdis* and claimed to be effective against *Fusarium* spp., although it is not registered in the United States. The objective of this study was to determine the efficacy of compounds extracted from a newly isolated soil microorganism on *Fusarium* spp. in asparagus.

METHODS AND MATERIALS

General Bioassay Procedure. Cultures of *Fusarium oxysporum* f.sp. *asparagi* (MSU-FOA 50), *Fusarium moniliforme* (MSU-FM F12), and *Gleosporium* spp. were grown on potato dextrose agar (PDA) (Smith et al., 1990) for 14 days. Cultures of *Aspergillus flavus* and *Candida albicans* were grown on YMG medium (yeast extract 4 g, maltose 10 g, glucose 4 g, agar 12 g, in 1 liter of distilled water) (Nair et al., 1989). Cultures of *Streptococcus pneumoniae*, *Staphylococcus aureus*, and *Escherichia coli* were grown on Emmons medium (neopeptone 10 g, glucose 20 g, agar 15 g in 1 liter of distilled water).

Cell or spore suspensions of the test organisms were made by adding 10 ml of sterile saline solution (0.85 g NaCl in 100 ml distilled water) to a culture dish and stirring with a glass rod. The cell concentration was adjusted to 10^6 colony forming units per milliliter (CFU/ml) using a hemacytometer. Bioassay plates were made by spreading evenly 100 μl of the cell suspension in Petri dishes containing 20 ml of the appropriate medium. Extracts were bioassayed by placing 100 μg of extract dissolved in 10 μl of dimethyl sulfoxide (DMSO) in the center of the bioassay plates and incubating at 27°C for four days. A zone of inhibition characterized by the absence of microbial growth was measured.

Isolation and Culture of ME2-27-19A. Spinks loamy sand soil (Psammetitic Hapludalf, sandy, mixed, mesic) was collected from an old asparagus field in Oceana County, Michigan. Eight actinomycetes were isolated from this soil by placing 10 g in 100 ml of normal saline and shaking for 30 min. The samples were diluted 1 : 1000 in normal saline solution and heat shocked at 60°C for 2 min to reduce the number of bacteria. Then, 100 μl of each sample was plated on water agar amended with cycloheximide (45 μg/liter) to eliminate fungi. The new actinomycetes were bioassayed on *Fusarium* bioassay plates. Strain ME2-27-19A was compared with six other actinomycetes with fungicidal activity,

which were isolated previously in the Bioactive Natural Products Laboratory at Michigan State University. Biochemical tests were conducted for ME2-27-19A using Biolog GN plates (Biolog, Inc., Hayward, California) to identify this organism.

Fermentation and Extraction. Cultures of ME2-27-19A were grown in 2-liter baffled-bottom Erlenmeyer flasks, containing 400 ml of A-9 medium (peptone 5 g, glucose 10 g, "Brer Rabbit green label" molasses 20 g in 1 liter of distilled water). The inoculated flasks were placed on a rotary shaker at 150 rpm and 27°C for eight days.

Microbial cells from 6 liters of fermentation broth were centrifuged (16,300 *g*). The cells were filtered under vacuum to remove additional medium. The cells then were homogenized with methanol-chloroform (MeOH:$CHCl_3$, 1.25 liters, 4:1 v/v) for 10 min and filtered through a sintered glass funnel under vacuum. The residue from the funnel was homogenized again with 1.25 liters of MeOH:$CHCl_3$ (4:1 v/v) and filtered. The filtrates were combined and dried under vacuum at 40°C.

Purification. The crude extract was purified by vacuum-liquid chromatography (VLC). Crude extract (2 g) was dissolved in MeOH, loaded on a silica gel column (100 g, 35–75 μm mesh) and eluted with $CHCl_3$:MeOH (1:1 v/v). Eight fractions (100 ml each) were collected. Fractions 1, 2, and 3–8 were combined into fractions 1, 2, and 3, respectively, based on similar R_f values from thin-layer chromatography (TLC). The column finally was eluted with 100% MeOH (600 ml) to remove any remaining compounds. All fractions including the MeOH fraction were bioassayed on PDA plates spread with FOA or FM. The most active fraction 3 (817.5 mg) was purified further by C-18 column chromatography and TLC as described below.

Purification of Fraction 3. Fraction 3 (0.4 g in 4 ml MeOH) was purified on a medium-pressure C-18 column (Chemco Scientific Co., Ltd., Osaka, Japan, 4 × 56 cm, 37–55 μm mesh). Six fractions (100 ml each) were collected with MeOH:H_2O (60:40 v/v, 25–30 psi, 2 ml/min flow rate). Finally, the column was eluted with 100% MeOH (500 ml). Fractions 3–6 were combined to obtain fraction III (40.2 mg), based on the R_f value at 0.52 on a C-18 TLC plate. All fractions were bioassayed on PDA plates spread evenly with FOA or FM, and the activity was located in fraction III. Fraction III was purified further on C-18 preparative TLC plates (500 μm thickness) using MeOH:H_2O (60:40 v/v) as the mobile phase. The bands were eluted with MeOH and dried under vacuum. The band with an R_f value of 0.52 afforded a pure amorphous solid (ME-144-4, 7.0 mg) that was active against FOA and FM.

Spectroscopic Analysis. The ultraviolet (UV) spectrum of ME-144-4 was measured on a Shimadzu (Kyoto, Japan) UV260 spectrometer and the ^{1}H NMR spectrum was determined on a Varian Gemini 500-MHz spectrometer in deuterated-DMSO (d_6-DMSO).

Preliminary Antimicrobial Bioassays. The crude extract of ME2-27-19A was bioassayed on plates spread with FOA, FM, *Aspergillus*, *Candida*, or *Staphylococcus*. ME2-27-19A extract also was bioassayed by adding 500 or 1000 mg in 0.5 ml of DMSO to 100 ml of warm (50°C) PDA before pouring the medium into Petri dishes. Plugs (5 mm) of PDA from fully grown FOA, FM, *Alternaria solani*, or *Sclerotinia sclerotia* plates were placed in the center of each Petri dish. Radial growth of the fungi was measured daily for seven days.

Crude extract of ME2-27-19A was chromatographed initially on silica thin layer chromatography (TLC) plates in $CHCl_3$: MeOH (2 : 1 v/v). The inhibitory bands were identified by pouring 10 ml of molten (50°C) YMG agar (amended with 6 g of agar in 1 liter of water) containing 10^5 CFU/ml of FOA, FM, *Aspergillus*, or *Candida* over the developed TLC plates. The plates were placed in a sterile moisture chamber and incubated at 27°C for four days.

Minimum Inhibitory Concentration. ME-144-4 was bioassayed in test tubes containing liquid Emmons medium to determine the minimum inhibitory concentration (MIC). ME-144-4 (2 mg) was dissolved in DMSO (200 μl) and serially diluted in the same solvent. A 20-μl aliquot of each dilution was mixed with 1.96 ml of Emmons medium. The tubes were inoculated with 20 μl of 10^5 CFU/ml solution of FOA or FM. The final concentrations were 0, 20, 40, 60 μg/ml in 2 ml of solution. The inoculated tubes were incubated on the shaker at 27°C and the results were recorded after four days. The lowest concentration of compound that totally inhibited growth of FOA or FM was recorded as the MIC for that species.

Insecticidal Assay. The insecticidal assay was conducted on fourth-instar mosquito larvae (*Aedes aegypti*). Mosquito larvae were hatched by placing eggs in 200 ml of degassed, deionized water and fed with liver powder. After four days, five to seven fourth-instar larvae in 975 μl of deionized water were placed in 4-ml test tubes. ME2-27-19A crude extract (0, 125, 250, 500, or 1000 μg) in 25 μl of DMSO was placed in the test tubes and left in the dark at room temperature. The numbers of dead larvae were recorded at 0, 2, 4, 6, 12, 24, and 48 hr. The experiment was repeated with 100 μg each of the chromatographic fractions.

Phytotoxicity. The herbicidal assay was conducted on Syn 4-56 asparagus, Curly cress (*Lepidium sativum* L.), and Campbell 1327 tomato (*Lycopersicum esculentum* L.) seedlings. The three fractions from VLC were dissolved in MeOH and placed on sterile filter paper in Petri dishes at 0, 500, and 1000 μg. The MeOH was allowed to evaporate and 1 ml of sterile water was added to each Petri dish. Ten seeds of each bioassay crop were placed in the Petri dishes. The Petri dishes were sealed with parafilm and incubated at 27°C. The root and shoot lengths of the seedlings were measured after four days for cress and 14 days for asparagus and tomato.

The experimental design was a randomized complete block design with three blocks. The data were analyzed by analysis of variance (ANOVA) using MSTAT-C (MSTAT-C, Michigan State University, East Lansing, Michigan). The means were separated by LSD and the relationships between treatments were analyzed by linear contrasts.

In Vitro Assay of ME2-27-19A Crude Extract on Asparagus and Fusarium. The effects of ME2-27-19A extract on FOA and FM in asparagus were determined by incorporating crude extract into 20 ml of partially cooled (50°C) Hoagland's agar (Hoagland and Arnon, 1938) in 100-ml test tubes. The final concentrations of the extract were 0, 100, 200, and 1000 μg/ml in 20 ml of agar. Asparagus seeds were surface-disinfected with 0.5% NaOCl (20% bleach) for 20 min, followed by infusion with benomyl (2.5 g) in acetone (100 ml) for 18 hr to eliminate *Fusarium* spp. (Damicone et al., 1981). The seeds were rinsed with acetone, followed by sterile deionized water, and dried on a sterilized clay tile (Stephens and Elmer, 1988). The seeds were germinated on 0.6% water agar. Germinated seedlings were transferred to the test tubes and grown in a growth chamber under 16-hr photoperiod with 15°C night temperature and 25°C day temperature. After three weeks, as the seedlings were initiating lateral roots, 0.1 ml of 10^7 CFU/ml solution of FOA or FM was added. The seedlings were grown for an additional five weeks and rated for *Fusarium* infection on a scale of 1–5, where 1 = healthy seedlings, 2 = slightly infected, 3 = moderately infected, 4 = severely infected, 5 = dead or dying seedling. The root and shoot dry weights also were measured.

The experimental design was a randomized complete block design with four blocks. The data were analyzed by ANOVA using MSTAT-C. The means were separated by LSD and the relationship between extract concentrations was analyzed by linear contrasts.

*Anti-*Fusarium *Assay of ME2-27-19A Extract in Sterile Soil.* ME2-27-19A extract was incorporated into sterilized Spinks loamy sand soil to determine the effects of ME2-27-19A on asparagus and FOA or FM in soil. Since purification required several steps and the whole plant experiment required large amounts of pure compound, an active-ingredient method was used. The content of the active ingredient in crude extract was calculated from the extraction procedure as follows: 2 g of crude extract separated by VLC yielded 812.5 mg of fraction 3. Separation of fraction 3 in the medium-pressure C-18 column yielded 40.2 mg of fraction III. Purification of fraction III by C-18 preparative TLC yielded 7.0 mg of pure ME-144-4, or 3.5 mg/g of crude extract.

Fusarium inoculum was prepared by adding a plug of FOA or FM grown on PDA to autoclaved fescue seed (*Festuca rubra* L.). The fungi were grown for three weeks with mixing each day. The seed was then air-dried and ground to pass a 2-mm sieve in a Wiley mill. Asparagus seedlings were surface-disin-

fected by the acetone-infusion method (Damicone et al., 1981) and germinated on water agar.

The soil was Spinks loamy sand soil (Psammetitic Hapludalf, sandy, mixed, mesic, pH 6.4, CEC 2 meq/100 g, BD 1.4–1.7 g/cm, OM 2–4%) and was autoclaved on three consecutive days. Crude extract was incorporated into dry soil to give 0, 100, 250, 500, and 1000 μg active ingredient (a.i.)/g of soil. Benomyl (50% a.i., wettable powder) was incorporated at the same active ingredient concentrations as the control. Fescue seed inoculum of FOA or FM were added to give 10^6 CFU/ml. Germinated asparagus seedlings were transferred to the treated soil and 1 ml of filter-sterilized, 100 ppm of 20N–8.7P–16.6K fertilizer solution was added. The seedlings were grown in the growth chamber for 10 weeks and rated for *Fusarium* infection on the 1–5 scale. The shoot lengths, number of shoots, shoot dry weights, and root dry weights also were measured.

The *Fusarium* population was counted by placing 10 g soil in 100 ml sterile saline solution and diluting serially. The 10^{-2} dilutions were plated on Komada's medium (Komada, 1975) and the number of colonies were counted after four days. Similarly, bacterial counts were conducted by plating the 10^{-6} dilution on PDA.

The experimental design was a randomized complete block with five blocks. Only four blocks were sampled for the microflora populations because of the time required to sample each block. All data were analyzed by ANOVA and means were separated by LSD. The extract concentrations were compared using orthogonal F tests.

RESULTS AND DISCUSSION

Strain ME2-27-19A was selected as the most inhibitory of the 14 isolates tested against FOA and FM. The microorganism was identified as a *Streptomyces* spp. by Dr. S.K. Mishra (Microbiology Laboratory, Krug International, Houston, Texas). The biochemical tests in the GN Biolog plates suggested that ME2-27-19A used Tween 40, Tween 80, α-D-glucose, methyl pyruvate, D-gluconate, D-glucuronate, and asparagine as carbon sources.

The wild-type ME2-27-19A produced 1 g/liter of crude extract in A-9 medium after eight days of fermentation. Fractions 2 and 3 from the VLC of crude extract were inhibitory to FOA and FM in vitro. The fraction that was eluted with 100% MeOH from the VLC column reacted with ninhydrin, indicating an amino group. This fraction had poor solubility in MeOH or $CHCl_3$ and caused much difficulty in earlier attempts to purify the active compound. Fraction III from the C-18 medium-pressure column was the most inhibitory to FOA and FM. Further purification of fraction III by C-18 preparative TLC was

limited by its solubility (30 mg/ml in MeOH). Purification finally was achieved by applying 21 mg per 20 × 20 C-18 plate (500 μm) and developing the plate in 60:40 (v/v) MeOH:H_2O.

The UV spectrum of ME-144-4 gave absorption maxima at 349, 331, and 316 nm and was indicative of one or more chromophores in the purified compound. The ^{1}H NMR experiment of VLC fraction 3 in deuterated DMSO revealed that it did not contain an aromatic moiety. The signal at δ6.25 appeared as a multiplet, suggesting that it represented olefinic bonds. The group of signals between 4.2 and 5.1 ppm indicated the presence of sugar or aliphatic protons attached to oxygenated carbon. The doublet at δ5.2 revealed the possibility of an anomeric proton of a hexose moiety. The multiplets from 3.0 to 4.0 ppm implied the presence of aliphatic or amino protons. Additional ^{1}H- and ^{13}C NMR experiments will be necessary to characterize the chemical identity of ME-144-4.

Bioassay. The crude extract inhibited growth of *A. flavus*, *C. albicans*, *F. oxysporum*, *F. moniliforme*, *Gleosporium* spp., and *S. aureus* at 100 μg/ml. The inhibition of FOA and FM was similar to that of nystatin but less than faeriefungin (Smith et al., 1990). Preliminary TLC agar overlay experiments gave zones of inhibition for the bands at R_f values of 0.10, 0.65, and 0.68. All three bands were visible under 366-nm UV light. The band at R_f 0.10 was more stable when left exposed to air on silica TLC plates and was selected for further purification. The minimum inhibitory concentration of ME-144-4 was 40 μg/ml for FOA and FM. Smith et al. (1990) found the MIC of faeriefungin was 12.5 μg/ml for FOA. ME-144-4 was not as effective as faeriefungin.

Crude extract of ME2-27-19A killed 95% of the mosquito larvae at 125 μg/ml after 12 hr. However, the insecticidal fractions from the VLC of crude extract were relatively nonpolar and different from the fraction that inhibited *Fusarium*, as demonstrated by the lack of insecticidal activity in the purified C-18 fractions. Since insecticidal compounds were not of direct interest in this project, the fractions were not investigated further.

The seedling assay for toxicity showed no differences in the root elongation of asparagus or tomato (Table 1). However, the root length of curly cress was decreased 42% by VLC fraction 3. The shoot length of asparagus was more than doubled by 1000 μg/ml of VLC fraction 3. The shoot length of tomato increased 21% with 1000 μg/ml of VLC fraction 1. Curly cress showed the least variation of these indicator plants.

Asparagus and Fusarium *Responses to ME2-27-19A Extract.* Although asparagus seedlings showed no inhibition from ME2-27-19A crude extract on filter paper, seedlings placed in Hoagland's agar containing 1000 μg/ml of crude extract failed to grow. When these seedlings were transferred to fresh Hoagland's agar, they resumed normal growth.

Asparagus seedlings grown in Hoagland's agar had higher disease ratings

TABLE 1. PHYTOTOXICITY FROM VLC FRACTIONS OF ME2-27-19A CRUDE EXTRACT[a]

	Fraction	Concn. (μg/ml)	Root length (cm)	Shoot length (cm)
Asparagus				
	1	0	2.7	0.7
		500	1.9	1.0
		1000	2.2	1.0
	2	0	2.4	0.8
		500	3.2	1.3
		1000	3.4	1.2
	3	0	1.4	0.7
		500	2.5	1.0
		1000	1.8	1.6
	$LSD_{0.05}$ (interaction)		NS	NS
Curly cress				
	1	0	1.9	1.6
		500	1.5	1.4
		1000	1.7	1.8
	2	0	1.9	1.6
		500	2.0	1.8
		1000	1.8	1.7
	3	0	1.9	1.6
		500	1.4	1.6
		1000	1.1	1.6
	$LSD_{0.05}$ (interaction)		0.5	NS
Tomato				
	1	0	1.2	3.3
		500	1.2	3.4
		1000	1.3	4.0
	2	0	1.0	3.6
		500	1.1	3.6
		1000	0.9	3.6
	3	0	1.0	2.9
		500	1.2	3.2
		1000	0.9	3.2
	$LSD_{0.05}$ (interaction)		NS	0.6

[a] Means represent the average of three replications.

when inoculated with FOA or FM than the control, as expected (Table 2). ME2-27-19A extract had no effect, except for inhibiting the asparagus at 1000 μg/ml. ME2-27-19A extract alone (200 μg/ml) caused a browning effect on the asparagus roots which increased the disease rating. FM-inoculated seedlings with 100 μg/ml of ME2-27-19A extract had a disease rating of 4.3, while the FM-inoculated seedlings with 200 μg/ml of extract had a rating of 3.7.

ME2-27-19A extract reduced the population of FOA and FM at 1000 μg/g in sterilized field soil (Table 3). It also reduced the shoot length by 92% and the number of shoots by 82% at 1000 μg/ml. Bacterial populations were not affected by either ME2-27-19A or benomyl.

Benomyl reduced the FM population to zero at 100 μg/g, but required 1000 μg/g to eliminate FOA (Table 4). Benomyl increased the number of shoots by 70%, suggesting that it removed apical dominance. Manning and Vardaro (1977) found a similar increase in shoot number from benomyl treatments. Benomyl has not been used for control of FOA and FM in asparagus because it produced no positive effects on yield over the control (Lacy, 1979).

ME2-27-19A does not compare favorably with benomyl for control of FOA or FM. While ME2-27-19A crude extract demonstrated some activity against FOA and FM, the purified compound was not sufficiently active to warrant further testing in the greenhouse or field. Although Me2-27-19A was not effec-

TABLE 2. EFFECTS OF ME2-27-19A CRUDE EXTRACT ON ASPARAGUS SEEDLINGS INOCULATED WITH FOA OR FM IN VITRO[a]

FUS	ACT concn (μg/ml)	Disease rating	Root dry wt. (mg)	Shoot dry wt. (mg)
O	0	1.0[b,c]	6.7[b]	19.4[b]
	100	1.3	4.4	16.9
	200	1.5	5.3	16.6
FOA	0	2.5	4.9	18.9
	100	2.7	4.6	13.4
	200	3.3	4.8	22.7
FM	0	2.7	5.0	19.0
	100	4.3	3.4	16.5
	200	3.7	6.0	20.2
$LSD_{0.05}$		1.3	2.5	8.0

[a] FUS = *Fusarium* spp.; ACT = actinomycete.
[b] Means represent the average of four replications.
[c] *Fusarium* infection rating based on the scale of 1–5, where 1 = healthy seedling, 2 = slightly infected, 3 = moderately infected, 4 = severely infected, 5 = dead seedling.

TABLE 3. EFFECTS OF ME2-27-19A CRUDE EXTRACT ON ASPARAGUS SEEDLINGS INOCULATED WITH FOA OR FM IN STERILIZED FIELD SOIL.

FUS	Extract concn (μg/ml)	*Fusarium* population (log cfu)	Bacteria population (log cfu)	Shoots (*N*)	Shoot length (cm)	Shoot rating
O	0	1.5[a]	7.2[a]	2.6[b,c]	9.9[b,c]	1.6[b,d]
	100	4.5	8.2	2.0	5.5	4.4
	250	0.0	8.2	1.2	4.4	3.6
	500	0.0	7.6	1.0	2.1	3.2
	1000	0.0	7.6	0.6	0.7	3.4
FOA	0	5.9	7.6	1.6	4.8	5.0
	100	5.5	7.7	1.2	3.3	5.0
	250	5.4	7.6	0.8	2.4	5.0
	500	5.5	7.8	0.8	1.8	4.8
	1000	1.5	7.3	0.4	1.0	4.2
FM	0	5.6	7.9	2.0	7.5	4.6
	100	5.7	7.4	1.2	3.2	5.0
	250	4.1	7.3	1.0	2.4	5.0
	500	2.8	7.7	0.4	1.1	4.8
	1000	2.7	7.8	0.4	0.7	4.6
$LSD_{0.05}$ (interaction)		2.4	1.3	0.8	1.8	1.2

[a]Means represent the average of four replicates.
[b]Means represent the average of five replicates.
[c]Linear F value significant at $P < 0.01$.
[d]Shoot rating based on a scale of 1-5, where 1 = healthy seedling, 2 = slightly stunted, 3 = moderately stunted, 4 = severely stunted or diseased, 5 = dead seedling.

TABLE 4. EFFECTS ON BENOMYL ON ASPARAGUS SEEDLINGS INOCULATED WITH FOA OR FM IN STERILIZED FIELD SOIL.

FUS	Benomyl concn (μg/ml)	Fusarium population (log cfu)	Bacteria population (log cfu)	Shoots (*N*)	Shoot length (cm)	Shoot rating
O	0	1.5[a]	7.2[a]	2.6[b]	9.9[b]	1.6p[b,c]
	100	0.0	7.5	3.6	8.6	1.6
	250	0.0	8.0	2.2	9.1	1.6
	500	0.0	7.7	3.2	7.5	2.0
	1000	0.0	7.5	3.8	6.2	2.6

TABLE 4. CONTINUED

FUS	Benomyl concn (μg/ml)	Fusarium population (log cfu)	Bacteria population (log cfu)	Shoots (N)	Shoot length (cm)	Shoot rating
FOA	0	5.9	7.6	1.6	4.8	5.0
	100	2.4	8.2	3.2	10.1	2.4
	250	1.6	7.9	3.0	10.4	2.2
	500	1.0	8.0	4.4	7.7	3.0
	1000	0.0	5.7	3.2	5.9	2.6
FM	0	5.6	7.9	2.0	7.5	4.6
	100	0.0	8.0	3.6	5.9	2.8
	250	0.0	8.0	4.2	6.2	2.6
	500	0.0	7.6	4.0	6.1	2.4
	1000	0.0	7.9	2.8	7.6	2.4

[a] Means represent the average of four replicates.
[b] Means represent the average of five replicates.
[c] Shoot rating based on a scale of 1-5, where 1 = healthy seedling, 2 = slightly stunted, 3 = moderately stunted, 4 = severely stunted or diseased, 5 = dead seedling.

tive against FOA or FM, this paper establishes a protocol that may be used for future attempts to control these pathogens.

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METHYL SALICYLATE AND (−)-(1*R*,5*S*)-MYRTENAL ARE PLANT-DERIVED REPELLENTS FOR BLACK BEAN APHID, *Aphis Fabae* SCOP. (HOMOPTERA: APHIDIDAE)

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Abstract—Methyl salicylate and (−)-(1*R*,5*S*)-myrtenal stimulate specific olfactory cells in the primary rhinaria on the sixth and fifth antennal segments, respectively, of the black bean aphid, *Aphis fabae*. In behavioral studies employing a linear track olfactometer, both compounds were repellent to *A. fabae* and also inhibited attraction to volatiles from its host, broad bean (*Vicia faba*). Methyl salicylate is associated with secondary metabolite-based defense in plants, and the monoterpenoid (−)-(1*R*,5*S*)-myrtenal is metabolically related to (−)-(1*S*,5*S*)-α-pinene, an abundant component of defensive resins produced by gymnosperms. It is argued that these two compounds are employed by *A. fabae* as indicators of nutritionally unsuitable or nonhost plants.

Key Words—Aphid, *Aphis fabae*, Homoptera, Aphididae, semiochemicals, chemical ecology, electrophysiology, salicylate, monoterpenoid.

INTRODUCTION

Evidence is accumulating that alate aphids employ volatile kairomones in host plant recognition prior to landing (Pickett et al., 1992). Studies on aphid olfactory receptors, particularly using single-cell recordings (SCR) coupled with high-resolution gas chromatography (GC) (Wadhams, 1990), have allowed identifi-

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cation of electrophysiologically active compounds, the behavioral roles of which are being examined (Campbell et al., 1993; Isaacs et al., 1993). The aphids *Brevicoryne brassicae* (L.) and *Lipaphis erysimi* (Kalt.), which specialize on plants in the Brassicaceae (= Cruciferae), are attracted in a linear track olfactometer to 3-butenyl isothiocyanate (Nottingham et al., 1991), a glucosinolate catabolite typical of this family (Ju et al., 1982). Similarly, (−)-*β*-caryophyllene, a ubiquitous plant component abundantly present in hops, *Humulus lupulus* L. (Cannabaceae), proved attractive to the damson-hop aphid, *Phorodon humuli* (Schrank), as was the fatty acid oxidation product (*E*)-2-hexenal (Campbell et al., 1993). The black bean aphid, *Aphis fabae* Scop., has also shown attraction to volatiles from its host, broad bean *Vicia faba* L. (Fabaceae = Leguminosae) (Nottingham et al., 1991). However, *A. fabae* and *P. humuli* also possess olfactory receptors for isothiocyanates (Nottingham et al., 1991; Pickett et al., 1992), although neither aphid colonizes brassicas, suggesting that these compounds could be employed as indicators of nonhost plants that may deter these aphids from landing. Indeed, olfactometer studies with alate *A. fabae* showed that 4-pentenyl and 3-butenyl isothiocyanates were repellent and also masked the attractancy of the host plant (Nottingham et al., 1991; Isaacs et al., 1993). For *P. humuli*, application of an isothiocyanate precursor decreased aphid numbers on hops in the field (C.A.M. Campbell, personal communication). With the cereal aphids *Rhopalosiphum padi* L. and *Metopolophium dirhodum* (Walk.), methyl salicylate, as a component of the rosaceous winter host, significantly reduced colonization by spring migrants on wheat and barley crops (Pettersson et al., 1994). Such masking of host attractancy by nonhost compounds has been documented in pioneering studies on the Colorado potato beetle, *Leptinotarsa decemlineata* Say (Thiery and Visser, 1987).

The olfactory receptors of a number of aphid species have been investigated by GC-SCR, using extracts from a range of host and nonhost plants (Pickett et al., in preparation). Methyl salicylate and (−)-(1*R*,5*S*)-myrtenal, a component of the resins produced by gymnosperms, were identified as having significant electrophysiological activity in many species. It was considered possible that these two compounds could have host-masking effects with *A. fabae*, which does not naturally colonize rosaceous plants or gymnosperms. In the present study, electrophysiological threshold concentrations for methyl salicylate and (−)-(1*R*,5*S*)-myrtenal were established for olfactory receptors of *A. fabae* and behavioral activity was investigated.

METHODS AND MATERIALS

Aphids. Alate virginoparous *A. fabae* were reared in crowded cultures on *V. faba* (cv. tick bean) at 15°C and 16 hr day length. For olfactometer studies, young adults were held over damp sand under glass tubes and starved for 24 hr prior to assays. Aphids were used only once.

Chemicals. Methyl salicylate and (−)-(1*R*,5*S*)-myrtenal were obtained from Aldrich Chemical Co. (purity 99% and 98%, respectively) and diluted in hexane for SCR studies. In olfactometer assays, compounds were placed in a 1- or 10-μl glass microcapillary (Drummond Sci. Co.) held at 45° in one arm of the olfactometer and released into an airstream of 500 ml/min. Release rates from the capillaries were calculated by measuring the movement of the meniscus over time using an eyepiece graticule and microscope (Isaacs et al., 1993).

Entrainment of Plant Volatiles. To ascertain whether methyl salicylate or (−)-(1*R*,5*S*)-myrtenal was present in the volatiles produced by *V. faba*, seedlings (cv. Sutton Dwarf, ca. 20 cm tall) in potting compost were contained in a glass culture vessel (Quickfit FV range, 1 liter) and air, dried and purified by passage through activated 5 Å molecular sieve and charcoal, was drawn at 1 liter/min over the plant material. Volatiles were entrained onto Porapak Q that had been purified by washing with ether (5 ml) and heating for 12 hr at 150°C under a stream of nitrogen. Collected volatiles were desorbed from the Porapak by elution with freshly distilled diethyl ether. The resulting extract was concentrated under a stream of nitrogen and stored in sealed glass ampoules at −20°C.

Gas Chromatography–Mass Spectrometry (GC-MS). GC employed an on-column injector and a 50-m × 0.32-mm-ID fused silica column with a cross-linked methylsilicone bonded phase (HP-1), 0.52-μm film thickness, linked directly to a 70–250 mass spectrometer and integrated data system (VG Analytical). Tentative identifications by GC-MS were confirmed by comparison with authentic samples and then by peak enhancement on GC (Pickett, 1990). Fragmentograms were constructed using the data system to select intensity values of designated ion masses typical of the spectra from compounds under analysis. Conditions: GC, 30°C for 5 min, 5°/min to 180°C isothermal; MS, electron impact at 70 eV, 230°C.

Electrophysiology. Recordings from cells associated with the olfactory receptors on the primary rhinaria of *A. fabae* alate virginoparae were made using tungsten microelectrodes. The indifferent electrode was placed in the first or second antennal segment, and the recording electrode was then brought into contact with the rhinarium until impulses were recorded. Permanent copies of the action potentials generated by the olfactory cells were obtained by standard methods (Wadhams et al., 1982). The stimulus was delivered into a purified airstream (1 liter/min) flowing continuously over the preparation. The delivery system, employing a filter paper in a disposable Pasteur pipet cartridge, has been described previously (Wadhams et al., 1982). The impulse frequency was determined as the number of impulses elicited during the first 1 sec after stimulus application.

Olfactometry. Experiments were carried out in a linear track olfactometer (Sakuma and Fukami, 1985) made of transparent Perspex and brass rods and based on a design modified for use with aphids. Groups of ca. 25 young adult

alate *A. fabae* were placed in a pot with Fluon-lined walls in the bottom of the olfactometer. Only those aphids that climbed the vertical rod, in response to an overhead light, were scored for their initial turning choice at the T junction of the rods, either towards odor-laden or control (odor-free) airstreams. For control runs, aphids were assayed without chemical in either arm. In all bioassays, total numbers of aphids turning left or right during a 10-min period were recorded, and the olfactometer was rotated 180° between runs to remove any directional bias. Air drawn though the apparatus was exhausted from the room, and the olfactometer equipment was washed between runs with a 1% (v/v) solution of Teepol detergent and then a 75% (v/v) methanol solution. The mean numbers of aphids orienting towards different treatments were compared using two-tailed related sample *t* tests.

Olfactometer assays to test the effect of nonhost chemicals on the orientation of *A. fabae* to *V. faba* (cv. Sutton Dwarf) odor employed 2 g of leaves freshly picked from greenhouse grown plants in one arm of the olfactometer, with damp filter paper (Whatman No. 1, 4.25-cm diameter) at the other inlet to equalize humidity. White paper was placed either side of the T junction to prevent orientation to visual stimuli from the plant material, and synthetic compounds were released as before from the same arm as the plant material. Aphids were assayed for their response to host odor with and without (−)-(1*R*,5*S*)-myrtenal and methyl salicylate.

RESULTS AND DISCUSSION

In alate virginoparae of *A. fabae*, electrophysiological responses to methyl salicylate are confined to cells in the distal primary rhinarium (sixth antennal segment) (Figure 1), while cells in the proximal primary rhinarium (fifth antennal segment) respond to (−)-(1*R*,5*S*)-myrtenal (Figure 2). The threshold level obtained for these two compounds (10^{-9} g) was two orders of magnitude lower than that observed previously for 4-pentenyl isothiocyanate (Nottingham et al., 1991). Responses for *A. fabae* in the linear track olfactometer to methyl salicylate and (−)-(1*R*,5*S*)-myrtenal are given in Tables 1 and 2, and show threshold repellency at release rates of 50 and 34 μg/hr, respectively, an order of magnitude lower than the behavioral threshold for the alkenyl isothiocyanates (Isaacs et al., 1993). *V. faba* leaf volatiles attracted *A. fabae* (Table 3) but, as shown previously with isothiocyanates, addition of threshold amounts of either methyl salicylate or (−)-(1*R*,5*S*)-myrtenal eliminated the response. To confirm that these two compounds are not produced by intact *V. faba*, entrained volatiles were analyzed in GC-MS by mass fragmentography at the abundant diagnostic ions *m*/*z* 92, 120, 152 and *m*/*z* 79, 107, respectively (Mass Spectrometry Data Centre, 1991). Neither methyl salicylate nor (−)-(1*R*,5*S*)-myrtenal could be detected in the sample.

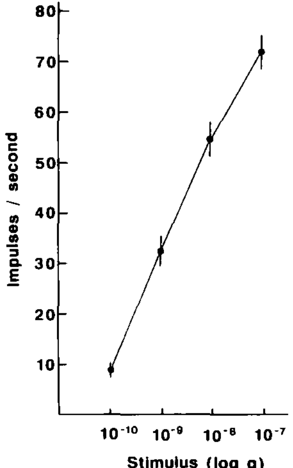

FIG. 1. Dose-response curve of *Aphis fabae* olfactory cell (distal primary rhinarium) to methyl salicylate. Means of five preparations ±SE.

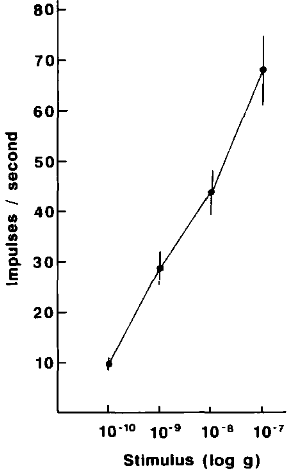

FIG. 2. Dose-response curve of *Aphis fabae* olfactory cell (proximal primary rhinarium) to (−)-(1*R*,5*S*)-myrtenal. Means of five preparations ±SE.

TABLE 1. RESPONSES OF ALATE *Aphis fabae* VIRGINOPARAE TO DIFFERENT RELEASE RATES OF METHYL SALICYLATE IN LINEAR TRACK OLFACTOMETER

Release rate (μg/hr)	Aphids (mean ± SE) moving towards		t	Significance (P)[a]
	Treatment	Control		
0	7.3 ± 0.9	9.0 ± 0.9	1.90	NS
30	7.3 ± 0.8	7.0 ± 0.9	0.37	NS
40	8.6 ± 0.6	9.9 ± 1.4	1.16	NS
50	6.3 ± 0.9	9.0 ± 1.1	2.67	<0.05
60	5.1 ± 0.6	7.6 ± 0.6	2.65	<0.05
70	5.0 ± 0.6	7.8 ± 0.5	2.76	<0.05
100	7.6 ± 1.2	11.0 ± 0.9	2.58	<0.05

[a] NS, not significantly different; related sample t test (eight replicates).

TABLE 2. RESPONSES OF ALATE *Aphis fabae* VIRGINOPARAE TO DIFFERENT RELEASE RATES OF (−)-(1*R*,5*S*)-MYRTENAL IN LINEAR TRACK OLFACTOMETER

Release rate (μg/hr)	Replicates (N)	Aphids (mean ± SE) moving towards		t	Significance (P)[a]
		Treatment	Control		
0	8	7.4 ± 0.4	7.1 ± 0.9	0.31	NS
8	8	8.4 ± 0.8	9.4 ± 0.6	1.25	NS
17	8	7.9 ± 1.0	8.9 ± 1.0	0.97	NS
25	8	9.3 ± 1.8	9.1 ± 1.5	0.09	NS
34	12	6.8 ± 0.5	11.8 ± 0.9	5.63	<0.001
42	12	6.3 ± 0.7	11.1 ± 0.7	5.29	<0.001
51	8	11.6 ± 0.9	14.8 ± 0.9	5.29	<0.001
67	8	6.1 ± 0.6	10.9 ± 1.2	4.09	<0.001
101	8	7.6 ± 1.3	12.9 ± 1.0	2.71	<0.05

[a] NS, not significantly different; related sample t-test.

Methyl salicylate (Figure 3A) is widely distributed as a component of certain plants, e.g., in the Rosaceae and Salicaceae. It is also a volatile metabolite of salicylic acid, a plant phenolic that is a known allelopathic agent (Balke et al., 1987) and a compound capable of inducing various secondary metabolite-based defense mechanisms, such as production of phenolics by the inducible phenylalanine ammonia-lyase (PAL) system (Ward et al., 1991). Thus, response to methyl salicylate would enable *A. fabae* to avoid nonhost plants typified by

TABLE 3. RESPONSES OF ALATE *Aphis fabae* VIRGINOPARAE TO HOST PLANT ODOR WITH AND WITHOUT THRESHOLD CONCENTRATIONS OF NONHOST CHEMICALS IN LINEAR TRACK OLFACTOMETER

Stimulus	Aphids (Mean ± SE) moving towards: Treatment	Aphids (Mean ± SE) moving towards: Control	t	Significance $(P)^a$
V. faba leaves	7.0 ± 0.6	4.6 ± 0.3	3.99	<0.05
V. faba + methyl salicylate (50 μg/hr)	8.6 ± 0.7	8.3 ± 0.9	0.44	NS
V. faba + (-)-(1*R*,5*S*)-myrtenal (34 μg/hr)	5.8 ± 0.5	6.8 ± 0.4	1.76	NS

[a] NS, not significantly different; related sample t test (eight replicates).

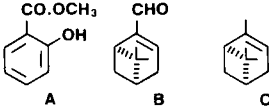

FIG. 3. Structures of methyl salicylate (A), (−)-(1*R*,5*S*)-myrtenal (B), and (−)-(1*S*,5*S*)-α-pinene (C).

a high release of this compound and may also give an indication of plants that are potential hosts but that have heightened phenolic-based defenses through induction by herbivore damage. Indeed, resistance in cultivars of the groundnut *Arachis hypogaea* L. (Fabaceae = Leguminosae) to the aphid *A. craccivora* Koch is known to arise from increased levels of the plant phenolic procyanidin (Grayer et al., 1992). Furthermore, Lima beans (*Phaseolus lunatus*) infested with the mite *Tetranychus urticae* released methyl salicylate and other compounds, which serve as foraging stimuli for the predatory mite *Phytoseiulus persimilis* (Dicke and Sabelis, 1988; Takabayashi et al., 1991; Bruin et al., 1992). Methyl salicylate is released from *V. faba* on damage by chewing insects (M.M. Blight and J.A. Pickett, unpublished), supporting the suggestion that this compound could function as a signal associated with damage or induced defense. It has been noted that in *H. lupulus*, the hop plant, levels of methyl salicylate are higher in damaged than in undamaged leaves, and for *P. humuli*, this compound inhibited attraction to host plant volatiles (Campbell et al., 1993).

(−)-(1*R*,5*S*)-Myrtenal (Figure 3B) is a monoterpenoid that is related, both structurally (Allard et al., 1958) and metabolically (Francke, 1986), to (−)-(1*S*,5*S*)-α-pinene (Figure 3C) and may be used by *A. fabae* to avoid nonhost plants typically containing such compounds. Indeed, the defensive resin secre-

tions produced by gymnosperms are known to attract adapted herbivores (Borden, 1974) and to repel unadapted organisms (Jacobson, 1990) by release of volatile monoterpenoids. Aggregation of pine bark beetles in suitable breeding sites is mediated by a complex of host- and insect-produced metabolites. Many of the latter are oxidation products of host monoterpenes, and it has been suggested that oxidation may represent a detoxification mechanism utilized by the insects for inter- and intraspecific communication (Francke, 1986).

At present, we can only speculate on the possible mechanisms for the masking effects observed with methyl salicylate and (−)-(1*R*,5*S*)-myrtenal in these experiments. Perception of these compounds, which is mediated by specialist olfactory receptors, could lead to a direct inhibitory input to the CNS. Alternatively, in the case of volatile components that are common to host and nonhost plants, these could signal a nonhost by being present at relative proportions typically different from those found in host species. The masking effect of host plant odor by repellent volatiles may be due to a direct interaction of the two conflicting cues. More detailed studies of plant volatiles and aphid neuroethology are required to define the mechanisms of masking.

CONCLUSIONS

A. fabae may avoid nonhost plants and plants with active induced chemical defenses after detection of their volatile components, and this study implicates methyl salicylate and (−)-(1*R*,5*S*)-myrtenal as having such a role. Nonetheless, studies continue on components that may be specific indicators of host acceptability, and future research will explore the effect of such compounds on aphid flight behavior.

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CHEMOSENSORY RESPONSES OF FEMALE ASIAN ELEPHANTS (*Elephas maximus*) TO CYCLOHEXANONE

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Abstract—Cyclohexanone, a naturally occurring component of male Asian elephant temporal gland secretion, was tested as a candidate elicitor of bioresponses from female Asian elephants (*Elephas maximus*). Four female Asian elephants were presented with synthetic cyclohexanone samples during a standardized bioassay. Four types of bioresponses, some or all of which may be important in intersexual communication, were monitored: flehmen, palatal pit area contact, scrub, and check responses. Cyclohexanone evoked persistent responses of all bioresponse types by two females, moderate response by a third female, and very few responses by a relatively unresponsive female. The results suggest that cyclohexanone may provide chemical information about to females male elephants, particularly regarding their state of musth.

Key Words—Cyclohexanone, Asian elephants, *Elephas maximus*, vomeronasal organ, pheromone, flehmen response, palatal pit area contact response, temporal gland secretion.

INTRODUCTION

Male Asian elephants experience an annual physiological change known as musth (Eisenberg et al., 1971; Jainudeen et al., 1972a). Musth occurs during approximately the same month(s) each year for an individual elephant but among males varies in duration (days to months) and time of year (Jainudeen et al., 1972a). The musth episode is characterized by increased aggressive behavior (McGaughey, 1963; Molamure, 1969; Jainudeen et al., 1972a), elevated serum testosterone levels (Jainudeen et al., 1972b; Rasmussen et al., 1984; Niemuller and Liptrap, 1991), and copious secretion of an aqueous, odoriferous fluid from

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the temporal gland, which is later followed by urine dribbling from the penis (McGaughey, 1963; Jainudeen et al., 1972a; Schmidt, 1978). We have observed flehmen responses by female Asian elephants to male temporal gland secretion (TGS) (Rasmussen, 1988). Based on these observations, we hypothesize that there are compounds present in TGS that are utilized by females for the detection of musth in males. Our long-term goals include the identification of possible male-to-female musth-signaling compounds present in temporal gland secretion.

Our initial approach to the identification of possible chemical signs in TGS was to bioassay randomly selected compounds that had been identified in TGS (Rasmussen et al., 1990; Perrin, 1994). During these preliminary bioassays, only one compound, cyclohexanone, evoked high frequencies of flehmen responses (i.e., responses per hour) by the females during repeated bioassays. In addition, cyclohexanone elicited high frequencies of self-directed palatal pit area contact, scrub, and check responses. The function of palatal pit area, scrub and check responses is unknown. However, we suspect that they have a role in intersexual communication based on observations of these responses to TGS and urine (Rasmussen et al., 1986; Rasmussen, 1988). Based on preliminary results, it was necessary to further investigate the possible biological activity of cyclohexanone. In this study, we quantified flehmen, self-directed palatal pit area contact, scrub, and check responses by four female Asian elephants to cyclohexanone.

METHODS AND MATERIALS

Animals. Four nonrelated female Asian elephants were utilized for these studies at the Metro Washington Park Zoo, Portland, Oregon. Their ages at the time of the studies were Belle, 42; Hanako, 29; Tamba, 22; and Susie, 35 years.

Bioresponse Definitions. Four bioresponses, flehmen, palatal pit area contact, scrub, and check, were recorded to placed cyclohexanone samples and control compounds. The bioresponses recorded are described as follows.

During the flehmen response the proboscideal process (the dorsal trunk tip) is placed in a liquid substance (often urine) to be assessed. The trunk is then curled and raised vertically where the trunk tip is placed on paired orifices of the incisive foramina located in the roof of the mouth. These orifices are contiguous with paired ducts that are confluent with the paired lumena of the vomeronasal organ (Ramussen et al., 1986; Rasmussen, 1988).

The palatal pit area contact response (PPACR) begins with placement of the proboscideal process in a liquid substance (often urine) to be assessed. The trunk is then raised to the side of the mouth where the trunk tip is placed on a series of small pits (Rasmussen et al. 1986; Rasmussen, 1988). These pits are located laterally at the junction of trunk and hard palate. Morphologically these

pits are cryptlike structures that are lined with stratified squamous epithelium containing many lymphocytes (Haight and Rasmussen, unpublished). Among the captive Asian elephants at the Metro Washington Park Zoo, PPACR is observed often; the function of this response is under assessment. Although PPACR can be of several types—self-initiated, sequentially multiconspecific, or in response to natural or placed samples—only the latter category was recorded for this study.

Scrub responses are characterized by an initial placement of the proboscideal process into a sample, either natural or placed, and an immediate flattening of the entire end of the trunk, especially the ventral region, on the sample. This is followed by a vigorous scrubbing motion using the whole trunk end. This response, often lasting several seconds, is usually longer in duration than the other responses (Rasmussen et al., 1986).

Check responses are defined as the placement of the proboscideal process into a sample, either natural or placed (Rasmussen et al., 1986; Rasmussen, 1988). By definition, check responses precede flehmen, PPACR, and scrub responses. Check responses may also be independent. During independent check responses the proboscideal process is placed into a sample and removed but no further trunk movements occur. For this study, only independently occurring check responses were quantified. Whether check and scrub responses are olfactory, chemosensory, tactile, or a combination of these is currently under investigation.

Presentation of Test Substances. All bioassays were conducted at 9:30 AM in a 0.3-ha sand yard. At one end was a covered concrete slab (6 × 10 ms) supported by five concrete posts. The samples for bioassay were placed on this slab after it had been hosed off and allowed to dry. The cyclohexanone and control samples were bioassayed simultaneously. Samples were poured adjacent to the upright posts in separate positions. For each bioassay we used two to three different concentrations of cyclohexanone. The presentation positions of the samples, the various concentrations of cyclohexanone and the controls, were changed and noted so that the positions of each sample were different for each bioassay. Placement of the samples next to the posts gave us the clearest view of the samples. After the samples were placed, the elephants were released, as a group, from the barn to the sand yard as part of their daily routine. All bioresponses performed by each elephant to the samples were recorded for 60 min by an observer who stood outside of the sand yard. A bioassay was considered complete only if each elephant voluntarily walked across the concrete slab at least five times to ensure ample exposure to the samples. Ten group bioassays were conducted within two months. Because of management procedures, Hanako was present only for six bioassays and Susie was present for nine bioassays.

We investigated possible low threshold concentrations for cyclohexanone

with one female Asian elephant (Tamba). Several months after group bioassays, the cow grouping had been changed. Our experimental conditions were dictated by these alterations. Only a solitary animal (Tamba) was available, at our requested time and location, for experimentation. We performed five bioassays, within two weeks, with this solitary animal. We suspected that low concentrations should elicit bioresponses since there are large fluctuations in concentration of the chemical components, including cyclohexanone, of male temporal gland secretion (Rasmussen et al., 1990).

Test Samples. During the 10 group experiments, we bioassayed cyclohexanone (analytical grade, 0.013% residue after evaporation, 0.020% water, purchased from Mallinckrodt) at estimated physiological concentrations (10, 20, 40, and 80 mM) based on previous analytical data. Each dilution of cyclohexanone was bioassayed three to 10 times. During the five bioassays with the solitary animal (Tamba) cyclohexanone concentrations of 0.10 mM and 1.00 mM were presented. Both concentrations were bioassayed five times.

Acetone has been used effectively as a solvent for several types of bioassays (Rasmussen et al., 1982, 1986). All samples for bioassay were dissolved in 10 ml of acetone prior to addition to 50 ml of acetate buffer, pH 6.0. The control samples for all bioassays included acetone (65 mM) and acetate buffer, pH 6.0 (200 mM).

Analysis of Data. A possible concentration effect of cyclohexanone on bioresponse frequency was analyzed. Linear regression was used to analyze the total number of bioresponses per bioassay to each concentration of cyclohexanone for each bioresponse type. This analysis was employed for group bioassays only.

Bioresponses to cyclohexanone versus controls, during group bioassays, for each elephant were statistically analyzed. Bioresponses to cyclohexanone occurred rarely, which resulted in high (50–100%) standard errors and nonnormal data. Standard errors are not reported due to lack of meaning. Because of nonnormal data, we used a one-tailed nonparametric sign test at a probability of 5%. The N value for this test was defined as the number of bioassays with the occurrence of at least one bioresponse to cyclohexanone (not including ties with the controls). An N value of at least five was required to utilize this test.

Gas Chromatographic Analysis of Cyclohexanone. We confirmed the purity of cyclohexanone, used in this experiment, by gas chromatographic (GC) analysis. A cyclohexanone GC standard (cyclohexanone dissolved in dichloromethane) was prepared and analyzed. In addition, we examined the possibility of cyclohexanone-derived side-product formation during bioassays, e.g., the reduction of cyclohexanone to cyclohexanol. For this experiment, a 40 mM cyclohexanone solution (cyclohexanone dissolved in 10 ml of acetone in 50 ml acetate buffer) was prepared and set outside in indirect sunlight for one hour (this step was performed to simulate sample placement on the covered concrete slab uti-

lized for bioassay). A 50-ml aliquot of the 40 mM cyclohexanone solution was immediately placed in a separatory funnel and extracted three times with 12.5 ml dichloromethane. The three 12.5-ml dichloromethane extracts were pooled, concentrated under nitrogen, and analyzed.

The gas chromatograph was a Hewlett Packard 5790A with a flame ionization detector (GC(FID). The GC-FID contained a DB-1, 0.32-mm × 60-m × 1.0-μm, polymethyl silicone-coated capillary column (J&W Scientific Inc.) and was connected to a Hewlett Packard 3392A integrator. On-column injection was used. The oven was temperature programmed from 32°C to 200°C at 4°C/min.

RESULTS

Gas Chromatographic Analysis of Cyclohexanone. The GC–FID chromatogram of the cyclohexanone standard showed only two peaks—a solvent (dichloromethane) peak and a cyclohexanone peak—thus demonstrating the purity of cyclohexanone. The GC–FID chromatogram of the 40 mM cyclohexanone solution, set in direct sunlight for one hour, showed two peaks. One peak was the solvent peak and the other had a similar retention time as cyclohexanone. Using peak enhancement, this peak was verified to be cyclohexanone. The results of this experiment demonstrated that side-product development during bioassays with cyclohexanone was unlikely.

Concentration Effect. During group bioassays, the concentration of cyclohexanone appeared to have no, or only a slight, effect on the frequency of each bioresponse types among the females. This is indicated by the low r^2 values (Table 1A). Even though the higher (40 mM and 80 mM) cyclohexanone concentrations were bioassayed more frequently, there was no distinct linear relationship between concentration and bioresponse frequencies.

The total numbers of bioresponses by the solitary animal (Tamba) to both the 0.10 mM and 1.00 mM cyclohexanone concentrations were similar (Table 1B). The single most important result with this experiment was the fact that an elephant did respond to cyclohexanone at these lower concentrations. During these bioassays, Tamba performed a total of six check responses, and zero flehmen, PPACR, and scrub responses to the controls. Responses to controls during group bioassays are presented in the next section.

Ten Bioassays with Four Female Elephants. The bioresponses to the four concentrations of cyclohexanone were pooled together for the analysis of group bioassay data because of the apparent negligible concentration effect of cyclohexanone on bioresponse frequencies (Table 1A).

Flehmen Response. The total number of flehmen responses to cyclohexanone by an individual animal ranged from zero to 14 (Figure 1). No flehmen

TABLE 1. TOTAL NUMBER OF BIORESPONSES TO CONCENTRATIONS OF CYCLOHEXANONE DURING 10 BIOASSAYS WITH A GROUP OF FOUR FEMALE ASIAN ELEPHANTS (A) AND FIVE BIOASSAYS WITH A SOLITARY FEMALE ASIAN ELEPHANT (B)

Cyclohexanone concentration	N^a	Flehmen	PPACR	Scrub	Check
A. Four elephants					
10 mM	3	2	0	1	3
20 mM	5	1	4	5	4
40 mM	10	12	5	7	23
80 mM	6	8	7	7	14
R^2		0.067	0.067	0.014	0.11
B. Solitary elephant					
0.10 mM	5	2	5	5	7
1.00 mM	5	1	4	5	7

[a] N = Number of trials concentration was bioassayed.

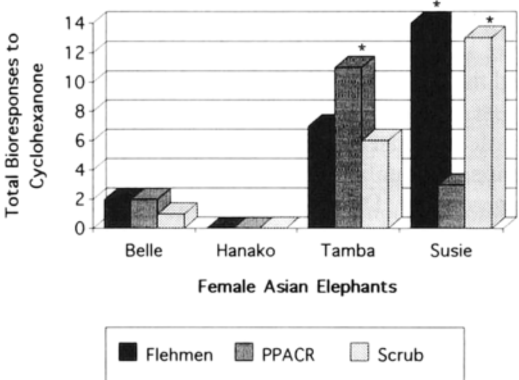

FIG. 1. Total number of flehmen, palatal pit area contact, and scrub responses for each elephant to cyclohexanone during 10 group bioassays. None of these responses occurred to the control compounds. Because of management procedures, Hanako was present for only six bioassays and Susie was present for nine bioassays. Susie's flehmen and scrub response totals to cyclohexanone versus controls were statistically testable, using a nonparametric sign test, and were significant (*$P < 0.05$). Tamba's PPACR to cyclohexanone was statistically testable and was significant (*$P < 0.05$) relative to controls.

responses occurred to the control compounds. The N value was high enough to statistically test one animal's (Susie) flehmen responses only. Susie performed at least one flehmen in six of nine bioassays and her flehmen frequency to cyclohexanone versus controls was significantly greater ($P = 0.016$, $N = 6$, nonparametric one-tailed sign test).

Palatal Pit Area Contact Response. The total number of PPACR by individuals to cyclohexanone ranged from zero to 11 (Figure 1). No PPACR were performed to the controls. The N value was high enough to statistically test PPACR of one elephant (Tamba) only. This animal exhibited at least one PPACR in seven of 10 experiments. Relative to controls, the PPACR frequency to cyclohexanone by this animal was significant ($P = 0.008$, $N = 7$).

Scrub Response. On an individual basis, scrub response totals to cyclohexanone ranged from zero to 13 (Figure 1). Zero scrub responses occurred to the control compounds. Only Susie's scrub response frequencies to cyclohexanone were statistically testable. This animal performed at least one scrub response in six of nine bioassays. Relative to controls, Susie's scrub frequency to cyclohexanone was significantly greater ($P = 0.016$, $N = 6$).

Check Response. Check responses were the most commonly occurring response measured and occurred to both cyclohexanone and the controls (Figure 2). During all bioassays, check response frequencies by each elephant to cyclohexanone were higher than those to the controls. The check response frequencies to cyclohexanone by two elephants (Tamba and Susie) were statistically testable. Relative to controls, both Tamba's and Susie's check response frequency to cyclohexanone were significant (Tamba, $P = 0.020$, $N = 9$; Susie, $P = 0.008$, $N = 7$). Notably, Hanako performed only one bioresponse during all bioassays. This was a check response to cyclohexanone during bioassay 8.

Temporal Distributions of Flehmen, PPACR, Scrub, and Check Responses. The unique, and perhaps most important, aspect of flehmen frequencies to cyclohexanone was the temporal distribution of responses (Figure 3). The temporal distribution of flehmen responses to cyclohexanone indicated that the flehmen responses to cyclohexanone were not novel substance responses. A novel substance response is defined as a bioresponse that occurs with an elevated fre-

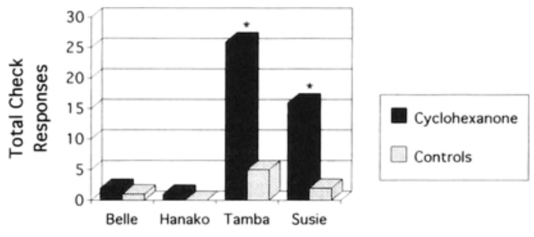

FIG. 2. Total number of check responses for each elephant to cyclohexanone and control compounds during 10 group bioassays. Hanako was present for six bioassays and Susie was present for nine bioassays. Tamba's and Susie's check response total to cyclohexanone were statistically testable, using a nonparametric sign test, and were significant (*$P < 0.05$) relative to controls.

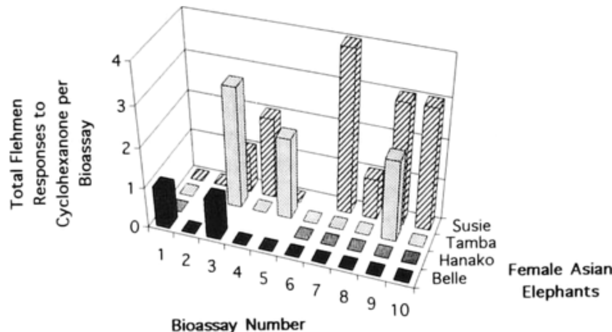

FIG. 3. Plot of temporal distributions of flehmen responses performed per bioassay to cyclohexanone by each elephant during 10 group bioassays. Hanako was present for six bioassays and Susie was present for nine bioassays.

quency (between three to six responses per hour) in the initial bioassay and that decreases to zero upon subsequent bioassays (usually by the third bioassay) (Rasmussen et al., 1986). Two elephant's (Tamba and Susie) flehmen responses to cyclohexanone were widely distributed. Susie was not present during bioassay 6 and this may have effected her flehmen frequencies during bioassays 7-10. Belle's flehmen response total to cyclohexanone was lowest relative to Tamba and Susie. The two flehmen responses that were performed by Belle occurred during the earlier tests. This was the inverse of the flehmen response pattern demonstrated by the other two elephants (Tamba and Susie). The distributions of PPACR, scrub, and check responses to cyclohexanone by the highly responsive animals (Tamba and Susie) were similar to their distributions of flehmen responses to cyclohexanone.

DISCUSSION

Cyclohexanone is the first synthetic, single compound demonstrated to elicit persistent bioresponses among elephants. In this particular instance, persistent occurrences of four distinct bioresponses to a naturally derived ketone have been demonstrated in a group of four female asian elephants at the Metro Washington Park Zoo, Portland, Oregon. The uniqueness of this finding is substantiated from results of previous bioassays with 30 other compounds that had been identified in male temporal gland secretion (Perrin, 1994). During these bioassays, the females either did not respond or responded with a novel substance response.

The flehmen response is a common chemosensory modality among mammals (Albone, 1984). Persistent flehmen responses to cyclohexanone indicate

that cyclohexanone may have some type of biological meaning to female elephants, at least the particular group of elephants tested here. The persistence of the other bioresponses—PPACR, scrub, and check responses—to cyclohexanone may also indicate some type of biological meaning for cyclohexanone, since these responses occurred simultaneously with flehmen responses. It is possible that flehmen, palatal pit area contact, scrub, and check responses may be interrelated during the processing of similar chemical information. Conversely, these responses may be separate responses, occurring at the same time, processing distinct chemical information.

On examination of the individuality of bioresponses to cyclohexanone, we hypothesized that dominance may have influenced the bioresponse patterns to cyclohexanone among this group. This hypothesis is supported by recent studies with cyclohexanone among 21 female Asian elephants at several different facilities (Perrin and Rasmussen, unpublished data). During these tests, dominant females performed bioresponses with a lower frequency than subordinate females. The greatest frequencies of bioresponses to cyclohexanone among our group occurred among Tamba and Susie, who were subordinate elephants relative to Belle and Hanako. The flehmen response pattern to cyclohexanone of the matriarch, Belle, was inversely related to the flehmen response pattern of the subordinate animals. In addition, the second most dominant elephant (Hanako) did not exhibit any flehmen, PPACR, or scrub responses to cyclohexanone, but performed one check response during the eighth bioassay. The response pattern by Hanako could possibly be explained by her dominance, or her individual, relatively nonresponsive trunk-to-mouth behavior to both naturally occurring and placed samples.

Although there was individual variation in response to cyclohexanone, the concentrations of cyclohexanone apparently had no or only slight effect on bioresponse frequencies during group bioassays. This result seems logical, since we bioassayed only a conservative cyclohexanone concentration range (an eightfold variation) among the group. The important information obtained from experiments with the solitary animal (Tamba) was that nonnovel substance bioresponses did in fact occur to low (10- and 100-fold lower than group concentrations) cyclohexanone concentrations. This indicates that there may be a very low threshold concentration for cyclohexanone. The results with Tamba as a solitary animal provide valuable information for the experimental design of future bioassays with cyclohexanone.

Concentrations of cyclohexanone similar to those bioassayed among our group of females have been bioassayed among male Asian elephants. During these experiments, one of three adult males demonstrated a novel substance response to cyclohexanone, while the other two showed no responses during 20 tests (10 each) over period of four years (Rasmussen, unpublished). Based on the positive results with female elephants and the lack of response by males,

we suggest that cyclohexanone is a male-to-female chemical cue that may signal musth cycles in Asian bull elephants.

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PROLONGED POSTSTRIKE ELEVATION IN TONGUE-FLICKING RATE WITH RAPID ONSET IN GILA MONSTER, *Heloderma suspectum*: RELATION TO DIET AND FORAGING AND IMPLICATIONS FOR EVOLUTION OF CHEMOSENSORY SEARCHING

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Abstract—Experimental tests showed that poststrike elevation in tongue-flicking rate (PETF) and strike-induced chemosensory searching (SICS) in the gila monster last longer than reported for any other lizard. Based on analysis of numbers of tongue-flicks emitted in 5-min intervals, significant PETF was detected in all intervals up to and including minutes 41–45. Using 10-min intervals, PETF lasted though minutes 46–55. Two of eight individuals continued tongue-flicking throughout the 60 min after biting prey, whereas all individuals ceased tongue-flicking in a control condition after minute 35. The apparent presence of PETF lasting at least an hour in some individuals suggests that there may be important individual differences in duration of PETF. PETF and/or SICS are present in all families of autarchoglossan lizards studied except Cordylidae, the only family lacking lingually mediated prey chemical discrimination. However, its duration is known to be greater than 2-min only in Helodermatidae and Varanidae, the living representatives of Varanoidea. That prolonged PETF and SICS are typical of snakes provides another character supporting a possible a varanoid ancestry for Serpentes. Analysis of 1-min intervals showed that PETF occurred in the first minute. A review of the literature suggests that a pause in tongue-flicking and delay of searching movements are absent in lizards and the few nonvenomous colubrid snakes

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tested. The delayed onset of SICS may be a specific adaptation of some viperid snakes to allow potentially dangerous prey to be rendered harmless by venom following voluntary release after envenomation and preceding further physical contact with the prey.

Key Words—Tongue-flicking, poststrike elevation in tongue-flicking, strike-induced chemosensory searching, gila monster, lizard, Squamata, Helodermatidae, snake origin.

INTRODUCTION

Poststrike elevation in tongue-flicking rate and strike-induced chemosensory searching occur in a wide variety of squamate reptiles (reviewed in Cooper, 1994a). In many highly venomous snakes, especially crotaline vipers, envenomation is followed by a predictable behavioral sequence (Chiszar and Scudder, 1980; Chiszar et al., 1983) having five major components: (1) voluntary release of the prey, which appears to be restricted to highly venomous snakes such as elapids and viperids, especially when attacking potentially dangerous prey (Radcliffe et al., 1980); (2) a pause before initiation of tongue-flicking and crawling, presumed to allow time for the venom to incapacitate the prey; (3) an increase in tongue-flicking rate; (4) locomotion while tongue-flicking (the latter two behaviors being necessary to relocate and follow the scent trail of the envenomated prey); and (5) scent-trailing to relocate the prey, which by the time of relocation is very likely to have died or become incapacitated.

The strike-release-trail strategy encompassing all five components appears to be unique to some highly venomous snakes including some viperids, but at least some of the components are more widely distributed among squamates (Cooper, 1994a). In particular, the increase in tongue-flicking rate and concurrent locomotion occur in many lizards and nonvenomous snakes (lizards reviewed by Cooper, 1994a; nonvenomous snakes by Cooper et al., 1989; Cooper, 1991a, 1992a; Burghardt and Chmura, 1993). Component 3 is called poststrike elevation in tongue-flick rate (PETF); components 3 and 4 combined are called strike-induced chemosensory searching (SICS). The increased tongue-flicking serves to sample chemical cues (presumably for delivery to the vomeronasal organ), and the movement serves to bring the squamate into a position to detect and follow the scent trail.

In species lacking a strike-release-trail strategy, it has been hypothesized that when prey has escaped, SICS may help relocate the lost prey or other individuals of the same prey type known to be in the vicinity (Cooper et al., 1989; Cooper, 1993; Burghardt and Chmura, 1993). The scant data available show that PETF and/or SICS occur in two families of snakes (Pythonidae, see Cooper, 1991a; Colubridae, see Cooper et al., 1989; Cooper, 1992a; Burghardt and Chmura, 1993) and numerous families of scleroglossan lizards, including

Teiidae, Lacertidae, Scincidae, Anguidae, Helodermatidae, and Varanidae (reviewed by Cooper, 1994a). SICS has not been demonstrated in any iguanian family, but PETF (and perhaps SICS) occurs in an iguanid (Cooper and Alberts, 1993).

The duration of PETF and SICS may be expected to vary widely among taxa, being prolonged in those groups of active foragers capable of scent-trailing as well as detection of prey chemicals in groups having prey that can be located reliably by scent-trailing (Cooper, 1994b). Among viperid snakes, the prey is usually killed and, barring removal by other predators, is reliably available for consumption for a relatively long time. SICS may last over 2 hr in crotalines in the laboratory (Chiszar et al., 1982, 1985). Among small insectivorous lizards, PETF and SICS are much briefer, presumably reflecting a combination of the difficulty of scent-trailing flying insects and greater profitability of searching other patches if brief search fails to detect additional prey. The greatest duration of PETF reported for a lizard is 25–30 min for the varanid *Varanus exanthematicus* (Cooper, 1993). Although *V. exanthematicus* feeds primarily on invertebrates in the field in Senegal (Cisse, 1972), perhaps due to availability, it readily consumes vertebrates, given the opportunity. SICS might have even greater duration in larger varanids that normally eat larger, more easily scent-trailed prey or in the other lizards that have exceptionally well-developed lingual–vomeronasal systems and consume prey that can be located reliably by scent.

In this paper we consider three components of chemosensory searching behavior in squamates. First, we present experimental data on the duration of PETF and SICS (components 3 and 4) in a helodermatid lizard, *Heloderma suspectum*. The gila monster is capable of discriminating prey chemicals from other chemicals (Cooper, 1989a) and exhibits PETF and SICS lasting a minimum of 10 min, the maximum possible time that could have been observed in a previous study (Cooper and Arnett, 1995). We predicted that SICS would be prolonged in the gila monster due to its prolonged active foraging over great distances (Bogert and Del Campo, 1956; Beck, 1990; Beck and Lowe, 1991) and to its diet. The gila monster consumes reptile eggs, eggs and young of ground-nesting birds, and young of small mammals (Bogert and Del Campo, 1956; Jones, 1983; Beck, 1990; Beck and Lowe, 1991). Such prey are unlikely to wander far if they initially escape or are lost. The lizards also might be able to locate juvenile rodents by following scent trails of parents to the nest. Support by prolonged PETF/SICS in varanoid lizards for the proposed varanoid ancestry of snakes is discussed. We review data on the occurrence of a pause after loss or release of prey before beginning to tongue-flick at elevated rates (component 2) and suggest that restriction of the pause to viperids may be a synapomorphic and integral part of the strike-release-trail strategy in that taxon. Finally, we briefly survey the status of knowledge regarding taxonomic distribution of the

five components of the strike-release-trail strategy and chemosensory search for prey in squamates.

METHODS AND MATERIALS

The duration of PETF was studied in eight adult gila monsters. Five were obtained from the Arizona Game and Fish Department and were maintained in the laboratory at Indiana University-Purdue University Fort Wayne (IPFW) for several months prior to testing to ensure habituation of the field-captured lizards to laboratory conditions. Several specimens initially refused to eat anything but eggs, but all were eating mice by the time the study was conducted. Each lizard was isolated in a 51 × 26 × 32-cm glass terrarium with a removable locking screen top. In the terrarium were a sand–gravel substrate and a water bowl. Heat lamps were provided to permit the lizards to thermoregulate. Fluorescent lighting cycled on a 12L:12D schedule. The lizards were fed albino mice (*Mus musculus*) once per week; water was continuously available.

The other three individuals were housed and tested at the Cincinnati Zoo (CZ). Prior to testing, these lizards were removed from their home cages and placed in separate 51 × 26 × 32-cm glass terraria having slate bottoms. No tops, sand, gravel, or water bowls were placed in the cages. Prior to the experiment, the lizards were fed mice once per week and had continuous access to water.

Experimental demonstrations of PETF and SICS in species that do not voluntarily release bitten prey typically incorporate four experimental conditions, including controls, for effects of the experimental context, the sight of prey, and mechanical disturbance caused by experimental removal of the prey from the predator's mouth (e.g., Cooper, 1989b, 1991b; Cooper et al., 1989). Because the presence of PETF and SICS already had been established and the mechanical disturbance condition was found to elicit much higher tongue-flicking rates than the other control conditions, the usual experimental protocol was abbreviated to establish the duration of PETF. As in a study of PETF in *Varanus exanthematicus* (Cooper, 1993), control conditions for effects of experimental context and sight of the prey were omitted.

The two conditions used in the present study were the strike and mechanical disturbance control conditions. In the strike condition a recently killed mouse held in forceps was presented to a lizard, which was allowed to approach, investigate, and bite the mouse. As soon as the lizard bit, the experimenter separated lizard and mouse by pulling steadily on the forceps. If the lizard did not release the mouse, the experimenter gently pushed against the lizard in the opposite direction with a second pair of forceps. To induce several individuals to release the prey, it was necessary to gently pull the mouse upward until the

front legs of the gila monster were lifted slightly off the substrate, which reliably induced voluntary release. To control for possible effects of mechanical disturbance caused by inducing the lizard to release the mouse, a mouse was presented as above and the gila monster was allowed to approach and investigate the mouse. However, when the gila monster opened its mouth and prepared to bite the mouse, the mouse was immediately removed, preventing oral contact, and the lizard was simultaneously pushed away from the mouse with another pair of forceps.

To begin a trial, an experimenter slowly approached a lizard's cage, carefully removed the lid and water bowl (if present), and moved away. Twenty minutes later, the experimenter returned and slowly positioned a mouse 20 cm anterior to the lizard's snout. As soon as a lizard released a bitten mouse or had been pushed after preparing to bite, the experimenter began recording tongue-flicks for 60 min at 1-min intervals. Observations were made between 1100 and 1530 hr at IPFW and between 0900 and 1630 hr at CZ. Ambient temperatures were 24–27°C at IPFW and 28–30°C at CZ.

Each individual was tested once per day in a repeated measures design in which the two stimulus conditions were presented in counterbalanced sequence. Due to extreme nonnormality indicated by the presence of many zero values of tongue-flicking in minutes near the end of the 60-min observation period, the statistical analysis was conducted using the nonparametric Wilcoxon matched-pairs signed-ranks test (Hollander and Wolfe, 1973). As the hypothesis tested was directional, all tests of significance were one-tailed with $\alpha = 0.05$ unless stated otherwise (Siegel, 1956). Data were analyzed for 1-, 5-, and 10-min intervals. The 1-min intervals were useful for determining the onset of PETF. The 5- and 10-min intervals allowed detection of somewhat smaller differences cumulated over longer intervals, especially when the initially high tongue-flicking rates had declined and PETF and SICS were waning. In addition, binomial tests were conducted to assess the significance of frequency of minutes in which an individual's tongue-flicking rate exceeded that of all individuals in the control group (Hollander and Wolfe, 1973).

RESULTS

Mean tongue-flicking rates were highest in both conditions in the initial minutes (Figure 1). They declined gradually thereafter, reaching zero in the control condition by minute 35. In the strike condition, the mean tongue-flicking rate decreased from values in the low 40s and high 30s per minute during the first 5 min to 3–7/min by minute 37, but some individuals continued tongue-flicking in every minute, and the mean rate showed no further detectable decline. Although many individuals failed to tongue-flick in the latter minutes, two indi-

viduals continued tongue-flicking at substantial rates even in the final few minutes. Movements were not recorded, but tongue-flicking and locomotion were closely associated throughout the observation period.

Analysis by 1-min intervals revealed significantly more tongue-flicks in the strike condition in the initial minute ($T = 3$, $N = 8$, $P < 0.02$). Significantly greater tongue-flicking rates were observed in the first three minutes (Table 1; min 2: $T = 1$, $N = 8$, $P < 0.01$; min 3: $T = 5$, $N = 8$, $P < 0.04$). Although the difference between conditions was not significant in minutes 4 and 5 due to elevated tongue-flicking in the control condition (Table 1; min 4: $T = 15$, $N = 8$, $P > 0.05$; min 5: $T = 7$, $N = 7$, $P > 0.05$), they were significant in 21 of the first 30 min. Although the difference was significant thereafter only in minute 34, more tongue-flicks were emitted in the strike condition in every one of the

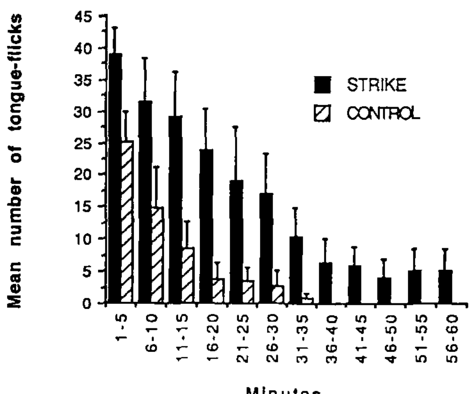

FIG 1. Mean numbers of tongue-flicks emitted by *Heloderma suspectum* in 5-min intervals in the strike and control conditions. Error bars represent 1.0 SE.

TABLE 1. MEANS, STANDARD ERRORS, AND RANGES OF NUMBERS OF TONGUE-FLICKS IN STRIKE AND CONTROL CONDITIONS FOR FIVE INITIAL 1-MINUTE INTERVALS

	Strike			Control		
Min	Mean	SE	Range	Mean	SE	Range
1	41.9	4.1	19–56	22.4	2.3	14–32
2	38.8	2.4	29–51	21.6	3.4	9–38
3	38.4	3.7	26–60	25.6	4.6	14–40
4	36.0	5.4	4–55	31.4	5.9	4–56
5	39.2	5.2	12–61	25.1	5.3	0–45

60 min. Of the two individuals that tongue-flicked in the strike condition during the final 10 min, one tongue-flicked in nine of the ten, the other in all 10. For each of these two individuals, we conducted a binomial test for the presence of PETF in minutes 51–60 using a null hypothesis that tongue-flicking rate by the individual and the maximum rate for any individual in the control group were equal. Both individuals tongue-flicked at significantly greater than control levels during the final 10 min ($P = 0.011$ and $P = 0.001$).

Analysis of data for 5- and 10-min intervals revealed the presence of SICS well after the last detectable time based on 1-minute intervals. In all 5-min intervals, up to and including minutes 41–45, tongue-flicking rates were significantly higher in the strike condition than in the control condition (Figure 1; 1–5: $T = 3$, $P < 0.02$; 6–10: $T = 1$, $P < 0.008$; 11–15: $T = 3$, $P < 0.02$; 16–20: $T = 3$, $P < 0.02$; 21–25: $T = 2$, $P < 0.024$; 26–30: $T = 1$, $P < 0.032$; 31–35: $T = 1$, $P < 0.032$; 36–40: $T = 0$, $P < 0.016$; 41–45: $T = 0$, $P < 0.032$; 46–50: $T = 0$, $P > 0.05$; 51–55: $T = 0$, $P > 0.05$; 56–60: $T = 0$, $P > 0.05$; due to tied values, effective sample sizes were eight in the first four intervals, seven in the fifth, six in the next three intervals, then five, four, four, and three in the last four intervals). Analyses for 10-min intervals were concordant with those for 5-min intervals in revealing significantly greater tongue-flicking in the strike condition in all intervals up to and including minutes 41–50, but not in the final 10 min (Figure 2; 1–10: $T = 1$, $P < 0.008$; 11–20: $T = 3$, $P < 0.02$; 21–30: $T = 1$, $P < 0.02$; 31–40: $T = 0$, $P < 0.016$; 41–50: $T = 0$, $P < 0.016$: 51–60: $T = 0$, $P > 0.05$; due to tied values, effective sample sizes were eight, eight, seven, six, six, and four). To further resolve the duration of PETF, the interval 46–55 was analyzed, revealing significantly greater tongue-flicking in the strike condition than in the control condition ($T = 0$, $P < 0.032$, after ties were removed, effective $N = 5$).

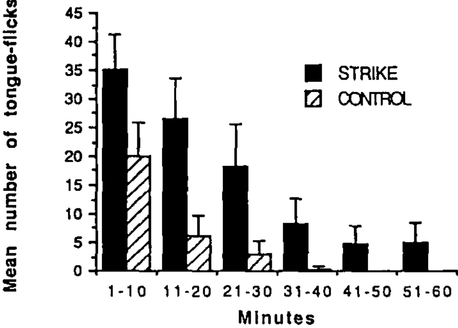

Fig. 2. Mean numbers of tongue-flicks emitted by *Heloderma suspectum* in 10-min intervals in the strike and control conditions. Error bars represent 1.0 SE.

There was substantial variation among individuals in duration of PETF. Numbers of individuals that tongue-flicked during 5-min intervals were zero in the control condition for all intervals beginning at 36–40. In the strike condition, the numbers of individuals tongue-flicking were six in minutes 36–50, five in minutes 41–45, four in minutes 46–50 and 51–55, and three in minutes 55–60. These data alone are sufficient to account for the pattern of significance observed in these intervals.

DISCUSSION

Duration of PETF. The prediction that PETF (component 3) is of longer duration in *H. suspectum* than in most other lizards was verified. Because movements and tongue-flicking were closely associated, SICS has a long duration, similar and perhaps identical to that of PETF. The analyses using five- and ten-min intervals showed that the minimum duration of which PETF was significant for the group was 41–45 min by the 5-min intervals and 46–55 min by the 10-min intervals. However, PETF may last even longer in some individuals, as shown by its presence during the final 10 min in two individuals. Thus, PETF is present for 46–55 min in the group, and some residual group effects remain after 60 min. PETF persists for at least 60 min and perhaps longer in some individuals.

High initial rates of tongue-flicking in both conditions reflect an immediate effect of mechanical disturbance and of biting prey on tongue-flicking. No baseline rates were measured in the present study, but tongue-flicking in the first minutes was far above baseline rates and above rates observed for the two additional control conditions in the demonstration of the existence of PETF in *H. suspectum* (Cooper and Arnett, 1994). Thus, there was no detectable pause before the onset of a high tongue-flick rate.

Although the temporal trends in tongue-flicking rates show a smooth decline for the 5- and 10-min intervals in Figures 1 and 2, the mean tongue-flicking rates in both conditions show some degree of independent waxing and waning from minute to minute (Table 1) as some individuals increased, decreased, or stopped tongue-flicking, sometimes momentarily, accounting for the numerous changes between significance and nonsignificance in the first 30 min. The data for 1-min intervals were useful primarily for detecting the onset of PETF and trends in the initial and final minutes.

PETF and SICS (components 3 and 4) last considerably longer in the gila monster than in any other lizard yet studied. As suggested above, the nature of the prey, which are immobile or have very limited mobility, greatly enhances the probability that a chemosensory search for prey lost in the immediate vicinity will eventually be successful. Prolonged PETF is thus favored. In addition, the

dispersed distribution of bird, mammal, and reptile nests requires chemosensory search over wide areas (Bogert and Del Campo, 1956; Jones, 1983; Beck, 1990, Beck and Lowe, 1991). An ability to follow prey scent trails would allow gila monsters to follow scent trails of adult rodents to nests containing edible young.

PETF and SICS occur in numerous families of autarchoglossan lizards (reviewed by Cooper, 1994a), but the duration of PETF among all the families studied is greater than 2 min only in Helodermatidae and Varanidae (Cooper, 1993, and this paper). In these two families, the duration is intermediate to that of other lizards and venomous snakes. Phylogenetic relationships among major families of autarchoglossan lizards in which PETF and/or SICS have been studied are shown in Figure 3. Although PETF occurs in all of these families except Cordylidae (Cooper, 1994a, unpublished data for Cordylidae), the only two

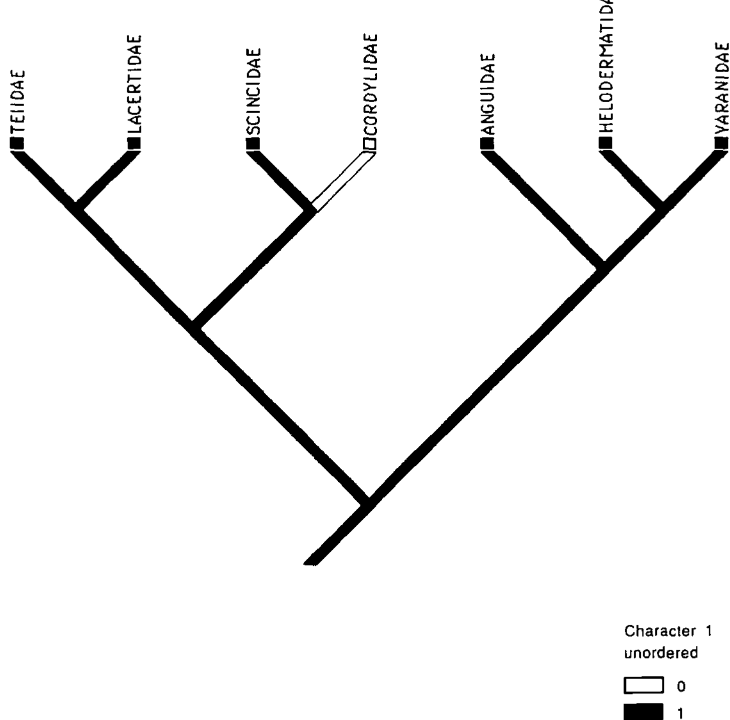

Fig. 3. The phylogeny of major families of autarchoglossan lizards for which data on PETF and/or SICS are available. Helodermatidae and Varanidae are the living families of Varanoidea, which probably includes snakes, which are the only squamates other than varanoid lizards known to have prolonged SICS.

families in which PETF lasts more than a few minutes, Varanidae and Helodermatidae, are very close relatives that constitute the living representatives of Varanoidea. Despite the limitation of data to one species from each of two families, the remarkable stability of chemosensory behaviors such as prey chemical discrimination within lizard families (Cooper, 1994a,c) suggests that long-lasting PETF may be a synapomorphy of varanoid lizards, at least of their extant families.

The phylogenetic relationship of snakes to other squamates remains poorly understood. It is widely accepted that snakes are included in Scleroglossa, which also includes autarchoglossans, gekkonoideans, and amphisbaenians (Estes et al., 1988). However, the relationship of snakes to other taxa within Scleroglossa is uncertain. On the basis of lingual structure, McDowell (1972) hypothesized a varanoid ancestry for snakes. Using more lingual characters and later a combination of lingual and other chemosensory characters, Schwenk (1988, 1993) presented evidence suggesting a sister group relationship between snakes and Varanidae. There is thus a possibility that long-lasting PETF may be a plesiomorphic heritage from varanoid lizards. Data on the existence and duration of PETF and SICS are needed in a wide variety of snake taxa. PETF has been observed to last for more than 2 hr in a colubrid (*Elaphe g. guttata*; Cooper, unpublished observations, $N = 2$), but almost no information is available for less advanced snakes. PETF occurs in a pythonid (Cooper, 1991a), but no data exist on its duration. Studies of scolecophidians and several henophidian families will be crucial to determination of the history of PETF and SICS in snakes.

The absence of a pause (component 2) of 1 or 2 min after prey was removed before gila monsters began to tongue-flick at elevated rates was consistent with findings for all lizards yet studied that exhibit PETF (Iguanidae, Cooper and Alberts, 1993; Lacertidae, Cooper, 1991b; Teiidae, Cooper, 1994; Scincidae, Cooper, 1992b; Gerrhosauridae, Cooper, 1992c; Anguidae, Cooper, unpublished data; Varanidae, Cooper, 1989b, 1993). The lack of delayed onset of PETF distinguishes PETF in lizards and colubrid snakes (Cooper et al., 1989; Cooper, 1992; Burghardt and Chmura, 1993) from that in viperid snakes (Chiszar and Scudder, 1980).

Components of Chemosensory Search for Prey. The five components of chemosensory searching by squamate reptiles outlined earlier as following oral contact with prey also contain elements that are used in searching for prey prior to chemosensory detection, prior to location of the prey after detection, and after swallowing induces elevated tongue-flicking rates. Thus, the components may be considered as a simple, general framework for investigation of chemosensory behaviors related to feeding.

Component 1, the voluntary release of prey, may occur occasionally in various taxa, especially in response to strong defenses. Nevertheless, reliable release of prey is known only in highly toxic viperid and elapid snakes (e.g.,

Chiszar and Scudder, 1980; Chiszar et al., 1983), presumably because the prey can be captured and ingested with high probability followed release because venom prevents their escape. Although phylogenetic relationships are poorly known, it is believed that Viperidae is the sister group of the remaining families of Caenophidia, the higher snakes (Dessauer et al., 1987), and that viperids are the oldest extant caenophidians (Cadle, 1988). The widespread occurrence of voluntary release in Viperidae suggests its origin within Viperidae or its common ancestor. Thus, voluntary release may be an adaptation to avoid injury that evolved in Viperidae and is a synapomorphy of many viperids that consume dangerous prey, especially rodents. However, its complete distribution in Viperidae is unknown, and voluntary release does not occur in several vipers that are rodent specialists (Chiszar and Radcliffe, 1989). One such species, the jumping viper, *Porthidium nummifer*, has skin folds that cover the facial pits and eyes as the prey is held, reducing the vulnerability of the head to injury (Chiszar and Radcliffe, 1989). Whether voluntary release in elapids is plesiomorphic or convergent is unknown and unlikely to be resolved until the phylogeny of caenophidian families is clarified. Determination of its presence or absence in Atractaspidae would also be useful.

Component 2, the pause in tongue-flicking and movement before initiation of SICS, is known only in Viperidae. It is absent in lizards and in the few species of nonvenomous snakes studied. PETF appeared rapidly in three species of colubrids (Cooper et al., 1989; Cooper, 1992a; Burghardt and Chmura, 1993). Only in the pythonid *Python regius* has a delay of PETF been observed (Cooper, 1991a). In that species the delay was much longer than that observed in crotaline snakes. Furthermore, the delay is not comparable to that of crotalines but is an artifact of the experimental design and analysis. The ball pythons began tongue-flicking at fairly high rates immediately in the strike condition, but also did so in a control condition for the effects of seeing the prey. In the first minute, they actually tongue-flicked at twice the rate performed in the mechanical disturbance control condition, but PETF was considered absent due to the lack of significantly greater tongue-flicking in the strike condition than in all of the control conditions.

Even in a highly venomous elapid snake, tongue-flicking rates appeared to be elevated in the first minute following release of envenomated prey (O'Connell et al., 1985). This immediate rise in tongue-flicking after biting in all taxa observed, except Viperidae, differs from the suspension of tongue-flicking and motionlessness by viperids for a minute or two after releasing prey (Chiszar and Scudder, 1980). Although data for additional taxa are needed to be conclusive, all existing data are consistent with the hypothesis that the delayed onset of tongue-flicking is a synapomorphy of some viperid snakes. That it may not characterize all viperids that exhibit SICS is suggested by the appearance of

elevated tongue-flicking in the first minute after release by *Lachesis muta* (Chiszar et al., 1989).

Despite the limited phylogenetic base, the data provide tentative support for the initial interpretation of the pause by Chiszar and Scudder (1980) as an adaptation to delay further contact with dangerous prey subsequent to envenomation until the prey has been rendered harmless by the venom. The delay is made possible by voluntary release and probably evolved in conjunction with it. Thus, the pause between envenomation and tongue-flicking is a functionally important characteristic of the highly specialized SICS of viperids.

Component 3, an increased tongue-flicking rate, is widespread among actively foraging lizards and nonvenomous snakes as well as in venomous snakes. Tongue-flicking serves to sample the environment for prey chemicals even before any prey has been detected in all families of actively foraging lizards. All of these families are able to distinguish prey chemicals from control chemicals (Cooper, 1994a,c). Lingually mediated prey chemical discrimination is an important means of detecting and locating prey. In the context of PETF and SICS, as well as after swallowing prey (Chiszar et al., 1980; Cooper et al., 1989), the increased tongue-flicking represents more intense chemosensory investigation, enhancing the probability of relocating the prey or other prey items of the same type.

Component 4, locomotion, is necessary to search a region for chemical cues and to follow any scent trails encountered. Tongue-flicking occurs at elevated rates during locomotion in lizards whether or not prey has been attacked recently (e.g., Burghardt et al., 1986). Movement alone may help locate prey by visual means, but movement must be combined with tongue-flicking to permit the lingual sampling of chemical cues needed for initial detection of prey and to detect and follow scent trails of prey already contacted.

Component 5, scent-trailing, may also occur in the context of the strike-release-trail strategy or prior to any direct contact with the prey. It has been observed in various venomous and nonvenomous snakes belonging to several major taxa (reviewed by Halpern, 1992). There is reason to believe that the chemosensory capacity for scent-trailing prey is widespread among autarchoglossan lizards. Prey chemical discrimination has been demonstrated in all autarchoglossan families studied except Cordylidae (references above plus Gerrhosauridae, Cooper, 1992c). Males of the scincid *Eumeces laticeps* can follow female pheromone trails by tongue-flicking (Cooper and Vitt, 1986). Because skinks possess tongues less specialized for chemical sampling (McDowell, 1972) and vomeronasal organs having a lower percentage of chemoreceptor cells in the sensory epithelium (Gabe and Saint Girons, 1976) than do members of several other autarchoglossan families, the chemosensory capacity for scent-trailing prey may exist in various families showing lingual specialization such

as forking and elongation and having high percentages of vomeronasal chemoreceptor cells.

The possible existence of such trailing ability has not been demonstrated in any lizard, but should be investigated due to its importance for both initial location of prey and relocation of lost prey. Whether scent-trailing, as opposed to local search in the vicinity of prey chemical cues, is profitable depends on several factors, including the density and mobility of prey. Scent-trailing should be favored when the prey is regionally scarce but locally dense, when there is a large energetic payoff per prey item, and when presence of the trail reliably indicates the continued presence of prey in the area. Due to their diets and the long durations of PETF, the lizards most likely to scent-trail prey are helodermatids and varanids. *Heloderma suspectum*, presumably using chemical cues, can detect eggs placed under sand (Bogert and Del Campo, 1956). There is anecdotal field evidence that *Varanus komodoensis* follows prey trails in the field (Auffenberg, 1981) and laboratory evidence that *V. bengalensis* uses chemical cues to detect hidden food (Auffenberg, 1984). Opportunities for studies of scent-trailing prey and the influence of foraging variables in lizards abound.

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STEREOCHEMICAL INVERSION OF PYRROLIZIDINE ALKALOIDS BY *Mechanitis polymnia* (LEPIDOPTERA: NYMPHALIDAE: ITHOMIINAE): SPECIFICITY AND EVOLUTIONARY SIGNIFICANCE

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Abstract—Pyrrolizidine alkaloids (PAs), acquired by adults or larvae of Danainae and Ithomiinae butterflies and Arctiidae moths from plants, protect these lepidopterans against predators and are biosynthetic precursors of male sex pheromones. The investigation of PAs in many species of wild-caught adults of Ithomiinae showed lycopsamine (**1**) [(7*R*)-OH, (2'*S*)-OH, (3'*S*)-OH] as the main alkaloid. In incorporation experiments, PA-free (freshly emerged) adults of the ithomiine *Mechanitis polymnia* were fed seven PAs: lycopsamine and four of its known natural stereoisomers—indicine (**2**) [(7*R*)-OH, (2'*R*)-OH, (3'*S*)-OH], intermedine (**3**) [(7*R*)-OH, (2'*S*)-OH, (3'*R*)-OH], rinderine (**4**) [(7*S*)-OH, (2'*S*)-OH, (3'*R*)-OH], and echinatine (**5**) [(7*S*)-OH, (2'*S*)-OH, (3'*S*)-OH], and two PAs without the 7-OH: supinine (**6**) [(2'*S*)-OH, (3'*R*)-OH] and amabiline (**7**) [(2'*S*)-OH, (3'*S*)-OH]. Males epimerized PAs **3**, **4**, and **5** mainly to lycopsamine (**1**). Females fed these same three PAs changed a smaller proportion to lycopsamine; their lesser capacity to modify PAs corresponds to their normal acquisition of already transformed PAs from males during mating rather than through visits of adults to plant sources of PAs. The alkaloids **1** and **2**, both 7*R* and 3'*S*, were incorporated without or with minimum change by males and females. Feeding experiments with **6** and **7** (males only) showed an inversion at the 3' center of **6** and no change in **7**. The inversion from 7*S* to 7*R* (probably via oxyreduction) may

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be closely related to the evolution of acquisition of PAs by butterflies and moths. Two hypotheses are discussed: (1) The ancestral butterflies are probably adapted to tolerate, assimilate, and use (7*R*)-PAs (most common in plants; all widespread 1,2-unsaturated macrocyclic PA diesters show this configuration). The development of (7*R*)-PA receptors in the butterflies could lead to a specialization on this configuration in two ways: to help find PA plants and to utilize these components in sexual chemical communication. A later appearance of (7*S*)-PAs in plants could have selected an enzymatic system for the inversion of this chiral center in order to continue producing (7*R*)-PA-derived pheromones. (2) The inversion would be due to the evolution of a enzyme system specialized in the transport of (7*R*)-PAs to the integument; the failure of this system to carry (7*S*)-PAs led to an enzymatic system to invert them to transportable (7*R*)-PAs. In this case, the 7*R* configuration is an effect and not a cause of (7*R*)-PA-derived pheromones. In both hypotheses, the partial inversion of the 3′-asymmetric center, when the butterfly was fed intermedine **(3)**, rinderine **(4)**, and supinine **(6)**, could be fortuitous due to the conformation of the molecule and/or the enzymatic system.

Key Words—Pyrrolizidine alkaloids, Lepidoptera, Nymphalidae, Ithomiinae, *Mechanitis polymnia*, stereochemical inversion, epimerization, evolution of PA acquisition.

INTRODUCTION

Pyrrolizidine alkaloids (PAs) (Figure 1) are well-known compounds with great importance in the chemical mediation of ecological interactions (Boppré, 1990). These substances are found in the N-oxide form in Danainae and Ithomiinae butterflies and Arctiidae moths, which obtain them from their larval host plants or sources visited by adults. These alkaloids protect the insects against predation and are utilized as precursors for pheromone biosynthesis (see Boppré, 1986, 1990, and references therein for review). Some Chrysomelidae beetles also sequester PAs from their food plants (Pasteels et al., 1990; Rowell-Rahier et al., 1991).

Two hypothesis have been published for the evolution of PA acquisition in butterflies. Edgar (Edgar, 1975, 1984; Edgar et al., 1974) suggested that ancestral larval host plants of the dananine–ithomiine lineage had both PAs and cardenolides, and the evolutionary diversification of these plants with loss of one or other, or both, led to the branching of the butterflies into their present state. Boppré (1978) argued that adult butterflies that overcame the PA barrier to take up and store these compounds had a selective advantage due to chemical protection; the utilization of PA derivatives in sexual chemical communication enhanced this selective advantage.

Study of the sequestration of PAs by moths show the inversion of (7*S*)-OH to (7*R*)-OH (Bell et al., 1984; Bell and Meinwald, 1986; Wink et al., 1988). Biller et al. (1994) showed that the grasshopper, *Zonocerus variegatus*,

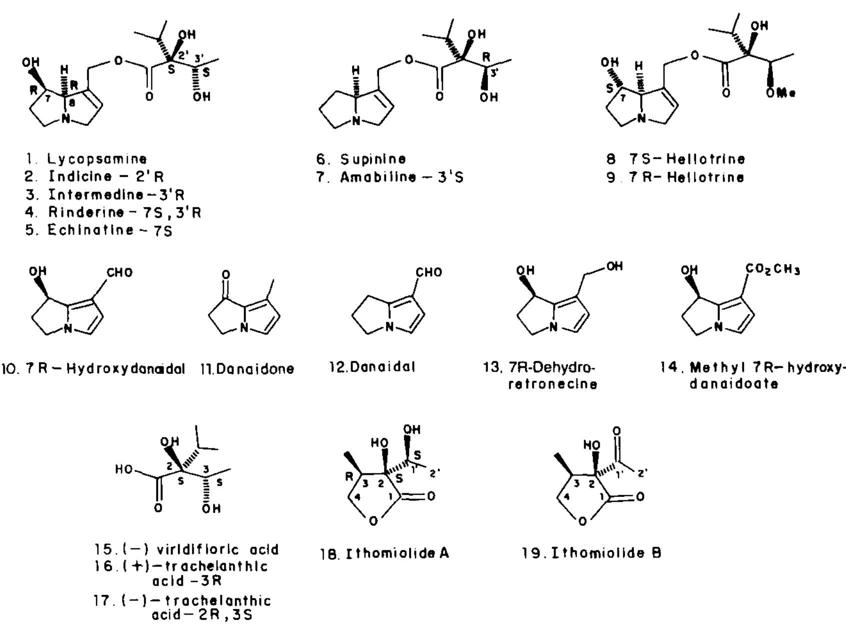

FIG. 1. Pyrrolizidine alkaloids and their derivatives.

sequesters PAs and modifies their configuration at C-3′ [intermedine (**3**) and rinderine (**4**) were changed to lycopsamine (**1**) and echinatine (**5**)], and C-7 [rinderine (**4**) to intermedine (**2**)]. Initial PA analyses of wild-caught adults (both sexes) of *Mechanitis polymnia* (Lepidoptera: Ithomiinae) by NMR and GC-MS showed that these butterflies presented mainly two PAs with the (7*R*)-OH configuration: lycopsamine (**1**) (range 60–100%) and intermedine (**3**) (0–40%) (Brown, 1984, 1985; Trigo, 1993). No traces of rinderine (**4**) or echinatine (**5**) [both (7*S*)-OH] were found, although the adults frequently visit *Eupatorium laevigatum* and *E. maximiliani* inflorescences, which have mainly these two PAs (Brown, 1985; Trigo, 1993). Both sexes of many other wild-caught Ithomiinae (42 species analyzed in 40 genera) and Danainae (four species analyzed in three genera) also showed mainly lycopsamine (**1**) or a mixture of this with intermedine (**3**); sometimes rinderine (**4**) or echinatine (**5**) and other nonlycopsamine type PAs were detected in variable amounts (Kelley et al., 1987; Trigo, 1993; Trigo et al., unpublished results; Freitas et al., unpublished results; Orr et al., unpublished results). References for other PA-specialist butterflies and moths show these alkaloids with only the (7*R*)-OH configuration (Edgar, 1975; Edgar et al., 1979; L'Empereur et al., 1989; Stelljes and Seiber, 1990; Nishida et al., 1991; Trigo et al., 1993).

Incorporation of PA monoesters (**1–7**) by adults of both sexes of *Mechanitis polymnia* was studied to verify stereochemical inversion in the 7 or 3′ hydroxyl-bearing chiral centers. The relation of this to the evolutionary acquisition of PAs by lepidopterans is also discussed.

METHODS AND MATERIALS

Incorporation Experiments. Eggs and larvae of *Mechanitis polymnia casabranca* Haensch were collected in the Campinas region, São Paulo State, Brazil, and reared in the laboratory on one of their larval host plants (*Solanum paniculatum* L., Solanaceae) to pupation. After emergence from the pupae, butterflies were kept in cages (45 × 30 × 25 cm) (five per cage). In captivity, the butterflies survived a maximum of three days after emergence. Males and females of freshly emerged adults (eight individuals of each sex) were starved one day and then individually fed a PA diet [50–200 μg of PA monoester free bases (see below) diluted in 3 μl of a saturated sugar solution]; the utilization of free bases represented no problem, since ithomiines visit decaying *Heliotropium* and *Eupatorium* plants that have these free alkaloids, and experiments of free-base incorporation by *Mechanitis* butterflies showed that these are rapidly N-oxidized (Trigo, 1993; Hartmann and Trigo, unpublished results). The following 100% pure PAs were isolated and purified from various Asteraceae and Boraginaceae plant sources, characterized by physical methods (NMR, EM) (Trigo, 1993), and utilized in the PA diet: lycopsamine (**1**) [(7*R*)-OH, (2′*S*)-OH, (3′*S*)-OH] from *Trichogonia gardneri*, indicine (**2**) [(7*R*)-OH, (2′*R*)-OH, (3′*S*)-OH] from *Heliotropium indicum*, intermedine (**3**) [(7*R*)-OH, (2′*S*)-OH, (3′*R*)-OH] from *Ophryosporus* sp., rinderine (**4**) [(7*S*)-OH, (2′*S*)-OH, (3′*R*)-OH] and echinatine (**5**) [(7*S*)-OH, (2′*S*)-OH, (3′*S*)-OH], and two PAs without the 7-OH: supinine (**6**) [(2′*S*)-OH, (3′*R*)-OH] and amabiline (**7**) [(2′*S*)-OH, (3′*S*)-OH] from *Eupatorium laevigatum*. Even after one day of starvation (second day in captivity), the butterflies did not always consume all 50 μg of PA diet, but they consumed all the pure sugar solution (without PAs). On the third day of captivity, the butterflies generally also refused the pure sugar solution. Attempts to feed butterflies on 200 μg of PA diet led to nonconsumption or refusal after partial ingestion. The incorporation of a mixture of labeled PA monoesters from *Eupatorium cannabinum* L. by adults of *Mechanitis polymnia* is about 20% of the ingested alkaloids (Trigo, 1993, Hartmann and Trigo, unpublished results). Assuming that the butterflies ingest all 50 μg of the PA diet, it would be difficult to analyze the isomer ratio in individual butterflies, since they would have only about 10 μg of PAs in their tissues, part of which would be lost by manipulation during PA purification. Other non-PA compounds present in the alkaloidal extract (from the insects or impurities of the solvents) also mask low amounts the PAs

in GC-MS analysis. Thus, 24 hr after the PA ingestion, the eight butterflies of each sex and diet were pooled, placed in MeOH, and stored at $-5°C$ for further PA purification.

Purification of PAs from Butterflies. The pooled butterflies were homogenized three times in 30 ml MeOH, filtered under vacuum, and the combined methanolic extracts were evaporated under low pressure and taken up in 6 ml 1 N $H_2SO_4:CH_2Cl_2$ (1 : 1). The acid layer was extracted two more times with CH_2Cl_2 (v/v), and the combined organic layers were set aside. The acid layer was reduced with excess Zn dust for 5 hr (the pH of the reaction was kept around 1–2), then filtered (gravity funnel), alkalinized with an excess of NH_3 (25%), and extracted three times with CH_2Cl_2 : MeOH (4 : 1). The combined organic layers were dried over Na_2SO_4 and evaporated under low pressure giving a total alkaloidal fraction (free bases + former N-oxides) that was directly analyzed by GC-MS.

GC-MS Analysis. The samples were analyzed on a Hewlett-Packard 5988 series II gas-chromatograph with a capillary column (WCOT, 15 m × 0.25 mm, DB-1, J&W Scientific) directly coupled to a selective mass detector (Hewlett Packard 5970). Conditions: injection temperature 250°C; temperature program 150–300°C, 4°C/min; split ratio 1 : 100, carrier gas He 0.7 bar, 1 ml/min; sample volume 1–2 μl, ionization energy 70 eV. The PAs utilized in the incorporation experiments were coinjected with each sample in a further analysis. The percentages of different diastereoisomers were calculated based on characteristic ions (*m/z* 138 for lycopsamine-like and *m/z* 122 for supinine-like PAs—see fragmentation patterns in Witte et al., 1993).

RESULTS

Feeding Experiments (Figure 2)

Lycopsamine **(1)** [*(7*R*)-OH, (2'*S*)-OH, (3'*S*)-OH*]. Males fed pure lycopsamine **(1)** incorporated most (86%) unchanged. Inversion of the 3'-OH occurred in small amounts (14%) giving intermedine **(3)**. Echinatine **(5)** was detected in trace amounts (< 1%), and there was no trace of rinderine **(4)**. Females fed pure lycopsamine presented the same pattern as the males, with less inversion to intermedine.

Indicine **(2)** [*(7*R*)-OH, (2'*R*)-OH, (3'*S*)-OH*]. Males and females fed pure indicine incorporated this alkaloid unchanged.

Intermedine **(3)** [*(7*R*)-OH, (2'*S*)-OH, (3'*R*)-OH*]. Males fed pure intermedine showed 62% unchanged and 38% hydroxyl inversion at C-3', giving lycopsamine **(1)** [(7*R*)-OH, (3'*S*)-OH]. Females fed pure **3** showed 91% unchanged and 9% of hydroxyl inversion at C-3' giving lycopsamine. Rinderine **(4)** and echinatine **(5)** were not detected in either sex.

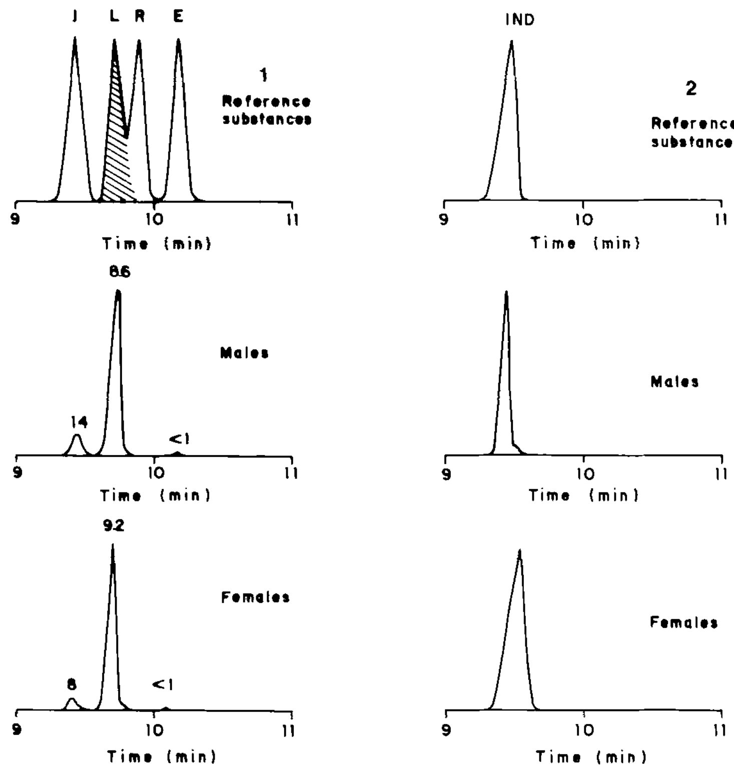

FIG. 2. GC-MS of PAs in males and females of *Mechanitis polymnia* in feeding experiments (dashed) with: **(1)** lycopsamine (L), **(2)** indicine (IND), **(3)** intermedine (I), **(4)** rinderine (R), **(5)** echinatine (E) (only selected peaks with abundant fragments at *m/z* 138 are shown), **(6)** supinine (S), and **(7)** amabiline (A) (only selected peaks with abundant fragments at *m/z* 122 are shown). Females were not analyzed for supinine and amabiline uptake.

Rinderine ***(4)*** [*(7S)-OH, (2'S)-OH, (3'*R*)-OH*]. Males fed pure rinderine **(4)** inverted two asymmetric centers giving lycopsamine **(1)** (58%). Inversion of the 7-OH only (26%) gave intermedine **(3)**, and echinatine **(5)** (16%) was produced by inversion of the 3'-OH. No rinderine was recovered. Females fed pure rinderine inverted both asymmetric centers to give lycopsamine (17%); inversion of the 7-OH gave intermedine (11%) and of the 3'-OH, echinatine (15%). Most of the rinderine (57%) remained unchanged.

Echinatine ***(5)*** [*(7S)-OH, (2'S)-OH, (3'S)-OH*]. Males fed pure echinatine **(5)** inverted mainly the asymmetric center at C-7, giving lycopsamine **(1)** (80%). Two inversions gave intermedine **(3)** (10%). Ten percent of unchanged echi-

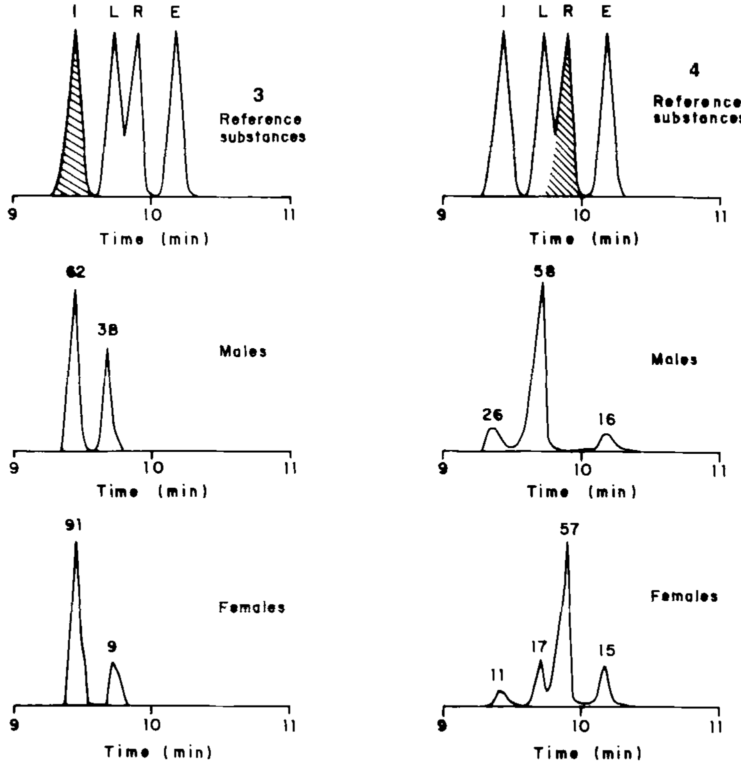

FIG. 2. Continued.

natine was found. Females fed pure echinatine showed a PA pattern similar to that in the rinderine feeding experiments: inversion of the 7-OH gave lycopsamine (48%); of the 7-OH and 3′-OH, intermedine (4%); and of the 3′-OH, rinderine (1%). Most of the echinatine (47%) remained unchanged.

Supinine (6) [*(2*′S*)-OH, (3*′R*)-OH*]. Males fed pure supinine changed 78% to amabiline, its 3′*S* epimer.

Amabiline (7) [*(2*′S*)-OH, (3*′S*)-OH*]. Males fed pure amabiline incorporated it unchanged.

DISCUSSION

The results show that males inverted the ingested (7*S*)-PAs to the preferred (7*R*)-OH configuration. Although no statistical analyses have been made due to pooled analyses of PAs by sex and diet, the results show that females modified

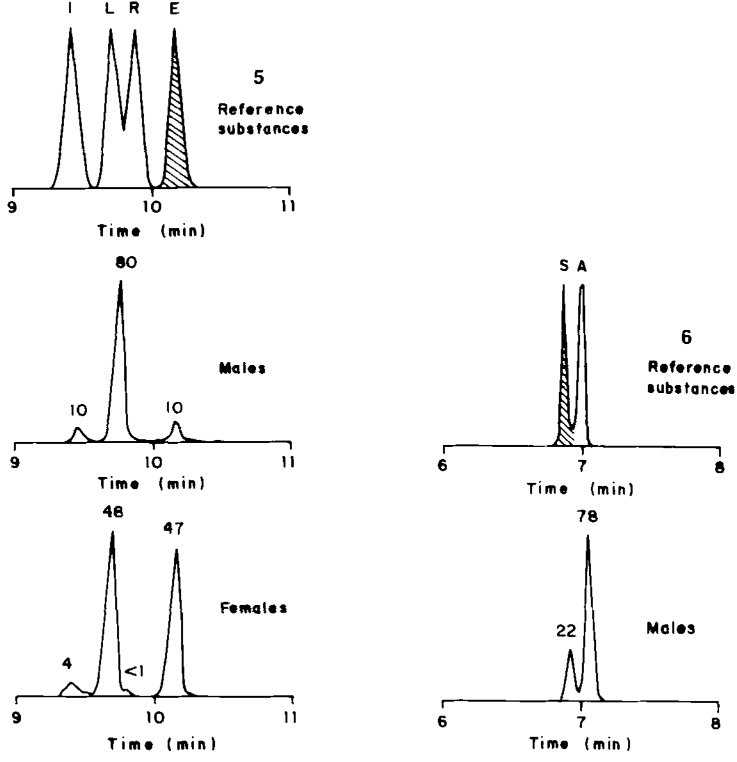

FIG. 2. Continued.

less of the ingested PAs to (7*R*)-OH than males. The overall inversion of the (7*S*)-OH (rinderine and echinatine) to (7*R*)-OH (lycopsamine and intermedine) was 84–90% (min–max) in males and 28–52% in females. In addition to inversion of the (7*S*)-OH to (7*R*)-OH, *M. polymnia* also inverted some of the (3′*R*)-OH to (3′*S*)-OH and vice versa. No inversion of the (2′)-OH of the esterifying acid was observed. Inversion at the 3′ asymmetric center in the necic acid has also been reported for the arctiid moth *Hyalurga syma* that fed on *Heliotropium transalpinum* leaves (Trigo et al., 1993).

The inversion at C-7 to the *R* configuration was not complete in the feeding experiments; the presence of (7*S*)-OH diastereoisomers has been observed in low amounts in Ithomiinae, and variable amounts have been found in wild-caught Danainae butterflies (Kelley et al., 1987; Trigo et al., unpublished results). This may be related to the kinetics of the incorporation/inversion of (7*S*)-OH PAs. The time (24 hr) between the PA ingestion and the chemical

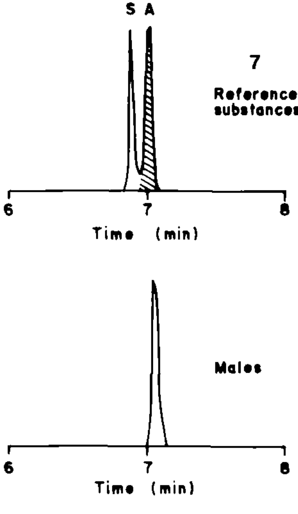

FIG. 2. Continued.

extraction of the butterflies for analysis may not have been sufficient for total inversion at C-7.

Taking into account the data obtained in our feeding experiments, the utilization of PAs in pheromone biosynthesis (Edgar, 1975, 1982; Edgar et al., 1971, 1973, 1976; Schulz, 1987, 1992; Schulz et al., 1988), the role of these alkaloids in the chemical protection of the butterflies and moths (Brown, 1984, 1985, 1987; Masters, 1990, 1992; Eisner and Eisner, 1991; Zikan-Cardoso, 1991), and the two hypotheses for the evolution of PA acquisition by lepidopterans proposed by Edgar (Edgar, 1975, 1984; Edgar et al., 1974) and Boppré (1978), we suggest two different scenarios that could explain the stereochemical inversion and also clarify some points about the evolution of PA acquisition by specialist lepidopterans.

The first scenario presumes that chemical communication plays a strong role in the stereochemical inversion and has the following steps:

1. Larvae of ancestral danaine/ithomiine butterflies acquired an ability to feed on PA-containing plants, overcoming the PA chemical barrier. This ability could be positively selected by freedom from competition on the PA plant food. Larvae of danaine and ithomiine groups that no longer feed on any PA plants have been shown to sequester PAs added to their diet (Rothschild and Edgar, 1978; Trigo and Motta, 1990). This supports ancestral utilization of PA host-plants by these groups, as in Edgar's hypothesis (Edgar, 1975, 1984; Edgar et al., 1974). Primitive ithomiines (*Tellervo*, *Tithorea*) still feed as larvae on PA

plants (Apocynaceae: Echitoideae), and store their alkaloids (Trigo and Brown, 1990; Orr et al., unpublished results; Trigo et al., in preparation).

2. These ancestral PA plants could have produced only 7*R* macrocyclic PAs, or 7*R*-PAs could be a ancestral feature in PA plants. In agreement with the first, Bremer et al. (1992) considered the tribe Senecioneae (Asteraceae) more primitive than Eupatorieae, based on morphological and chloroplast DNA data. Most Senecioneae contain 7*R* macrocyclic diester PAs, while the more derived Eupatorieae produce mainly 7*R* and 7*S* open monoesters (Culvenor, 1978; Mattocks, 1986; Rizk, 1991; Trigo, 1993, Hartmann and Witte, in preparation; Trigo et al., unpublished results). It is probable that the 1,2-unsaturated macrocyclic PAs (the most common PAs in plants; Hartmann and Witte, in preparation), also known in Leguminosae and Echitoideae, were initially encountered by ancestral butterflies in their potential food plants. The 114 known 1,2-unsaturated macrocyclic PAs have exclusively the 7*R* configuration (Hartmann and Witte, in preparation).

3. Lepidopterans that incorporated PAs (always of 7*R* configuration), as larvae and probably also as adults, became chemically protected against predators, with an increase in fitness (see also Boppré's hypothesis). PAs probably are active against a wide spectrum of predators (spiders—Eisner, 1982; Brown, 1984, 1985; Masters, 1990; Eisner and Eisner, 1991; Trigo et al., 1993; Orr et al., unpublished results; mantids—Trigo, personal observation; lizards—Masters, 1992; birds—Zikan-Cardoso, 1991; Masters, 1992; monkeys—Coimbra-Filho, 1981).

4. The utilization of (7*R*)-PA plants by the ancestral lepidopterans would develop in both sexes specific 7*R*-PA receptors for the recognition of these plants. Volatile dihydropyrrolizines (**10–14**) might be responsible for the attraction of both sexes to the ancestral larval food plants (see also Edgar's hypothesis) in the same way that the 7*R* (**10**) or nonchiral (**11**) dihydropyrrolizines today attract mainly males and also females of Danainae and Arctiidae (Schneider et al., 1975; Wunderer et al., 1986; Bogner and Boppré, 1989; reports for Ithomiinae are unknown). Krasnoff and Dussourd (1989) showed that (7*S*)-(+)-hydroxydanaidal attracts both sexes of three species of arctiid moths significantly more than its 7*R* enantiomer (**10**), however.

5. The chemical protection and the development of specific receptors to PAs introduced some new components into this scenario. Males with large amounts of PAs to give to females during mating produced more progeny due to protection of the female and the eggs, and thus would be preferred by females that could recognize this quantity during courtship. Thus, adult males would also seek PA sources, utilizing the same PA receptors. Since the PAs themselves (macrocyclics, diesters, and monoesters) are not volatile, males that developed a chemical communication system, similar to the plants, that signaled to females a large amount of PAs in their tissues would be favorably selected. Again,

volatile dihydropyrrolizines would be the most reliable chemical signals, since they are necessarily derived from PAs. In *Utetheisa ornatrix* (Arctiidae), Conner et al. (1981) suggested that dihydropyrrolizines were utilized by females as a measure of the capacity of PA incorporation by males; these PAs, on the other hand, also represent a measure of the potential capacity of chemical defense (Conner et al., 1981) or the potential amount of PAs transferrable from males to females in mating, where females could be using these PAs in their own defense or in the defense of their offspring by chemical protection of eggs (Dussourd et al., 1988). Thus, a high amount of dihydropyrrolizines might be produced, since these compounds would have the function to give a measure of the capacity of PA incorporation. In evolutionary time, adult females would come to obtain these alkaloids, already in the 7*R* configuration, from males during mating. This acquisition would also leave more time available for them to search for larval host plants. Literature data show that females of most ithomiines rarely visit PA sources (Pliske, 1975a,b; Trigo, 1993; Trigo et al., in preparation) and obtain these alkaloids from males via mating (Brown, 1985).

6. Due to diversification and competition, butterflies colonized other host plants without PAs; in the Danainae case, Asclepiadaceae, Moraceae, and others to a lesser degree, and in the Ithomiinae, Solanaceae (see also Edgar's hypothesis). These two butterfly subfamilies maintained their dependence on PAs for chemical defense and chemical communication, and thus were forced to seek PAs as adults in other plant sources. A parenthesis must be opened at this point. Analyses of PAs in wild-caught Danainae show that, contrary to ithomiines, some populations of wild-caught *Lycorea*, *Ituna*, and *Danaus* species present a high content of (7*S*)-1,2-unsaturated monoester PAs (Kelley et al., 1987; Trigo et al., unpublished results). Thus, along evolutionary time, the danaine branch could have lost the capacity for efficient (7*S*)-PA inversion, but this needs more study.

7. The rise of (7*S*)-PA plants increased the complexity of this system. Males that were able to recognize these plants would increase their fitness, since they would have a competition-free space to sequester PAs. However, 7*S*-PA derivatives might be poorly recognized by females as a PA measurement, making males with these derivatives less able to attract females.

8. Males that were able to invert (7*S*)-PAs to 7*R*, in order to produce the attractive (7*R*)-dihydropyrrolizines would have a selective advantage in mating. Schulz (personal communication) verified the presence of only (7*R*)-PA derivatives in scent organs of several Danainae and Ithomiinae species. Why the lepidopterans would invert the configuration of the PAs and not only those of the derivatives is a unanswered question of biosynthetic cost–benefit ratios. Schulz et al. (1993) showed that the biosynthetic path of (7*R*)-hydroxydanaidal production by male *Creatonotos transiens*, when fed (7*S*)-heliotrine, starts with an epimerization at C-7 by an oxidoreduction process, followed by a dehy-

drogenation step to produce an unstable pyrrole alkaloid, the subsequent hydrolysis of the ester function; the final oxidation produces hydroxydanaidal. Other PA derivatives, the butyrolactones **18** and **19,** were found in costal fringes of some ithomiines, always with absolute configuration related to (−)-viridifloric acid **(15),** the necic acid of lycopsamine (Schulz, 1992).

In a second scenario, the physiological features of probable enzymes responsible for the accumulation of PAs in the integument and PA stereochemistry and conformation might play a major role in the stereochemical inversion of hydroxyl-bearing chiral centers.

1. Just as in the first hypothesis, larvae of the ancestral danaine-ithomiine group were adapted to (7*R*)-PA host plants. Due its particular conformation, the (7*R*)-PA feature could have selected, in the early PA lepidopterans, the production of an enzyme system responsible for transport to the integument, which would approach only on the less hindered side of the pyrrolizidine ring (Figure 3a). In specialist insects (e.g. *creatonotos*), PAs are taken up passively as free bases from the midgut to the hemolymph, where they are N-oxidated and accumulate both there and in the integument (Hartman, personal communication).

2. Again, as in the first hypothesis, chemical protection and chemical communication due to PAs enhanced the fitness of males that had high PA content.

3. Along evolutionary time, the insects could encounter other larval host plants with both 7*R* and 7*S* configuration; the probable conformation of some (7*R*)-PA open monoesters would allow an enzyme system to approach only on the less hindered face of the pyrrolizidine ring (the convex side) (Figures 3b–d). MacKay et al. (1983) found a "pseudomacrocyclic conformation" in crystals of lycopsamine (Figure 3b) and intermedine (Figure 3c), and Giordan et al. (unpublished results) found, by molecular mechanics methods, a probable H bond between the hydrogen of a (7*R*)-OH and the esterified oxygen group (Figure 3d). The 7*S* monoesters would be too hindered to be handled by the metabolic enzymes specific to (7*R*)-PAs, since approach would be impeded by any of several possible blocking groups: the 7-OH in pseudoequatorial position (Figure 3e), an H bond between the 7-OH proton and the N (Figure 3f), or a "pseudomacrocyclic conformation" like the 7*R* monoester PAs involving an H bond between a hydroxyl of esterifying acid and the (7*R*)-OH (Figure 3g). Wodak (1975) showed that (7*S*)-heliotrine **(8)** has a "pseudomacrocyclic conformation" with an H bond between the 2′-OH of the esterifying-acid and the 7-OH of the ring (Figure 3g). Giordan et al. (submitted) show, by molecular mechanics and ab initio calculations, that an H bond between the proton of the secondary hydroxyl with N can occur in the (7*S*)-necine base heliotridine *endo*-conformer (Figure 3f).

4. Butterflies that developed an enzymatic process to invert the asymmetric center 7*R* to 7*S* obtained an adaptive advantage, since they could exploit sources with both 7*R* and 7*S* configuration. The inversion of (7*S*)-OH to (7*R*)-OH was

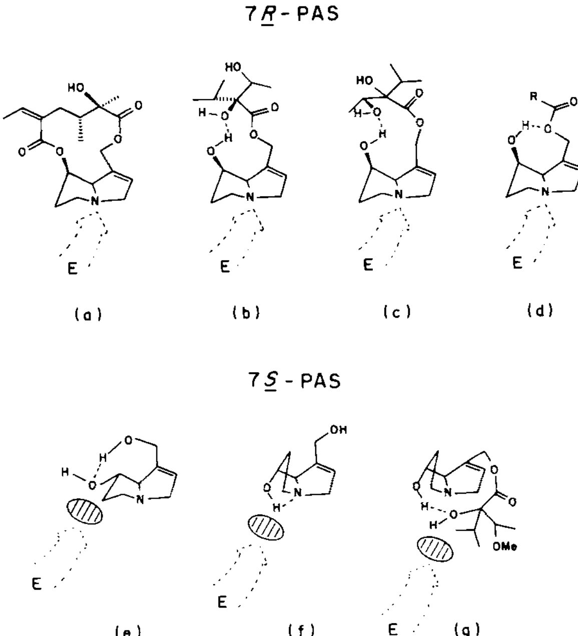

FIG. 3. Conformation of some pyrrolizidine alkaloids: (a) senecionine (modified from Mackay and Culvenor, 1982), (b) lycopsamine and (c) intermedine (modified from Mackay et al., 1983), (d) retronecine esters (modified from Giordan et al., in preparation), (e) *exo*-heliotridine, (f) *endo*-heliotridine (modified from Giordan et al., submitted), (g) heliotrine (modified from Wodak, 1975), and hypothetical enzyme system (E) approach in the less hindered side of the pyrrolizidine ring for PA transport to accumulation sites.

first reported for the arctiid moth *Creatonotos transiens* artificially fed (7*S*)-heliotrine **(8)** (Bell et al., 1984; Bell and Meinwald, 1986; Wink et al., 1988). The latter authors showed that males inverted (7*S*)-heliotrine to (7*R*)-heliotrine **(9)** more than females. They related the inversion at 7-OH with pheromone production by males, since the hydroxydanaidal found in the coremata of these arctiids shows the (7*R*)-OH configuration. In this evolutionary scenario, we propose also the inversion at 7-OH with male pheromone production and suggest that the (7*R*)-OH dihydropyrrolizine production is an effect and not a cause of the inversion at C-7 in the former PAs.

5. As in the first hypotheses, females would not need a high capacity for PA inversion at C-7, since they can obtain these alkaloids, already in 7*R* configuration, from males during mating.

Both scenarios can explain the stereochemical inversion at C-7, but they do not clarify the inversion at C-3′ when *Mechanitis polymnia* is fed intermediate **(3)**, rinderine **(4)**, and supinine **(6)**, and its maintenance or small inversion when fed lycopsamine **(1)**, indicine **(2)**, echinatine **(5)**, and amabiline **(7)**.

We propose that PA monoesters change their conformation by binding with the enzymatic system as shown in Figure 4. If the oxidoreductive system acts preferentially on (7*S*)-OH (not at 7*R*), this particular PA conformation will also favor the oxyreduction of the (3′*R*)-OH (Figure 4). In the same way, intermedine and supinine in this hypothetical "enzyme system" will also change the 3′ asymmetric center to give lycopsamine and amabiline, respectively.

Both scenarios, presented above show lacunae, which must be filled in by future observations and experiments. Studies on Ithomiinae and other PA insects

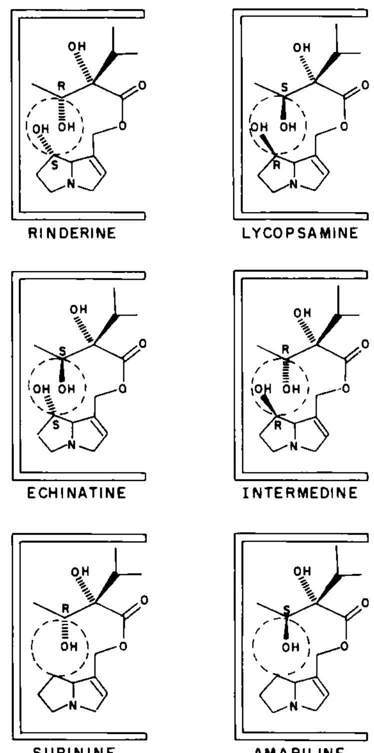

FIG. 4. Probable change of conformation of rinderine, lycopsamine, intermedine, echinatine, supinine, and amabiline in relation to a binding with a hypothetical oxyreductase enzyme (oxyreductive site at dashed line).

focused on (1) PA and PA-derivative composition in other PA-insects and their PA plant sources, (2) PA incorporation experiments with them, (3) the role of PA derivatives in the attraction of PA insects, (4) the mechanism of PA uptake and accumulation, (5) the phylogeny of these insects in relation to their chemistry, and (6) PA conformation in natural systems, will help to fill in some of these lacunae.

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INTERACTIONS BETWEEN *Alloxysta brevis* (HYMENOPTERA, CYNIPOIDEA, ALLOXYSTIDAE) AND HONEYDEW-COLLECTING ANTS: HOW AN APHID HYPERPARASITOID OVERCOMES ANT AGGRESSION BY CHEMICAL DEFENSE

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Abstract—Foraging females of the aphid hyperparasitoid *Alloxysta brevis* were attacked by honeydew-collecting workers of the ant *Lasius niger* at the first encounter. However, ants abandoned their attacks quickly, and foraging *A. brevis* remained unmolested for a subsequent time interval of approximately 5 min, which is long enough for the hyperparasitoid to oviposit successfully. Furthermore, freshly killed intact *A. brevis* were disregarded by ants, while decapitated specimens were readily removed. We present evidence that *A. brevis* females release a mandibular gland secretion, which contains 6-methyl-5-hepten-2-one, actinidin, and unidentified iridoids, in response to an ant attack. This secretion functions both as a measure of self-defense if the female is seized by an ant worker and as a repellent, which prevents ant attacks during subsequent encounters. This is the first evidence for chemical defense in a hymenopterous parasitoid. It enables *A. brevis* females to hyperparasitize ant-attended aphids that constitute a major proportion of their hosts and significantly reduces mortality by ectohyperparasitoids.

Key Words—Hymenoptera, Alloxystidae, hyperparasitoids, ants, Formicidae, interactions, aggression, chemical defense, 6-methyl-5-hepten-2-one, actinidin, biological significance.

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INTRODUCTION

Predators and parasitoids of aphids and coccids are often heavily attacked by honeydew-collecting ants and therefore have to concentrate their foraging efforts in unattended colonies (Way, 1954, 1963; Bartlett, 1961; Banks, 1962; Bradley, 1973; Völkl, 1992). However, a number of recent studies showed that ant protection is incomplete for their mutualistic partners. A number of predators and parasitoids gain access to ant-protected resources by various morphological and behavioral adaptations and/or by chemical camouflage (Stary, 1966; Eisner et al., 1978; Takada and Hashimoto, 1985; Völkl and Mackauer, 1993; Milbrath et al., 1993; Dettner and Liepert, 1994). Primary parasitoid species that overcome ant defense benefit from ant attendance by a significantly reduced hyperparasitism, since ant aggression often prevents successful oviposition by the hyperparasitoid females (Flanders, 1951; Kamijo and Takada, 1983; Völkl, 1992; Cudjoe et al., 1993).

The alloxystid wasp *Alloxysta brevis* (Thomson) is a common hyperparasitoid of the aphidiid wasp *Lysiphlebus cardui* (Marshall), a primary parasitoid of the black bean aphid *Aphis fabae* Scop. (Evenhuis, 1978; Völkl, 1990). *Alloxysta* species are endohyperparasitoids and parasitize the aphidiid larvae within the living aphid (Gutierrez and van den Bosch, 1970; Sullivan, 1987, 1988). Since *L. cardui* prefers to oviposit into ant-attended colonies (Stary, 1987; Völkl, 1992), foraging *A. brevis* females are often confronted with honeydew-collecting ants that attack the colony intruders (Banks 1962; Way, 1963; Völkl and Mackauer, 1993). In *A. fabae* colonies attended by the ant *Lasius niger* L., *L. cardui* is significantly less hyperparasitized by *A. brevis* than in unattended ones, but nevertheless the endohyperparasitoid was commonly found in *L. niger*-attended colonies and represented the most frequent hyperparasitoid species in that system (Völkl, 1992). Thus, we hypothesized that *A. brevis* has evolved strategies to overcome ant aggression.

In the present study, we analyzed the interactions between honeydew-collecting *L. niger* workers and foraging *A. brevis* females. We present evidence that this hyperparasitoid gains access to ant-attended aphid colonies by applying a defense secretion that probably originates from the mandibular glands. We will show that this secretion has no intraspecific effects on foraging females, as suggested by Micha et al. (1993) for the closely related species *Alloxysta victrix* (Westwood), but acts interspecifically. We discuss the selective advantage of the defense for immature and adult stages of *A. brevis*.

METHODS AND MATERIALS

Insect Culture. A laboratory stock of *A. fabae* was kept on potted creeping thistles *Cirsium arvense* L. (Scop.) in a plant-growth chamber at 21 $\pm$ 0.5°C, approx. 70% relative humidity, and 16L:8D. A laboratory culture of *A. brevis*

was established from a single mated female reared from an *L. cardui* pupa collected on thistles near Bayreuth, Bavaria, Germany. The culture was kept on *A. fabae* feeding on creeping thistle *C. arvense* via *L. cardui* as primary parasitoid. Emerged *A. brevis* were kept in a climate chamber at 5°C and regularly fed with a water–honey solution until they were used in the experiments. Each female was used only once.

To obtain ant-attended host colonies for laboratory experiments, potted goosefoot plants (*Chenopodium* spec.) with small mixed-aged *A. fabae* colonies (ca. 20–40 aphids) were kept together with a small colony (ca. 100 workers) of the ant *L. niger*, which was established in a terrarium (70 cm long × 35 cm deep × 35 cm high) within the growth chamber. The ants heavily tended the *A. fabae* colonies, which formed their only carbohydrate supply. Ant activity was assumed to resemble the situation in nature.

Interactions between Alloxysta brevis *Females and Foraging* Lasius niger. We analyzed the interactions between foraging *A. brevis* females and honeydew-collecting *L. niger* workers in a climate chamber at 20°C, 8000 lx, and 60% relative humidity. We used very old *A. brevis* (>30 days), which had no previous opportunity for oviposition. Since these females had a high oviposition pressure, we expected that they would leave the host plant less often in response to an ant attack than young females (<15 days old) did in the field (G. Hübner, unpublished data). All experiments were carried out on *L. niger*-attended *Chenopodium* plants (see above). Most leaves and flowers of the host plants were cut to create a simply structured environment where we expected a maximum number of encounters between *A. brevis* and *L. niger*.

In general, we released single *A. brevis* females ($N = 11$) onto the main stem of a host plant, at a distance of about 1–3 cm from the *A. fabae* colony. To avoid provoking attacks by *L. niger*, we took care that no ants were within about 5 cm of the point of release. We observed the following interactions, distinguishing between several behavioral patterns (see below). A trial was completed when the parasitoid left the plant and did not return within the next 5 min.

In encounters with foraging or ovipositing *A. brevis*, the ants were either aggressive (attacking—the ant tried to seize the wasp with its mandibles) or displayed one of three nonaggressive behavioral patterns (ignoring—the ant passed the wasp at a distance of <2 mm or walked over the wasp's body without making physical contact; touching—the ant made physical contact with the wasp but otherwise did not respond either aggressively or nonaggressively; antennal tapping—the ant made physical contact with the wasp and tapped it at least twice with the antennae).

A. brevis could respond to approaching or attacking ants with the following behaviors: drop off—the wasp dropped off the plant; avoidance—a wasp tried to avoid physical contact with an approaching ant but did not leave the plant;

cowering—the wasp showed no movement after being touched by ant, pressing its body and antennae close to the plant surface; ignoring—the wasp continued its activities without showing any obvious response to a contact with an ant.

The threshold time for a successful oviposition of *A. brevis* is approximately 3 min (Völkl, unpublished observations; Singh and Srivastava, 1987). Therefore, we defined all ovipositor insertions of *A. brevis* of more than 3 min as ovipositions.

Response of Lasius niger *to Dead* Alloxysta brevis. Females of *A. brevis* were killed by freezing them at −30°C and subsequently storing them at −30°C. A dead hyperparasitoid was carefully placed in close vicinity to an aphid colony into a leaf axil, which was frequently passed by *L. niger* workers. Subsequently, we observed the first 15 contacts between ants and the test individuals. We distinguished two broadly different patterns of ant behavior: disregarding—ants did not seize the dead *A. brevis* even if they tapped them frequently with the antennae; removal—the ant seized *A. brevis* with its mandibles and removed it from the plant.

We set up the following experimental designs: In design 1, we tested *A. brevis* females (N = 10) within 15 min after being thawed. In design 2, we decapitated the frozen females (N = 14) in a climate chamber at 5°C directly after having removed them from the freezer and tested subsequently only the thorax/abdomen of *A. brevis* within 15 min after the head was removed. In design 3, thawed *A. brevis* females (N = 15) were stored in open glass Petri dishes at 20°C and 40% relative humidity for two days before being tested. As a control (design 4), we tested the response of *L. niger* to freshly killed specimens of the aphidiid species *Trioxys angelicae* (Haliday) (N = 15), which is treated in a very aggressive way by this ant both in living and dead condition (Liepert and Dettner, 1993; Völkl and Mackauer, 1993).

Interactions between Foraging Alloxysta brevis *Females.* We set up three experiments to analyze interactions between foraging *A. brevis* females for an evaluation of the potential role of the mandibular secretion in intraspecific communication, as proposed by Micha et al. (1993). In the first set of experiments, we released two females (A and B) simultaneously onto a cut leaf stem of a potted *Chenopodium* spec. leading into an unattended aphid colony (25–30 individuals). This ensured that the searching females encountered each other when approaching the aphid colony. We observed the following interactions between females, distinguishing between three behavioral patterns: ignoring—wasps did not display any obvious behavioral change during an encounter and continued on their searching path; cowering—the wasp showed no movement after being touched by the conspecific female, pressing its body and antennae close to the plant surface; avoidance—a wasp changed its searching direction in response to an encounter with a conspecific. A trial was completed when both parasitoids had left the plant.

In the second set of experiments, we released female A as described above. After 60 sec, we seized female B with a pair of forceps and held it for about 5 sec in front of female A's head (distance <2 mm). This seizing seems to elicit the release of mandibular gland secretion since females are not attacked by ants after such a treatment (G. Hübner and W. Völkl, unpublished data). Thus, female A should have been confronted with a natural amount of mandibular gland secretion. Subsequently, we observed female A until it left the plant.

In the third set of experiments (=control), patch residence times of singly foraging *A. brevis* in unattended *A. fabae* colonies were observed.

All females were 15–30 days old and had no previous opportunity for oviposition.

Analytical Procedures. The chemical analyses were carried out as combined gas chromatography–mass spectrometry (GC-MS) studies. The system used consisted of a Carlo Erba GC 6000 Vega series 2 equipped with a split-splitless injector and coupled to a Finnigan MAT ion trap detector ITD 800. For all GC-MS analyses, a Chrompac CP SIL 19CB 0.32-mm-ID × 12.5-m fused silica capillary column coated with a 0.2-μm film was used. We applied samples by solid injection using complete heads or thorax/abdomen of *A. brevis* and a temperature of 230°C at injection. The GC was temperature programmed from 40°C (3 min isothermically) to 100°C (heating rate: 5°C/min) and from 100°C to 275°C (heating rate: 7°C/min). Samples were analyzed by the ITTS program (Finnigan MAT). Recorded substances were compared with known mass spectra using the National Bureau of Standards (NBS) library and a library of the Department of Animal Ecology II, University of Bayreuth.

Authentic 6-methyl-5-heptene-2-one (Fluka) and actinidin were used isolated from defensive-gland-containing abdominal tips of the rove beetle *Philonthus carbonarius* (Dettner, 1983).

RESULTS

Interactions between Alloxysta brevis *and* Lasius niger *Workers.* The pattern of interactions between *A. brevis* and *L. niger* showed a typical sequence (Figure 1). The foraging wasp was readily located and attacked by an ant worker. *A. brevis* escaped from this attack by dropping off the plant in 28% of occasions. In 72% of occasions, the ant seized the wasp with its mandibles (Figure 2A) for 0.5–4 sec but "spat it out" subsequently, normally at the place where the wasp was attacked. Only one female was carried away for a few millimeters. Neighboring ant workers did not display any obvious response to this interaction. *A. brevis* was never seriously wounded or killed. Females either continued their search directly after the attack or dropped off, returned, and continued their search elsewhere on the plant. After an attack, *A. brevis* was not treated aggres-

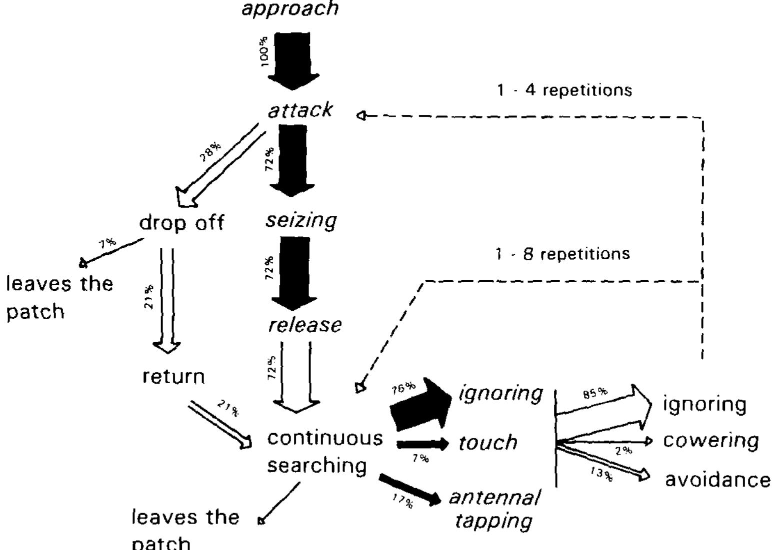

FIG. 1. Scheme of the typical interaction sequence between *Alloxysta brevis* and *Lasius niger*. Black arrows and italic letters indicate ant behavior, open arrows, and Roman letters indicate wasp behavior.

sively by *L. niger* workers (mean ± SE: 4.0 ± 0.6 nonaggressive contacts per time interval; range: 1–8 contacts) for some minutes, independent of whether they were encountered by the same ant that had attacked them previously or by another ant. These wasps ignored the ants in 85% of occasions (Figure 1) and were able to forage and oviposit unmolested (Figure 2B).

The last nonaggressive contact after an attack was observed, on average, after 248 ± 43 sec. After 310 ± 58 sec, an ant responded aggressively again when encountering an *A. brevis* female.

A. brevis remained for 717 ± 163 sec on the *Chenopodium* plants. During this time we observed on average 2.3 ± 1.0 sequences with an ant attack followed by an interval with exclusively nonaggressive interactions (range: 1–4). Ovipositions of *A. brevis* were observed during eight intervals (=32%).

Response of Lasius niger *to Dead* Alloxysta brevis. Foraging *L. niger* workers ignored most of the killed *A. brevis*, which were exposed within the first 15 min after being thawed (design 1; Table 1). Ant behavior changed significantly towards decapitated hyperparasitoids (design 2) or hyperparasitoids that were presented two days after being thawed (design 3). Here, ants displayed an aggressive behavior and removed the majority of the dead individuals at the

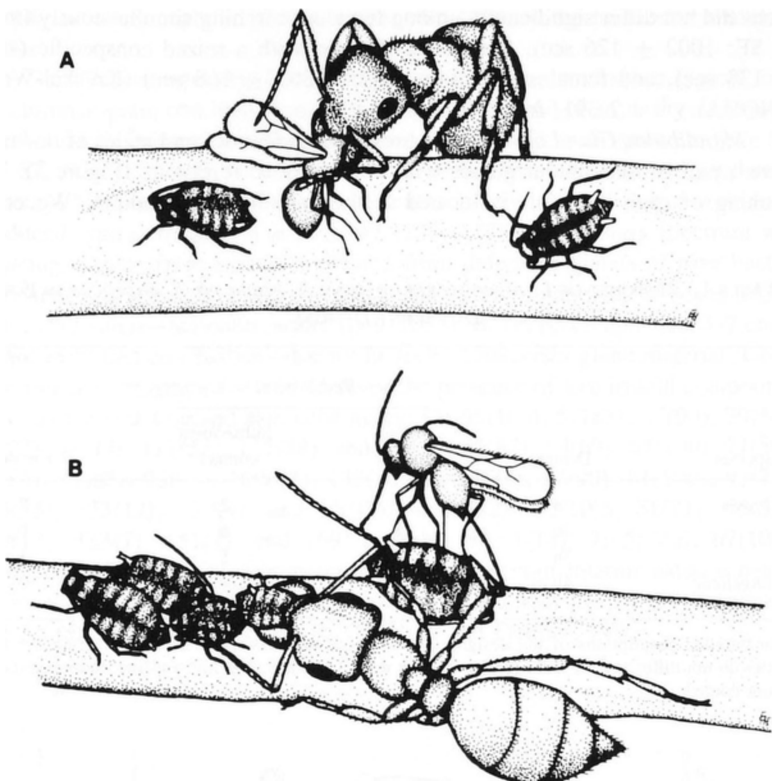

FIG. 2. (A) A worker of *Lasius niger* has seized an *Alloxysta brevis* female with its mandibles; (B) the ovipositing *Alloxysta brevis* female is ignored by the *Lasius niger* worker shortly after an earlier attack.

first contact (Table 1). *T. angelicae*, which was used as a control, was also quantitatively removed (design 4).

Interactions between Foraging Alloxysta brevis *Females.* Simultaneously searching females had in average 3.1 ± 1.2 contacts during a common stay on a host plant. Wasps mainly ignored conspecific females (44% of all contacts), but avoidance (32%) and cowering (16%) were also observed regularly. However, a female never left a plant directly in response to an encounter with a conspecific or within the following 60 sec. There was also no obvious response of *A. brevis* females to conspecifics that were held in front of their head with a pair of tweezers. The wasps either ignored them (N = 5) or changed their path to avoid physical contact (N = 8) but never left the plant. The average patch

times did not differ significantly among females searching simultaneously (mean ± SE: 1002 ± 126 sec), females confronted with a seized conspecific (1056 ± 138 sec), and females searching singly (1302 ± 168 sec) (Kruskal-Wallis ANOVA: $\chi^2 = 2.391$, $N = 39$, $P = 0.301$).

Mandibular Gland of Alloxysta brevis. Both females and males of *A. brevis* have a pair of mandibular glands with large bilobed reservoirs (Figure 3). The opening of each reservoir is located at the base of the mandible. We could

TABLE 1. RESPONSE OF *L. niger* TOWARDS DEAD *A. brevis* OR *T. angelicae* IN FOUR DIFFERENT DESIGNS[a]

Species	Design	Removed at: First contact	Removed at: Subsequent contacts	Not removed
A. brevis	1a	1	2	7
	2b	8	6	0
	3b	9	5	1
T. angelicae	4b	11	4	0

[a] For detailed descriptions of the designs, see Methods and Materials. Designs sharing the same letter do not differ at $P < 0.05$ (Fisher's exact test) if "removed at first and subsequent contacts" were pooled.

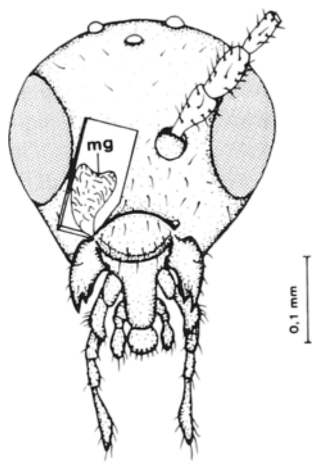

FIG. 3. Head of *Alloxysta brevis* (frontal view) and position and size of the mandibular gland reservoir (mg).

identify no other cephalic glands or fine structures of the macerated secretory tissues.

Gas Chromatography-Mass Spectrometry. Figure 4 shows a typical total ion chromatogram obtained by solid injection of the head of an *A. brevis* female. Compound **1** exhibited a molecular mass of 126. Its EI mass spectrum (*m/z* 55, 58, 83, 93, 108, 109, 11) and retention time was identical with authentic 6-methyl-5-hepten-2-one. The second main compound of the *A. brevis* head produced typical fragments at *m/z* 147, 132, 117, and its mass spectrum was indistinguishable from actinidin isolated from defensive glands of rove beetle, *Philonthus carbonarius* (Dettner, 1983; *Alloxysta*—compound **2:** scans 1039–1043; *Philonthus*—actinidin: scans 1049–1055). *A. brevis* components **3–7** could be not identified conclusively due to the lack of *Alloxysta* gland material. Common intensive fragments at *m/z* 81 favor the presence of five iridoid compounds of unknown structure and stereochemistry. [**3:** 55(100), 58(83), 67(90), 79(58), 81(97), 93(47), 111(53), 135(28), and 150; **4:** 55(87), 58(69), 67(100), 71(50), 79(53), 81(85), 93(38), 109(41), 135(33); **5:** 55(38), 67(90), 81(100), 95(71), 113(55), 123(12), 139(5), and 153(2); **6:** 55(22), 67(100), 81(71), 95(59), 109(18), 123(2), 151(1), and 169: probably M+1(14); **7:** 55(23), 67(100), 81(60), 95(26), 109(10)]. Compounds **3–7** showed fragmentation patterns nearly identical with unknown rove beetle isomers of iridodial **(3, 4)**, dihydronepetalactone **(5)** and iridomyrmecine **(6, 7)** (Huth and Dettner, 1990).

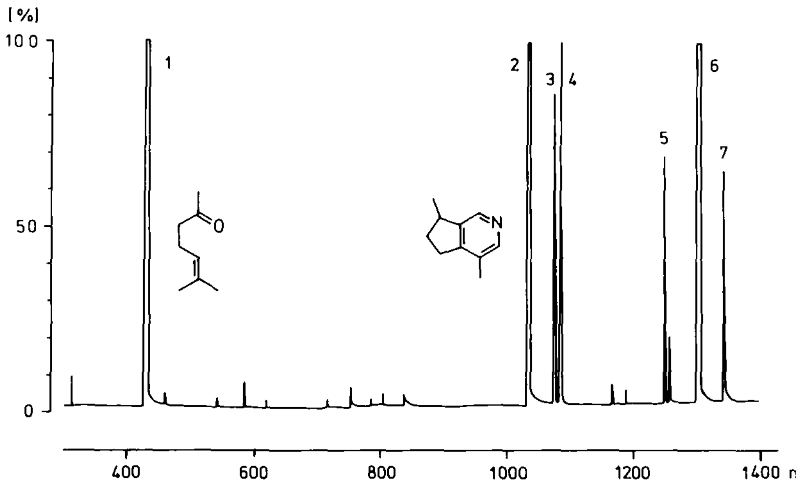

FIG. 4. Total ion current chromatogram (relative intensity, %, n = scan number) obtained by solid injection of one *Alloxysta brevis* female head. Compounds identified: **1,** 6-methyl-5-heptene-2-one; **2,** actinidine; **3–7,** unknown iridoids.

These peaks occurred consistently in all replicates (N = 8 females). Essentially the same pattern was obtained from males (N = 3). None of these seven compounds was found in the thorax/abdomen samples of the identical *A. brevis* females that were analyzed by the solid injection technique.

Only 6-methyl-5-heptene-2-one and actinidin could be identified in a headspace sample of 30 freshly thawed *A. brevis*.

DISCUSSION

Insects living in close association with ants need to overcome ant attacks to survive and obtain access to ant-attended resources. This can be achieved, for example, by chemical camouflage, by imitating the ants' alarm pheromone, or by applying defense secretion (for reviews, see Hölldobler and Wilson, 1990; Dettner and Liepert, 1994). For example, parasitoids and predators of ants avoid detection by biosynthesizing a cuticular hydrocarbon profile nearly identical to that of their host or prey, respectively (e.g., Vander Meer et al., 1989; Howard et al., 1990a,b; Stanley-Samuelson et al., 1990). Similarly, the aphid parasitoid *L. cardui* was assumed to mimic cuticular hydrocarbons of its host *A. fabae* to gain access to ant-attended aphid colonies (Liepert and Dettner, 1993; Völkl and Mackauer, 1993).

A. brevis is an obligatory hyperparasitoid whose main hosts, larvae of *Lysiphlebus* species, are commonly guarded by honeydew-collecting ants (Stary, 1987; Völkl, 1992, 1994; Mackauer and Völkl, 1993). Foraging hyperparasitoid females were readily attacked and caught by ant workers but were released quickly and subsequently ignored by the ant worker for about 5 min. Similarly, intact dead *A. brevis*, where the mandibular gland reservoirs had opened during thawing (as perceived by a significant agreeable odor) were ignored by ants during the first 15 min after being thawed. By contrast, both decapitated *A. brevis*, which were freshly thawed, and intact individuals thawed two days before the experiment were carried off the plant by *L. niger* in the same manner as dead *T. angelicae* were removed (see also Liepert and Dettner, 1993). These results present evidence that *A. brevis* survives ant attacks by chemical defense with volatile substances.

The cephalic extracts of *A. brevis* contained seven variably volatile components, some of which are known to show a pronounced repellency and fumigant effect against arthropods. Actinidin, commonly found, e.g., in the pygidial glands of rove beetles (Dettner, 1983; Huth and Dettner, 1990) and in the anal glands of dolichoderine ants (Wheeler et al., 1977), is known to have excellent fumigant activity against adults of *Drosophila melanogaster* (Dettner et al., 1992). In addition, this nitrogen-containing iridoid shows a pronounced knockdown effect against last-stage *Calliphora* larvae (Dettner, unpublished data).

Another widespread volatile repellent/fumigant is 6-methyl-5-hepten-2-one, a main compound of the cephalic and the headspace samples of *A. brevis*. This volatile ketone is a common exocrine defense compound of insects (Blum, 1981; Davies and Madden, 1985). Obviously, it may act as an alarm pheromone or as an allomone in a number of ant species (Blum, 1981), but was not reported for *L. niger* up to now (Bergström and Löfqvist, 1970; Hölldobler and Wilson, 1990). This may explain why the response to the mandibular gland secretion was restricted to the attacking or encountering ant, while conspecific ants foraging in the same aphid colony did not obviously change their behavior.

Although compounds **3–7** could not be conclusively identified, iridodial, dehydronepetalactone, and iridomyrmecin may act as effective defensive compounds against ants (Jefson et al., 1983), and iridomyrmecin may even have insecticidal properties (Pavan, 1975). At the same time, iridodial can act as a fixative, to retard evaporation of more volatile gland constituents (Pavan and Trave, 1958; Dettner et al., 1985).

6-Methyl-5-hepten-2-one was also reported as an exocrine secretion of the closely related *Alloxysta victrix* (Westwood) by Micha et al. (1993), who interpreted this ketone as a sex and spacing pheromone. In our experiments, average patch times of *A. brevis* did not differ significantly between experiments where wasps were searching alone or in the presence of conspecifics, and encounters between foraging *A. brevis* usually resulted in ignoring. Wasps never left the patch in response to an encounter with a conspecific, even if this female had released mandibular secretion. Hence, we have no evidence that 6-methyl-5-hepten-2-one acts as a spacing pheromone for *A. brevis*. Our assumption is supported by Matejko and Sullivan (1979), who observed regularly up to five females of *Alloxysta megourae* (Ashmead) ovipositing simultaneously into the same aphid.

The only possible sources for these defensive compounds are the mandibular glands, which have large reservoirs (Figure 3). This assumption is supported by three arguments: (1) They obviously represent the only exocrine glands within the head capsule. (2) Compounds **1–7** could be only identified from cephalic but not from abdominal extracts. (3) Only dissection of the minute *A. brevis* heads produced spreading in water when the mandibular glands were destroyed. This effect was not observed during dissection of abdomens.

We hypothesize that *A. brevis* females release mandibular gland secretion only in response to an ant attack as an act of self-defense. However, after application, the secretion continues to function as a repellent for approximately 5 min, which prevents attacks by subsequently appearing ants. 6-Methyl-5-hepten-2-one and actinidin are volatile substances. As natural fumigants they evaporate quickly (Dettner et al., 1992). However, iridodial and other unidentified compounds of the secretion may act as fixatives and prolong any protective effect.

The large variances observed between particular periods of ignoring may result from different amounts of glandular secretion being released in response to an ant attack. The large reservoirs may provide the opportunity to store a considerable quantity of gland secretion and enable the hyperparasitoid female to release secretion several times, even in comparatively short intervals. Thus, *A. brevis* can adjust its chemical defense in response to the frequency of ant attacks.

The possibility of chemical defense against ants provides selective advantages both for foraging *A. brevis* adults and for their developing progeny. First, the mandibular gland secretion seems to have a knockdown effect on the attacking ant, and foraging females are therefore not killed by ants. This leads to increased adult survival of the hyperparasitoid. Second, adults gain access to ant-attended resources, which constitute a major proportion of their hosts (Stary, 1970, 1987; Völkl, 1992), and successful ovipositions are possible during the period of ignoring. Despite reduced oviposition numbers in comparison to unattended colonies (Völkl, 1992), this may lead to an increased overall reproductive success, which may be especially important early and late in the season when the few available hosts are heavily attended by ants. Third, aphid mummies containing immature *Alloxysta* larvae that still feed within their aphidiid host can be attacked by ectohyperparasitoids (mainly *Dendrocerus* spp. and *Asaphes* spp.), which are intrinsically superior and kill the endohyperparasitoid larva (Sullivan, 1972; Matejko and Sullivan, 1984). The probability of an ectohyperparasitoid oviposition into an *A. brevis*-hyperparasitized mummy is roughly proportional to the relative frequency of emerging *A. brevis* if compared to the number of emerging aphidiid wasps (Sullivan, 1972; Matejko and Sullivan, 1984; Walker and Cameron, 1981). Recalculating data from Völkl (1992), we find a significantly reduced offspring mortality of *A. brevis* due to ectohyperparasitoid attacks in ant-attended colonies (unattended mummies: $33.5 \pm 4.9\%$ mortality, $N = 41$; *L. niger*-attended mummies: $9.9 \pm 1.4\%$, $N = 100$, $t = 6.19$, $P < 0.001$). Furthermore, *A. brevis* should benefit from the protection against predators, which may consume *A. brevis* eggs together with parasitized aphids (Stary, 1970; Völkl, 1992).

Adult aphidophagous ladybird beetles deter ants by "reflex bleeding," whereby they release alkaloids only for self-defense (Tursch et al., 1971; Pasteels et al., 1973; Eisner et al., 1986), but this does not enable them to exploit ant-attended resources (Way, 1963; Völkl, 1990). The chemical defense of *A. brevis*—the first evidence for chemical defense in hymenopterous parasitoids—not only increases adult survival but also leads to an increased reproductive success, since it allows this hyperparasitoid to oviposit successfully into ant-attended aphids where its progeny is protected against natural enemies.

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COMPARISON OF WALKING LOCOMOTORY REACTIONS OF TWO FORMS OF *Callosobruchus maculatus* MALES SUBJECTED TO FEMALE SEX PHEROMONE STIMULATION (COLEOPTERA: BRUCHIDAE)

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Abstract—A comparison of the walking locomotory reactions of flightless and flight form *Callosobruchus maculatus* males when subjected to an air current scented with female sex pheromone was undertaken in a tubular olfactometer. The pheromone was delivered to the males either as short pulses or as a continuous flow. To analyze the males' reactions, three behavioral sequences were defined (sequence 1: male sensitivity/arousal; sequence 2: male reactivity; sequence 3: male progression and source location). Although flightless and flight form males were sensitive and reactive in all experiments, their locomotory displacement differed depending on the stimulus conditions. The flightless form males' response remained roughly the same whatever the stimulus conditions (i.e., they always reached the pheromone source). In contrast, the flight form males displayed a markedly reduced response when subjected to a continuous stimulation, indicating that intermittent on–off pheromone stimulation is required in order to sustain their upwind walking progress. This effect could be the result of sensory adaptation and/or habituation in the central nervous system of the flight form, requiring a flickering signal that is unnecessary for the flightless one.

Key Words—*Callosobruchus maculatus*, Coleoptera, Bruchidae, flightless form, flight form, tubular olfactometer, walking behavior, intermittent stimulation.

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INTRODUCTION

The orientation of insects to a distant odor source has long been a matter for study and is best documented in flying male moths encountering a windborne plume of female sex pheromone, either under laboratory conditions, in a wind tunnel (Marsh et al., 1978; Kennedy et al., 1980, 1981; Bell and Tobin, 1982; Baker and Kuenen, 1982; Kennedy 1983, 1986; Willis and Baker, 1984; Willis and Arbas, 1991; Vickers and Baker, 1992), or in the field (David et al., 1983; Ono and Ito, 1989; Willis et al., 1991). The main difficulty remains in relating precisely, in real time, the distribution of the odor to the semiochemical-mediated behavior (Marsh et al., 1978). Wright (1958) was the first to point out the filamentous nature of odor and stated that an insect entering an odor plume "would perceive the odor not as a continuous sensation of gradually increasing strength, but rather as a series of pulses or alterations of high and low odor intensity as it passed through the many odor trails that make up the cloud." More recently, Murlis and Jones (1981) described quantitatively the fine structure of an odor plume by simulating it with an ion generator and by monitoring the ion concentration at various points downwind from the ion source. It appeared that the ion plume (= odor plume) is detected as a series of pulses of different durations and concentrations (Murlis and Jones, 1981; Murlis, 1986; Murlis et al., 1992). Not surprisingly, locomotory behavior, as a reaction to odor (prey, host, sexual partner, or food) is reliant upon the intermittent nature of the signal; indeed, several studies have confirmed Wright's original notion that the discontinuous structure of odor stimulation provided by the filamentous plume is somehow important for orientation (Marsh et al., 1978; Pouzat, 1978; Kennedy et al., 1980, 1981; Willis and Baker, 1984; Vickers and Baker, 1992).

The cowpea weevil *Callosobruchus maculatus* (Coleoptera: Bruchidae), a phytophagous insect present in all tropical zones, is a serious pest of pulses, especially the cowpea *Vigna unguiculata* (L.) Walp. During the biological cycle of *C. maculatus*, two distinct adult forms are known to occur, differing in their morphology, physiology, and behavior; these are referred to as the flightless form, which is sexually mature on emergence, and the flight form, which emerges in reproductive quiescence (Utida, 1954). The former is only found in stored beans, the latter is the migrant form, which leaves the bean stores and infests the fields of *Vigna* sp.

The existence of a volatile sex pheromone produced by the mature females of this beetle has been reported by Rup and Sharma (1978); this pheromone attracts the sexually active males (Qi and Burkholder, 1982; Bilal, 1987). The present study was undertaken to compare by olfactometric techniques the locomotory response of the flightless and flight forms of *C. maculatus* males subjected either to a continuous or a flickering pheromone stimulation; the

pheromonal stimulation was provided either as an airstream continuously scented with pheromone or as short pulses interspersed with clean air.

METHODS AND MATERIALS

Rearing. C. maculatus, obtained from a crop infestation in Burkina Faso, were reared in the laboratory on seeds of *Vigna unguiculata* (Black Eyes California variety), at 12:12-hr light-dark, temperatures of 40°C (light) and 25°C (dark), and 30% relative humidity. These conditions were chosen as being closest to those found in the natural state. Furthermore, these "high" temperatures prevented attack by entomophagous acari. The flightless form and the flight form were produced by culturing seeds with respectively low or high larval density (Utida, 1954).

Experimental Insects. Males and females were selected at random from culture cages. To obtain unmated adults, seeds showing emergence opercula were isolated in small boxes and regularly observed. Immediately after emergence, the imagos were sexed, then males and females were kept separate under a 25:17°C, 12L:12D regime until the test. The emerging males of the flight form do not immediately react to the pheromone of sexually active females, and the emerging females of the flight form do not produce/release the sex pheromone (Bilal, 1987). The beetles of the flight form were given host plant seeds for several days in order to suppress their quiescence. Pouzat et al. (1989) demonstrated that the presence of the host plant over a seven to 10-day period was sufficient to trigger sexual activity. All the experiments were conducted with virgin 9 to 10-day-old flight form males to ensure that they were sexually mature and with 3 to 4-day-old flightless form males. Flightless form males are short-lived and age more quickly than the flight form males kept in the same environmental conditions. Up to the time of the test, the beetles were fed with a sucrose solution ad libitum.

Olfactometer. The apparatus used in these experiments was described by Chaibou et al. (1993). Air was introduced through a system with two parallel channels (called the odor delivery system) (Figure 1), enabling either a current of pure air or a current of air laden with the test odor to be sent through the tube. Each of these two channels consisted of a removable glass cartridge (6 cm long, 0.8 cm ID). One of these cartridges was empty (i.e., clean channel), the other contained the odor source (i.e., odor channel). An electrovalve (Chatelain, unpublished design) enabled air to be alternately delivered into the clean channel and into the odor channel, thus delivering the odor in the form of puffs. The electronic system that controlled the gate enabled the duration and frequency of the odor puffs to be controlled. The air speed, monitored by the flowmeter, averaged 0.3 m/sec in the olfactometer.

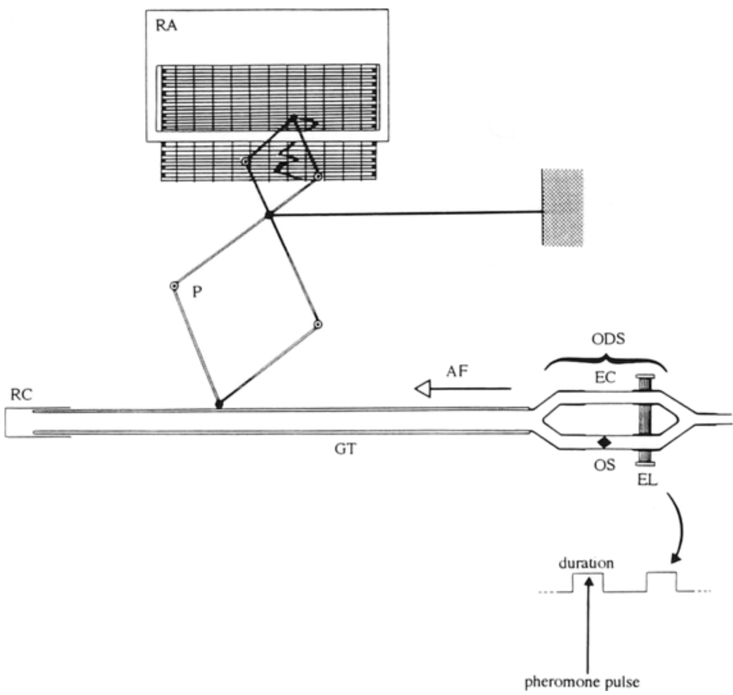

FIG. 1. Diagram of the olfactometer used in the experiments (different parts are not the same scale). AF: air flow (purified and humidified), EL: electrovalve, EC: empty cartridge, GT: glass tube (length = 90 cm), ODS: odor delivery system, OS: odor source (group of females), P: pantograph, RA: recording apparatus, RC: release cage.

The displacement of the insect in the olfactometer was recorded with a pantograph on a recording paper running at a constant speed of 4 cm/min. This system does not provide a record of the actual track of the insect but does indicate its location in the olfactometer and its "gross" displacements.

Pheromone Source. The odor source was a group of five virgin flightless form females age 3–5 days, placed in the cartridge inside the odor delivery system (Figure 1). In previous studies, we showed, by cross-attractivity tests between the two forms, that the sexually active males, whichever their form, were similarly excited whether the sexually active females releasing the pheromone were of the flight or flightless form (Lextrait, 1990).

Experimental Procedure. Tests were performed in a controlled area at 25°C, between the fifth and eighth hour of the photophase, the moment of peak sexual pheromone production (unpublished data). The temperature at which the observations were made differed from that used during rearing because higher tem-

peratures could lead to hyperactivity in some individuals, making interpretation of behavior difficult, whereas, at 25°C, they displayed a "normal" activity and were easier to handle; furthermore, this was the temperature of the photoperiod in which the adults were kept (see above).

Each male was released singly in the olfactometer from a small plastic cage fitting the downwind end of the olfactometer, under a continuous flow of clean air. For each test, the reaction of each male to the pure air stream (4 min) and to an air flow permeated with pheromone (4 min) was observed. The male was then subjected again to pure air (4 min) to record its reaction to the removal of the stimulation. To analyze the male response during pheromone stimulation, three behavioral sequences were defined (Figure 2). The first one is an arousal reaction characterized by vertical antennal movements; a male displaying such a behavior was said to be sensitive. The second sequence was the departure from the release cage; a male was said to be reactive when it left the release cage and entered the olfactometer. The third sequence is a sustained upwind progress in the olfactometer. The number of males successful in locating the source was noted (reaching the 15-cm point of the upwind end of the olfactometer was scored as "source location"). Two sets of temporal data were recorded (Figure 2): (1) latency time, which is the time spent by the male in the release cage until its entrance into the olfactometer; and (2) time spent to reach the source.

The pheromone was delivered to the beetle according to the following three stimulus conditions: (1) 50 msec pulses every 2 sec, (2) 1-sec pulses spaced 1 sec apart (i.e., both conditions had a period of 2 sec), (3) an airstream constantly permeated with pheromone. To avoid any risk of contamination, the olfactometer was flushed with pure air between the tests. Between each experiment it was washed in hot water, rinsed with alcohol, and dried for 24 h in an oven at 90°C.

Statistical Analysis. In all experiments, differences between behavioral cat-

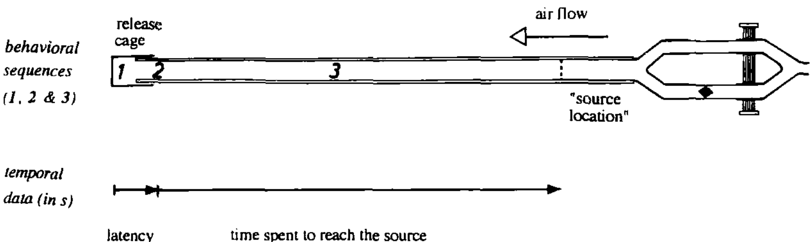

FIG. 2. Parameters used to characterized the male response to female sex pheromone. Sequence 1: male sensitivity (arousal); sequence 2: male reactivity (departure from the release cage); sequence 3: male progression and source location.

egories were compared using a chi-square test of independence with Yates' correction. Latency time until departure from the release cage and time spent to reach the source were compared using the nonparametric Mann-Whitney U test (Sokal and Rohlf, 1969). For all statistical tests, the differences were significant at the 0.05 probability level.

RESULTS

The flightless form insects of our strain never fly. Thousands of flightless form males having the morphological and color characteristic have never shown any attempt to take off. In the present work, all the flightless form males were actually flightless. Regarding flight form males, they sometimes tried to take off in the olfactometer, but this behavior was quite exceptional (less than 1%).

Reaction to Pure Air. In a control airstream, the males of both forms were inactive: the males' displacements were restricted to the downwind half of the olfactometer and 50–80% of them remained in the release cage.

Reaction to the Pheromone Stimulation. In the first sequence, the pheromone onset triggered a characteristic behavioral response displayed both by flightless and flight form males: the male pricked up its antennae, which made up and down sweeping movements. Such a sequence was always observed. In the second sequence, between 87% and 97% of the males, according to the experiment concerned, left the release cage. All the males were therefore reactive (Table 1). In the third sequence, source location, the results varied greatly depending on the male form and the stimulus conditions (Table 1). Of the

TABLE 1. EFFECTS OF STIMULUS CONDITIONS ON DEPARTURE FROM RELEASE CAGE (SEQUENCE 2) AND SOURCE LOCATION (SEQUENCE 3)

Form	Stimulus conditions	*N*	Departure (%)[a]	Source location (%)[a]
Flightless males	pulses duration: 50 msec, period: 2 sec	26	92a	85ab
	pulses duration: 1 sec, period: 2 sec	23	96a	91ac
	continuous flow	27	93a	67bd
Flight males	pulses duration: 50 msec, period: 2 sec	30	87a	73bc
	pulses duration: 1 sec, period: 2 sec	30	97a	47de
	continuous flow	30	97a	27e

[a] Percentages in the same column having no letters in common are significantly different (chi-square test of independence with Yates' correction, significant level: $P < 0.05$).

flightless form males, 85–91% reached the pheromone source when the stimulation was delivered as pulses, and 67% succeeded in a continuous odor flow. However, there was no significant difference between the three treatments (Table 1). Of the flight form males, 73% reached the source when stimulated with 50-msec pulses, but only 47% when stimulated with a 1-sec pulse ($P < 0.05$). Of the flight form males subjected to a continuous odor flow, 27% were successful in locating the source. The stimulus conditions clearly influenced the upwind displacement of the flight form males. A continuous stimulation did not enable most of them to locate the source.

In the olfactometer, the flightless form males moved upwind with a fairly straight track, still displaying vertical sweeping antennal movements whatever the stimulation conditions (Figure 3A, and C). When the flight form males left the release cage, they began a short due-upwind progress. The further upwind they progressed, the more tortuous the path became: more and more lateral displacements were observed, more or less perpendicular to the direction of the odorous airflow (Figure 3B). This type of displacement was observed in each of the three stimulus conditions, but when the stimulation consisted of 1-sec odor puffs or was a continuous odorous flow (Figure 3D), the lateral movements in the olfactometer became so frequent that there was no further upwind progression.

Temporal Data. Even though all the males were sensitive and reactive, the latency time of the flightless form males was always shorter than that of the flight form males ($P < 0.05$) (Table 2). For each form, the latency time was not significantly different regardless of whether the stimulation was continuous or consisted of 1-sec puffs every 2 sec. For 50-msec puffs, the latency time was higher (Table 2).

Both flightless and flight form males were quicker in locating the source when the pheromone was delivered as pulses (Table 2). However, the time spent by the former in reaching the source always remained shorter than that of the flight form males, except in the case of continuous stimulation, where the performances of the males actually reaching the source were not significantly different (Table 2).

Reaction to Removal of Stimulation. When stimulation was stopped, the males, now subjected to a flow of pure air, moved in every direction (lateral movements, upwind and downwind displacements). They made several U-turns in the olfactometer with a marked tendency to return downwind.

DISCUSSION

Subjected to a flow of odorless air, all *C. maculatus* males showed a limited locomotory activity and no anemotactic behavior. Pheromonal stimulation elicited an instant reaction (first sequence = arousal) and triggered an onset of

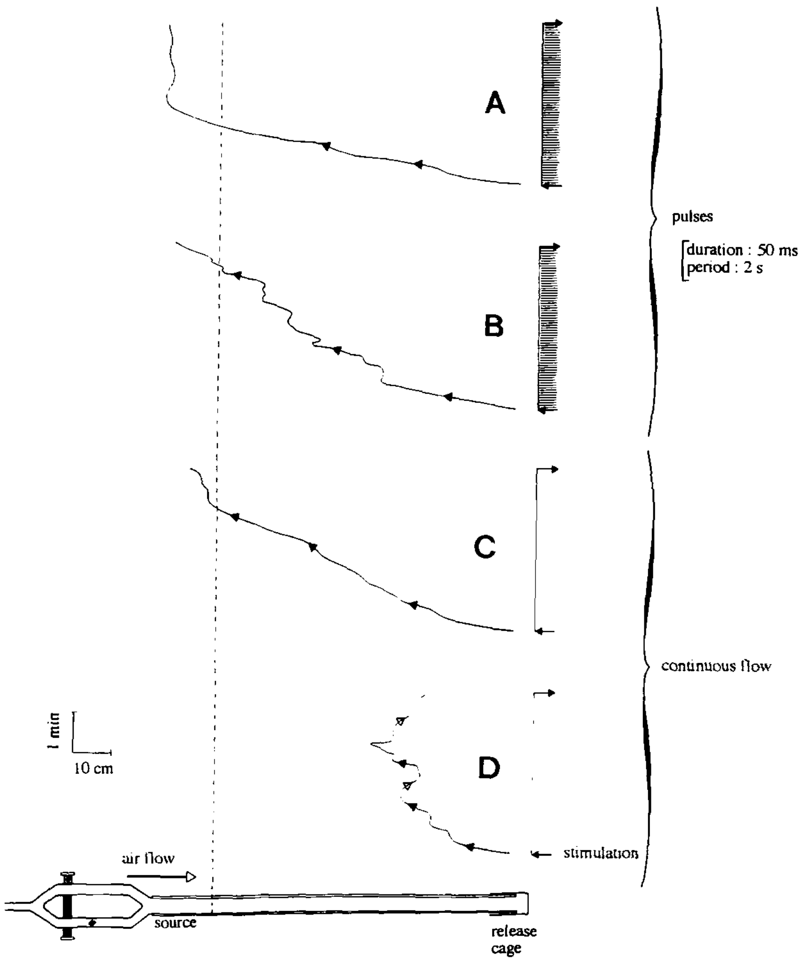

FIG. 3. Representation of males displacement depending on the stimulus conditions. Tracks A and C: flightless form males; tracks B and D: flight form males;—◀—: upwind displacement;—▷—: downwind displacement.

locomotory response. The flight and flightless form males presented a characteristic antennal reaction, which showed they formed a homogeneous batch with respect to their sensitivity.

The flight form males were also all reactive (second sequence = departure from the release cage), but they remained slower to react to the odorous flow and to orient towards the source under the three stimulus conditions studied.

TABLE 2. MEAN VALUES (±SE) OF LATENCY TIME TO DEPARTURE FROM RELEASE CAGE AND TIME SPENT TO REACH SOURCE

	Latency time[a]		Time to reach source[a]	
Stimulus conditions	Flightless males	Flight males	Flightless males	Flight males
Pulse duration: 50 msec, period: 2 sec	25.63 ± 4.89a (24/26)	40.54 ± 6.61c (26/30)	65.27 ± 5.29a (22/26)	107.41 ± 7.09c (22/30)
Pulse duration: 1 sec, period: 2 sec	7.32 ± 1.63b (22/23)	21.86 ± 6.48d (29/30)	70.86 ± 7.02a (21/23)	103.57 ± 13.45c (14/30)
Continuous flow	7.36 ± 1.56b (25/27)	23.62 ± 7.52d (29/30)	87.94 ± 20.73b (18/27)	122.50 ± 18.9bc (8/30)

[a] For each parameter latency time and time to reach source, means in the same column or in the same line having no letter in common are significantly different (Mann-Whitney U test, significant level: $P < 0.05$). The ratio in parenthesis indicates the number of males leaving the release cage or the number of males reaching the source relative to the number of males in each experiment.

The locomotory reactivity threshold of these males appeared higher than that of the flightless form males. Both flightless and flight form males showed a delayed reaction (high latency time) when stimulated by 50-msec pulses: this might be due to the dilution of the odor on its arrival at the downwind end of the olfactometer: in the boundary layer the airspeed is slowed down, which lengthens the odor puffs and then dilutes odor [relative to a continuous odor flow, the dilution factor would be 1/40 if the blending of the odor puffs (50-msec duration) with clean air puffs (1950 msec) was complete: the concentration of the odor at the downwind end of the olfactometer would be only 2.5% of the concentration of a permanently odorized air].

The displacement of the flight form males towards the pheromone source differed from those of the flightless form males; their locomotory behavior, characterized by an upwind progression with numerous lateral movements more or less perpendicular to the direction of the odorous airflow, is not unlike the one observed in flying moths subjected to a pheromone stimulation, i.e., chemoanemotactic progression punctuated by numerous zigzags (Marsh et al., 1978; Kennedy et al., 1980, 1981; Baker and Kuenen, 1982; Kennedy, 1983, 1986; Willis and Baker, 1984). The only work comparing maneuvers used by walking versus flying males during pheromone stimulation was performed on *Grapholita molesta* by Willis and Baker (1987). They showed that while walking upwind toward a pheromone source, males exhibited none of the temporally regular zigzag that characterized their tracks when flying upwind to a pheromone source. The results obtained on *C. maculatus* are not congruent: when they are only allowed to walk, the flight form males do not move in a straight path. The difference in the time spent to reach the source reflected also the difference in

the progress of the two kinds of males. It remains that the olfactometer used in these experiments, first, did not allow the analysis of the orientation mechanisms used by the insect but only enabled the demonstration of a possible attraction manifested by a locomotory displacement upwind toward the odor source; and, second, only enabled mimicking the interrupted plume structure. Furthermore, in our study, flight form individuals were only allowed to walk and could not, therefore, display the behavioral sequences induced by pheromone perception in flight. In fact, it seems that the necessary intermittent stimulation is normally provided by the fluctuations of concentration within the pheromonal plume itself (Wright, 1958; Murlis and Jones, 1981) and also by the incursion and excursion into and out of the pheromonal plume caused by the zigzagging upwind path observed in the flying moths (Marsh et al., 1978; Kennedy et al., 1980, 1981; Baker and Kuenen, 1982; Kennedy, 1983, 1986; Willis and Baker, 1984). It is therefore necessary to study the flight behavior of *C. maculatus*, for which it will be necessary to redesign and adapt a wind tunnel in which the boundary of the odorous zone will be unambiguously known.

A high percentage of flightless form males were able to locate the odor source under the three experimental conditions. The more discontinuous the pheromonal stimulation, the more easily the flight form males oriented towards and reached the source. When the stimulation was in the form of 1-sec puffs, only half the flight form males managed to locate the odor source; however, under our experimental conditions, it was possible that a stimulation of 1-sec puffs was equivalent to a homogeneous odor flow in the downwind part of the olfactometer (the puffs tend to lengthen due to the slowing down of the airspeed in the boundary layer, with the effects that at the downwind end of the olfactometer the male might perceive the stimulation as a continuous odorous flow). When the pheromone was delivered as 1-sec pulses or as a continuous flow, the upwind progress toward the source stopped; the flight form male seemed unable to maintain a sustained reaction to the odor.

Kramer (1986) performed a behavioral study with *Bombyx mori* and observed a decrease in the linear velocity and a reduction in the walking response when the pheromone was continuously delivered. This experiment, conducted on a servosphere, is relatively similar to our own: the insect, while being able to move in any direction, was continuously subjected to the stimulation as it cannot get out of the odor flow. This is the sole study equivalent to our own that we have been able to find in published data. The reduction in the responsiveness to a continuous stimulus has been mentioned in male Lepidoptera stimulated by the female sex pheromone (Traynier, 1970; Bartell and Lawrence, 1973; Sower et al., 1973; Kennedy et al., 1980, 1981; Kuenen and Baker, 1981; Willis and Baker, 1984; Kramer, 1986; Vickers and Baker, 1992) and more rarely in Coleoptera subjected to the sex pheromone or the aggregation pheromone (Vick et al., 1973; O'Cellachain and Ryan, 1977).

Some studies have focused on the consequences of a pheromone preexposure on subsequent behavioral sequences such as wing fanning (Traynier, 1970) or walking/flying initiation and displacement (Bartell and Lawrence, 1973; Sower et al., 1973; Vick et al., 1973; O'Cellachain and Ryan, 1977; Kuenen and Baker, 1981; Liu and Haynes, 1993). A markedly reduced responsiveness was observed after preexposure. Other more recent works investigated the direct influence of a constant pheromone stimulation on flight orientation relative to an odorous air current. Observations have shown also that in a wind tunnel, a continuous stimulation elicited within a few seconds a reduction/cessation of the upwind flight (Kennedy et al., 1980, 1981; Willis and Baker, 1984; Kramer, 1986; Baker et al., 1988).

The reduction of the locomotory response could occur because of peripheral sensory adaptation or due to a central mechanism, i.e., habituation, or a combination of both. The neurophysiological causes of response reduction have not been generally investigated, although mechanisms had been proposed. In fact, the term "habituation," generally defined as "a gradual decrease in the intensity of a reflex response to a monotonously repeated stimulus" (Kandel, 1976 in Kuenen and Baker, 1981), has been commonly used by authors to describe the decrease in responsiveness following continuous stimulation without precise references to neurophysiological mechanisms (Traynier, 1970; Sower et al., 1973; Vick et al., 1973; O'Cellachain and Ryan, 1977). Adaptation has been suspected as a cause of arrestment of upwind progress to a continuous pheromone stimulation by Kennedy et al. (1980, 1981), without further explanation. Bartell and Lawrence (1973) attributed the observed effects to an inhibition in some integrative center in the central nervous system as the effects were of long duration and not significantly dependent upon the responses at the time of preexposure. Kuenen and Baker (1981) attempted to determine whether reduced responsiveness to pheromone was due to sensory adaptation or habituation by correlating electroantennogram (EAG) response patterns with altered behavioral patterns in preexposed moths, *Trichoplusia ni*. It appeared that EAG response was reduced concurrent with exposure, so that receptor adaptation occurred. These results confirmed those of Payne et al. (1970). In the same way, Baker et al. (1988) attempted to gain further insight into the relationships between receptor output and the change from upwind flight to arrestment in *Agrotis segetum* by recording single antenna neurons and reached the same conclusion. This "adaptation" was not due to an overloading of the sensory system by an excessive amount of pheromone: the findings of Kennedy et al. (1981) and Willis and Baker (1984) showed that, when a pheromonal plume was superimposed onto the uniformly permeated airstream, the moths were able to resume their upwind progress. Concentration fluctuations also seemed to be necessary to maintain the response, which would indicate that a central mechanism is operating. Although adaptation of receptors was observed during pheromone exposure, the subsequent effects

were different according to the species, the receptor responsiveness being recovered within tens of seconds (Payne et al., 1970; Kuenen and Baker, 1981) to tens of minutes (Kaissling, 1986; Baker et al., 1988). In certain cases, the behavioral responsiveness remained altered after previous pheromone exposure, recovery requiring many minutes or even hours, but the authors were not always precise about what happened at the peripheral level (Traynier, 1970; Bartell and Lawrence, 1973; Sower et al., 1973; Vick et al., 1973; O'Cellachain and Ryan, 1977), except Kuenen and Baker (1981), who showed that habituation was probably the cause of a reduced behavioral response.

The first electroantennogram studies in *C. maculatus* showed that the response of flight form males to the odor of females persists during the whole stimulation (stimulus duration between 1 and 32 sec) (unpublished data). However, these results are not conclusive because in conditions of prolonged stimulation, obtaining an electroantennogram response does not prove that there is no sensory adaptation, as this technique only allows the recording of antenna slow potentials, not the action potentials (see, for example, Kaissling, 1986). Further investigations by single unit recording are necessary to elucidate this aspect.

In *C. maculatus*, the present behavioral differences may be interpreted as adaptations to the usual living conditions of the two imaginal forms. The flight form males are well adapted to natural stimulation; in an odor plume of female pheromone, these migrant males encounter frequent and extensive changes of odor concentration (Murlis and Jones, 1981; Murlis, 1986; Murlis et al., 1992). In contrast, flightless form males indefinitely reproducing under storage conditions are not subjected to this kind of stimulation and apparently no longer require it. It may be hypothesized that in storages, male and female encounters occur very easily owing to the density of the insect population.

The findings reported here have some significance regarding the employment of pheromones in pest management. Indeed, disruption of intraspecific communication by saturation of the environment with synthetic pheromone could be considered; but further information about the processes of adaptation/disadaptation and habituation/dishabituation are required for a possible control.

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RESPONSE OF *Dendroctonus brevicomis* LE CONTE (COLEOPTERA: SCOLYTIDAE) TO DIFFERENT RELEASE RATES AND RATIOS OF AGGREGATION SEMIOCHEMICALS AND THE INHIBITORS VERBENONE AND IPSDIENOL

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Abstract—The response of western pine beetle, *Dendroctonus brevicomis* Le Conte, to different release rates of the aggregation semiochemicals (attractants) *exo*-brevicomin, frontalin, and myrcene and the inhibitors verbenone and ipsdienol was investigated. Release rates of verbenone ranging from 0.18 mg/24 hr to 1.2 mg/24 hr did not result in significant reductions in mean trap catch of *D. brevicomis*. In contrast, very low release rates of ipsdienol (0.02–0.4 mg/24 hr) significantly reduced trap catch compared to controls. The combination of verbenone and ipsdienol, released at rates above 0.09 and 0.02 mg/24 hr, respectively, resulted in significantly lower trap catches of *D. brevicomis* in attractant-baited traps. Results of an experiment testing a factorial combination of different release rates of verbenone and attractants suggest that response is not ratio-specific. The response curve of *D. brevicomis* to the levels of verbenone was similar across all levels of attractants, while the response to equivalent ratios of attractants to verbenone was not similar, suggesting that the behavior of the beetles is primarily influenced by the absolute release rate of verbenone.

Key Words—Coleoptera, Scolytidae, *Dendroctonus brevicomis*, western pine beetle, aggregation, verbenone, ipsdienol, inhibitors, antiaggregants, dose-response.

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INTRODUCTION

Western pine beetles, *Dendroctonus brevicomis* Le Conte, colonize ponderosa pine, *P. ponderosa* Lawson, and Coulter pine, *Pinus coulteri* D. Don, via semiochemical-mediated aggregation behavior (Miller and Keen, 1960; Wood, 1982). Females initiate the colonization process, releasing the pheromone *exo*-brevicomin {(+)-*exo*-7-ethyl-5-methyl-6,8-dioxabicyclo-[3.2.1] octane} as they feed and construct galleries (Silverstein et al., 1968; Stewart et al., 1977). Both sexes, but primarily males, are attracted to (+)-*exo*-brevicomin, and this attraction is synergized by host tree monoterpenes, predominantly myrcene (7-methyl-3-methylene-1,6-octadiene) (Bedard et al., 1969). Males produce the pheromone frontalin {(−)-1,5-dimethyl-6,8-dioxabicyclo-[3.2.1] octane}, which attracts additional females (Kinzer et al., 1969; Vité and Pitman, 1969; Stewart et al., 1977).

Verbenone (4,6,6-trimethylbicyclo-[3.1.1]hept-3-en-2-one) inhibits the response of *D. brevicomis* to aggregation semiochemicals (attractants) in the field (Bedard et al., 1980; Tilden and Bedard, 1988; Paine and Hanlon, 1991). Natural sources of verbenone include biosynthesis by *D. brevicomis* (the majority is produced by males) (Byers et al., 1984), autoxidation of α-pinene, a monoterpene constituent of pine resin (Hunt et al., 1989), and possibly, as has been shown for other species of *Dendroctonus*, biosynthesis by microorganisms introduced into the wood by the beetles (Brand et al., 1976; Hunt and Borden, 1990). The enantiomeric composition of verbenone depends on the composition of its precursor, α-pinene (Vanderwel and Oehlschlager, 1987). The enantiomeric composition of α-pinene in conifer resin varies; the (−)-enantiomer predominates in ponderosa pine in California (Byers, 1983).

In addition to verbenone, racemic ipsdienol (2-methyl-6-methylene-2,7-octadien-4-ol) inhibits the response of *D. brevicomis* to attractants in the field (Byers, 1982; Paine and Hanlon, 1991). Male *D. brevicomis* produce (+)-ipsdienol in small amounts, primarily during their initial contact with host resin (Byers, 1982; Byers et al., 1984). However, (+)-ipsdienol is also a principal component of the aggregation pheromone of the sympatric bark beetle, *Ips paraconfusus* Lanier (Silverstein et al., 1966), while another sympatric species, *I. pini* (Say), produces primarily the (−)-enantiomer in California (Birch et al., 1980). Both species of *Ips* are potential competitors with *D. brevicomis* for the same host tree species, and *D. brevicomis* is inhibited from responding to its own pheromones by the presence of *I paraconfusus* boring in logs (Byers and Wood, 1980).

Behavioral inhibition is thought to have evolved in scolytid bark beetles in response to intraspecific competition or host tissue that has deteriorated in quality. Certain inhibitory semiochemicals, such as verbenone, are produced in increasing quantities as colonization progresses and have been hypothesized to

be the chemical cues responsible for termination of attack on fully colonized trees (Borden et al., 1987). Raffa and Berryman (1983) suggest that mass attack is terminated when a critical ratio of inhibitory to attractive semiochemicals is reached as production of aggregation pheromones ceases following exhaustion of host resin supply. In agreement with this hypothesis, the response of *D. brevicomis* to artificially varied ratios of attractants and verbenone suggested that the ratio, rather than the absolute release rate of verbenone, may be an important factor influencing behavioral inhibition (Tilden and Bedard, 1988). The number of beetles responding to traps baited with two levels of attractants and five levels of verbenone [60% (+)-/40% (−)-] was significantly reduced only when the ratio of (+)-*exo*-brevicomin to verbenone was approximately 1 : 1 (Tilden and Bedard, 1988). However, in another study, in which verbenone release rates were varied and the rates of attractants held constant, ratios of (+)-*exo*-brevicomin to verbenone as low as 1 : 0.07 (for 14% (+)-/86% (−)-verbenone, lower still for 69% (+)-/31% (−)-verbenone) significantly reduced the numbers of *D. brevicomis* caught compared to traps releasing the attractants alone (Paine and Hanlon, 1991).

The general objective of the present study was to determine whether the relative release rates of attractants and inhibitors have a greater influence than the absolute release rates of inhibitors on the response of *D. brevicomis* to attractants. The specific objectives were to: (1) confirm for 14% (+)-/86% (−)-verbenone and determine for both racemic ipsdienol and the combination of verbenone and ipsdienol the effects of low release rates on attraction of *D. brevicomis* to aggregation semiochemicals, and (2) determine whether there is a threshold ratio of attractant to verbenone, above which beetles will be inhibited and below which they will not.

METHODS AND MATERIALS

Verbenone and Ipsdienol at Low Release Rates. The response of *D. brevicomis* to a range of low release rates of verbenone, ipsdienol, and the two inhibitors combined was tested in September 1991, at a mixed conifer site dominated by *P. coulteri* near Palomar Mountain, San Diego County, California (elevation 1370 m). Lindgren 12-unit funnel traps (Phero Tech Inc., Delta, British Columbia), suspended from 2-m-tall steel stakes and spaced at least 20 m apart in lines, were baited with the attractants alone (control) or with a combination of attractants plus 14% (+)-/86% (−)-verbenone, racemic ipsdienol, or both inhibitors. Verbenone was released at 0.09, 0.18, 0.6, or 1.2 mg/24 hr (at 24°C); ipsdienol at 0.02, 0.05, 0.2, or 0.4 mg/24 hr (at 24°C); and the attractants *exo*-brevicomin (racemic) at 2.6 mg/24 hr (at 24°C), frontalin (racemic) at 2.4 mg/24 hr (at 20°C), and myrcene at 95 mg/24 hr (at 25°C)

(all release rates are approximate; chemical purity of compounds >98%, compounds and release devices obtained from Phero Tech Inc., Delta, British Columbia, Canada). Combinations of verbenone and ipsdienol were tested by combining in rank order the release rates of each inhibitor compound as tested singly (i.e., the lowest pair of rates, the next lowest pair, etc.). Because *D. brevicomis* are attracted to primarily (+)-*exo*-brevicomin and (−)-frontalin (Wood et al., 1976), the release rates of the active enantiomers were approximately half the rates specified for the racemic mixtures. The presence of the antipodes in the racemic mixture does not synergize or inhibit response to the active enantiomers (Wood et al., 1976).

All treatment combinations were replicated in two blocks. Initially, the treatments were assigned randomly to trap positions within the block, then the treatments were shifted sequentially to a new position each day for 12 days, such that each of the six treatments occupied each trap position twice during the experiment. A 3-cm × 3-cm piece of dichlorvos-impregnated plastic was placed in the collection cup of each trap to kill captured beetles, which were collected from the traps each day.

Trap catch counts were transformed by $\log_{10}(x + 1)$ prior to analysis to correct for nonnormality and unequal variances. Analysis of variance for split-block designs was performed using the blocks × treatments term as the F-test denominator for treatment effects. The inhibitor treatment means were compared to the control (without inhibitor) means using Dunnett's procedure ($\alpha = 0.05$) (Steel and Torrie, 1980).

Varying Ratios of Attractants to Verbenone. The response of *D. brevicomis* to a range of low release rates of ipsdienol indicated that there was no strong dosage–response with that compound; therefore, only verbenone was tested in subsequent relative release rate experiments. To determine whether there is a threshold ratio of release rates of attractants to verbenone that determines whether beetles will be inhibited, a series of ratios were tested. This was achieved by presenting a factorial combination of three levels of release of attractant blend (low, intermediate, and high) with five different levels of verbenone, ranging from very low to very high. The release rates tested were: 14% (+)-/86% (−)-verbenone at 0.05, 0.27, 1.2, 6, and 30 mg/24 hr; and the attractants racemic *exo*-brevicomin, racemic frontalin, and myrcene at 0.6, 0.6, and 67 mg/24 hr (at 24°C) (low level); 2.6 (at 24°C), 2.4 (at 20°C), and 95 (at 25°C) mg/24 hr (high level), respectively (all release rates are approximate; chemical purity >98%, Phero Tech Inc., Delta, British Columbia, Canada). Again, because the racemic mixtures of *exo*-brevicomin and frontalin were used, the actual release rates of the active enantiomers were approximately half the rates specified above. The factorial combination of the three levels of attractants with the five levels of verbenone produced the (approximate) ratios 1 : 0.2, 1 : 1, and 1 : 4 of attractants (*exo*-brevicomin and frontalin) to verbenone for all three levels

of attractants. In addition, approximate ratios of attractants to verbenone of 1 : 0.04 for the intermediate and high levels of attractants, 1 : 20 for the low and intermediate levels of attractants, 1 : 100 for the low level of attractants, and 1 : 0.008 for the high level of attractants were produced. Each level of attractants was presented without verbenone as a control.

The experimental design consisted of a split-plot, with the levels of attractants as the main plots, the levels of verbenone as the subplots, and the days as replicates. The verbenone treatments were assigned randomly to trap positions within the main plots, the rerandomized daily for 21 days from September 9 to 30, 1992. The Palomar Mountain site was again used, with the traps spaced at least 25 m apart.

Analysis of variance for split-plot designs (Steel and Torrie, 1980) was performed on the data, and Dunnett's procedure ($\alpha = 0.05$) was used to compare the means of the verbenone treatments to the control means within each level of attractants.

RESULTS

Verbenone and Ipsdienol at Low Release Rates. All release rates of ipsdienol caused significant reductions in mean daily trap catch compared to the catch at traps baited with attractants alone ($F = 461.4$; $df = 4, 4$; $P < 0.001$) (Table 1). Similarly, the combination of verbenone and ipsdienol significantly reduced trap catch at all release rates compared to the attractant-baited control ($F = 30.72$; $df = 4, 4$; $P = 0.003$) (Table 1). However, the treatment effects for low release rates of verbenone were not significant at the 95% confidence levels ($F = 6.00$; $df = 4, 4$; $P = 0.055$) (Table 1).

Varying Ratios of Attractants to Verbenone. There were significant differences in mean trap catch among the levels of attractants ($F = 18.77$; $df = 2, 40$; $P < 0.001$) and among the different levels of verbenone ($F = 39.57$; $df = 5, 300$; $P < 0.001$) (Table 2). The attractants × verbenone interaction was not significant ($F = 0.99$; $df = 10, 300$; $P = 0.448$) (Table 2), indicating that the response curve to the five levels of verbenone was similar for all three levels of attractants (Figure 1). Trap catch was reduced significantly compared to controls (attractants without verbenone) at the 6.0 and 30.0 mg/24 hr release rates of verbenone for all levels of attractants and at the 1.2 mg/24 hr release rate at the low level of attractants only (Figure 1). Within all levels of attractants, the mean number of beetles caught by the 6.0- and 30.0-mg rates of verbenone were nearly the same, suggesting that there may be a threshold for high release rates of verbenone above which higher rates do not elicit greater inhibition (Figure 1). Furthermore, the lower release rates of verbenone (i.e., 0.05 and 0.27 mg/ 24 hr) had no significant effect on beetle response compared to the controls

TABLE 1. MEAN DAILY CATCH OF *Dendroctonus brevicomis* ARRIVING AT TRAPS BAITED WITH *exo*-BREVICOMIN, FRONTALIN, AND MYRCENE (EFM), ALONE OR WITH INHIBITORS VERBENONE AND IPSDIENOL, SEPTEMBER 1991[a]

Treatment[b]	N	Mean (SE)[c]
EFM alone (control)	24	144 (26.2)
EFM + verbenone		
0.09 mg/24 hr	23	144 (25.1)
0.18 mg/24 hr	24	108 (23.1)
0.6 mg/24 hr	24	100 (16.9)
1.2 mg/24 hr	23	81 (15.8)
EFM alone (control)	24	158 (24.2)
EFM + ipsdienol		
0.02 mg/24 hr	24	69 (11.8)*
0.05 mg/24 hr	24	65 (9.3)*
0.2 mg/24 hr	24	50 (6.9)*
0.4 mg/24 hr	24	53 (10.9)*
EFM alone (control)	24	107 (14.2)
EFM + verbenone		
0.09 + ipsdienol, 0.02 mg/24 hr	24	45 (5.9)*
0.18 + ipsdienol, 0.05 mg/24 hr	24	41 (6.6)*
0.6 + ipsdienol, 0.2 mg/24 hr	24	10 (1.3)*
1.2 + ipsdienol, 0.4 mg/24 hr	24	7 (1.1)*

[a] Data transformed data by $\log_{10}(x + 1)$; untransformed data presented with means rounded to nearest whole number.
[b] *exo*-brevicomin released at 2.6 mg, frontalin released at 2.4 mg, and myrcene released at 95 mg/24 hr.
[c] Means within an experiment (separated by lines) followed by an asterisk are significantly different from control mean, analysis of variance and Dunnett's procedure, $\alpha = 0.05$.

TABLE 2. ANALYSIS OF VARIANCE OF DAILY CATCH OF *Dendroctonus brevicomis* IN FUNNEL TRAPS RELEASING *exo*-BREVICOMIN, FRONTALIN, AND MYRCENE AT THREE LEVELS ALONE AND WITH VERBENONE RELEASED AT FIVE LEVELS, SEPTEMBER 1992[a]

Source of variation	df	Mean square	F	P
Replications	20	0.867	3.20	0.001
Attractants (factor A)	2	5.079	18.77	<0.001
Error (a)	40	0.271		
Verbenone (factor B)	5	3.456	39.57	<0.001
Attractants × verbenone	10	0.087	0.99	0.448
Error (b)	300	0.087		

[a] Data were transformed by $\log_{10}(x + 1)$ prior to analysis.

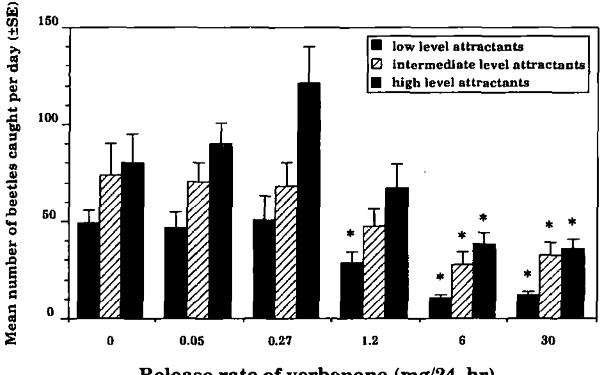

FIG. 1. Response of *D. brevicomis* to funnel traps releasing three levels of attractants (*exo*-brevicomin, frontalin, and myrcene), and five release rates of verbenone or no verbenone (controls). Bars topped by an asterisk indicate verbenone treatment mean is significantly lower than control mean within that level of attractants (Dunnett's procedure, $\alpha = 0.05$). Analyses were performed on transformed data, untransformed means presented.

(Figure 1). At the high level of attractants, the two lowest rates of verbenone appeared to increase beetle response, although the increase was not significant (two-sided Dunnett's procedure, $\alpha = 0.05$) (Figure 1). The proportion of control trap catch caught by each verbenone treatment was not similar for equivalent ratios of attractants to verbenone (Figure 2). There was no clear threshold ratio of attractants to verbenone that resulted in significant reductions in trap catch across all three levels of attractants; however, all treatments releasing a 1:4 or greater ratio caught significantly fewer beetles than controls, and only one lower ratio, 1:1 at the high level of attractants, resulted in a significant reduction in beetle response (absolute release rate of verbenone was 6.0 mg/24 hr) (Figure 2).

DISCUSSION

Our results confirm that when verbenone is released at low rates relative to those of attractants, the number of *D. brevicomis* responding to attractants is not significantly reduced compared to when attractants are released alone. In contrast, extremely low (e.g., 0.02 mg/24 hr) release rates of ipsdienol caused significant reductions in the numbers of beetles trapped. These results suggest that there may be differences between the neurophysiological threshold concentrations of ipsdienol and verbenone perceived by *D. brevicomis*, as has been

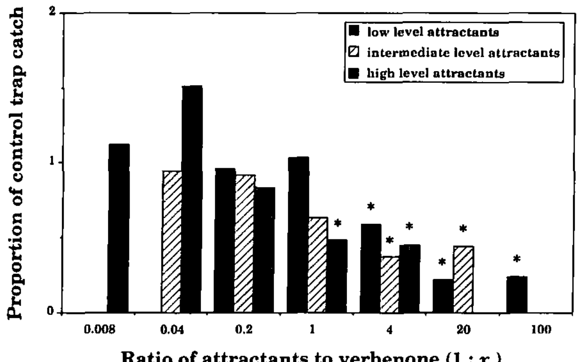

FIG. 2. Comparison of proportion of *D. brevicomis* caught by three release levels of attractants and five release rates of verbenone. Categories are the ratio of attractants (*exo*-brevicomin and frontalin) to verbenone represented by the treatment; proportion = mean number of beetles caught by verbenone treatment/mean number of beetles caught by control. Bars topped by an asterisk indicate verbenone treatment mean is significantly lower than control mean within that level of attractants (Dunnett's procedure, $\alpha = 0.05$). If a bar is not shown for a level of attractants, that particular ratio was not tested at that level.

found in other scolytid species. Male *I. paraconfusus* exhibited significantly greater antennal sensitivity to the (*R*)-(−)- enantiomer of ipsdienol, produced by the sympatric species *I. pini*, than to the (*S*)-(+)- enantiomer, a component of their own aggregation pheromone blend (Light and Birch, 1982). Both sexes of *I. paraconfusus* were inhibited from responding to natural sources of attraction by (*R*)-(−)-ipsdienol or either enantiomer of verbenone, but antennal sensitivity was lower for both enantiomers of verbenone than for either enantiomer of ipsdienol in both sexes (Light and Birch, 1979, 1982; Light, 1983). *Dendroctonus ponderosae* Hopkins exhibited a lower sensitivity to racemic verbenone at the antennal level than to their aggregation pheromones (Whitehead, 1986). A lower sensitivity to verbenone than to aggregation pheromone compounds and the putative allomone ipsdienol in *D. brevicomis* could explain the results obtained in the present study: low release rates of verbenone may not cause behavioral changes, while high sensitivity to allomonal compounds such as ipsdienol results in modification of behavior at very low release rates of the compound. However, because responses to verbenone and ipsdienol have not been studied at the electrophysiological level in *D. brevicomis*, differential response to verbenone and ipsdienol at the central nervous system level cannot be ruled out.

Greater behavioral sensitivity to ipsdienol may have evolved as a result of a greater risk of reduced fitness due to interspecific competition (indicated by

the presence of ipsdienol) than to intraspecific competition (verbenone released from attacked tree in later stages of colonization). Interspecific competition between *I. paraconfusus* and *I. pini* was shown to have a greater effect on larval survival than intraspecific competition within each species (Light et al., 1983), while pheromone-induced attack by *I. pini* on trees naturally attacked by *D. ponderosae* greatly reduced mean progeny production in *D. ponderosae* (Rankin and Borden, 1991).

The extremely low trap catches obtained when verbenone and ipsdienol are released together at release rates ≥ 0.6 and 0.2 mg/24 hr, respectively confirm the results of Paine and Hanlon (1991) and suggest that an additive inhibitory effect may be occurring when these two inhibitors are released together above certain minimum rates. Neither verbenone nor ipsdienol, even when released at high rates relative to attractant release rates, completely eliminate the response of *D. brevicomis* to sources of attraction. This could be due to inherent variation among individuals in a population in response to a given stimulus (Borden et al., 1986), with the result that not all beetles are inhibited by any one compound. A possible explanation, then, for the additive effects of combining verbenone and ipsdienol is that individuals that would not be inhibited by one stimulus are inhibited by the other and vice versa.

There was no clear threshold ratio of attractant blend to verbenone that resulted in significant reductions in trap catch for all three levels of attractants. Instead, our results suggest that the level of behavioral inhibition in *D. brevicomis* is primarily influenced by the absolute release rate of verbenone. Inhibition of beetles of concentrations of verbenone above a certain level may have evolved due to the association of high levels of verbenone with a deteriorated or fully colonized host, and the response is elicited regardless of the level of attractants being perceived. However, our results suggest that at low levels of attractants, a lower absolute rate of verbenone becomes significantly inhibitory. This may simply be due to the lower degree of variation in trap catch associated with lower release rates of attractants, resulting in the mean trap catch reduction being statistically significant. Alternatively, because a lower concentration of aggregation pheromones is less attractive to beetles, a greater proportion may be inhibited by a lower level of verbenone.

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Glycine max SIGNALING OF ENVIRONMENTAL STRESS: DYNAMICS OF INDUCIBLE AROMATIC ALLELOCHEMISTRY

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Abstract—Treatment of Davis soybeans (*Glycine max* (L.) Merrill) with a proven stress elicitor, iodoacetic acid, which binds specifically with sulfhydryl groups, induced a defensive phytochemistry, which included increased concentrations of aromatic compounds. High-performance liquid chromatography resolved groups of peaks, including one that contains the major allelochemical daidzein and several that contained relatively nonpolar compounds. Some peak groups were increased quantitatively, while others were decreased by iodoacetic acid elicitation. The more significant differences in methanol-extractable HPLC-resolved aromatic metabolites between elicited and nonelicited *G. max* apical leaves occurred at 24, 48, and 72 hr after elicitation.

Key Words—Induced resistance, aromatic metabolites, phenylpropanoids, HPLC profiles, allelochemicals, *Glycine max*, soybean, iodoacetic acid.

INTRODUCTION

Environmental stresses can alter phytochemical defenses, i.e., allelochemicals, against insects and microorganisms (Sequeira, 1990; Ebel, 1986). Such stresses, or elicitors, may alter these defenses through changing the redox state of thiol-disulfide receptor protein in the plasma membrane around the plant cell (Neupane and Norris, 1990, 1991a,b, 1992; Liu et al. 1992; Norris, 1994). Some proven chemical elicitors for such a redox-dependent receptor include: iodo-

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acetic acid, L-ascorbic acid, α-tocopherol, *N*-ethylmaleimide, and reduced glutathione (Neupane and Norris, 1990, 1991a,b, 1992; Wingate et al. 1988).

The predominant inducible allelochemicals in *G. max* include flavonoids such as daidzein, coumestrol, genistein, and glyceollins (Hedin and Waage, 1986; Fischer et al., 1990; Sequeira, 1990; Sharma and Norris, 1991). These inducible defensive compounds have proven antixenosic and/or antibiotic effects on several insect herbivores: e.g., the soybean looper, *Pseudoplusia includens* (Walker); cabbage looper, *Trichoplusia ni* (Hübner); Mexican bean beetle, *Epilachna varivestis* Mulsant; velvetbean caterpillar, *Anticarsia gemmatalis* Hübner; corn earworm, *Helicoverpa zea* (Boddie), and tobacco budworm, *H. virescens* (F.) (Hart et al., 1983; Binder and Waiss, 1984; Reynolds and Smith, 1985; Chiang et al., 1986, 1987; Lin et al., 1990; Lin and Kogan, 1990; Neupane and Norris, 1990, 1991a,b; Sharma and Norris, 1991). A mixture of aromatic phytochemicals, rather than a single compound, is involved in the inducible *G. max* resistance to herbivores (Binder and Waiss, 1984; Chiang et al., 1987; Neupane and Norris, 1990; Wheeler and Slansky, 1991).

The Davis cultivar of *G. max* demonstrates a dynamic allelochemical phenylpropanoid response to environmental stresses, which alters the feeding preference of some oligophagous and monophagous insects (Norris et al., 1988). The dynamic Davis defensive chemistry apparently involves mostly quantitative changes (Chiang et al., 1987; Norris et al., 1988; Neupane and Norris, 1990, 1991a,b, 1992). The objective of the present study was to qualify and quantify changes in the aromatic allelochemicals in Davis as inducible by the exoelicitory (i.e., environmentally stressful) sulfhydryl-binding agent, iodoacetic acid (IAA).

METHODS AND MATERIALS

Plants. Thiram-treated seeds of Davis soybean (*G. max*) were germinated in moist, coarse vermiculite at 27°C under a photoperiod of 16L:8D hr in plastic trays (27.5 × 27.5 × 5.5 cm). Thiram (tetramethylthiuram disulfide; Science Products Company, Inc., Chicago, Illinois) was used as a seed protectant to reduce potential pathological stresses in the young plants. Distilled water was applied daily to maintain the moisture of the vermiculite. After seven days, the seedlings had a hypocotyl length of 1–3 cm and were transplanted into a support medium, Jiffy Mix (Jiffy Products of America, West Chicago, Illinois), which is a mixture of shredded sphagnum moss and horticultural-grade (pathogen-free) vermiculite, in 15.5 × 15-cm pots. Plants were grown in the University of Wisconsin-Madison Biotron under the controlled conditions detailed in Table 1.

The light intensity at 100% illumination averaged 695 μE. The light intensity was measured with a Li-Cor Quantum Radiometer Photometer with a quantum probe placed 15 cm above the plant canopy. Relative humidity remained

TABLE 1. DAILY GROWTH CHAMBER CONDITIONS

Time (hr)	Temperature (°C)	Fluorescent lights[a]	Incandescent lights
0400	23	20	20
0420	25	20	40
0440	27	40	70
0500	28	100	100
1700	26	40	70
1720	24	40	40
1740	22	20	20
1800	21	0	0

[a]Percent of maximum light intensity.

at 70%. Half-strength Hoagland's solution (Hoagland and Arnon, 1938) (115 ml) was delivered to each pot every 6 hr at 0600, 1200, 1800, and 2400 hr. The Hoagland's solution was stored in a 200-liter reservoir and was delivered by a pump connected to a timer. The pump operated for 5 min at each delivery.

When the unifoliate leaves were fully expanded and the first trifoliate leaf was beginning to expand, the plants were attached to bamboo stakes. When the plants had grown to the V7 stage (Fehr et al., 1971), the soil was treated with iodoacetic acid (Aldrich Chemical Company, Inc., Milwaukee, Wisconsin) at the rate of 1.0 kg (AI)/ha dissolved in 15 ml of distilled water. The treatment was applied in a small trench located at a 5-cm radius around the plant base. The control plants received 15 ml of distilled water in the trench. The treatment and all daily harvests were conducted at 1230 hr. The leaves were harvested from five different plants of each treatment at each time, i.e., 0, 24, 48, 72, 96, and 120 hr after treatment. Thus, no plant was sampled more than once, so induction from such wounding was not a factor. The leaves were pinched from the plants at the base of the petiole, and they were separated into unifoliate, basal (nodes 1, 2, and 3), apical (nodes 4 and above), and axillary leaves. Immediately after harvest, the leaf tissue was quick-frozen in a −10°C freezer, lyophilized, and then sealed in a darkened glass jar in a desiccator at room temperature. Leaves used for the fresh- and dry-weight analyses were placed in preweighed jars (Kimble 40 × 80-mm), weighed, dried in an oven at 100°C for 36 hr, and reweighed.

HPLC Analyses. Five plants were harvested at each time from each treatment; however, only those three whose weight was closest to the mean weight of the five plants were analyzed, except at 24 hr when four and five plants were used from the treatment and control, respectively. The lyophilized leaf tissue was first ground with a mortar and pestle, and then 0.5 g was homogenized in

40 ml 100% methanol with a Tekmar Tissuemizer (Tekmar Company, Cincinnati, Ohio) for 60 sec at 55% maximum speed. The homogenized tissue–methanol mixture was vacuum filtered using a Buchner funnel and Whatman No. 1 paper. The filtrate was collected and stored at 5°C. The tissue residue was added to 100 ml 100% methanol in an Erlenmeyer flask and was placed on a shaker for 16 hr. Two 16-hr extractions of each residue with methanol were conducted. All methanol filtrates per sample were pooled, reduced to 2 ml by rotoevaporation at 35°C, and stored in a sealed vial at −5°C. Each concentrated extract was filtered through a Gelman Nyaflo 0.45-μm filter, and the volume was adjusted to 7.5 ml by either adding methanol or reducing the volume with a stream of nitrogen gas. Twenty-microliter injections of each extract were analyzed by HPLC using a Beckman Ultrasphere ODS column (4.6 × 250-mm). The elution was with 2% acetic acid–acetonitrile (90:10) for 5 min and then with a linear gradient to 100% acetonitrile in 15 min. The total chromatographic time was 30 min. The elution was monitored with a UV detector at 254 nm and the peak area was integrated with a Spectra Physics 4400 integrator. Each sample's analysis is an average of at least three replicate injections.

All statistical analyses used SAS (SAS Institute, 1985). All analyses to compare means used the estimate statement within the general linear model procedure.

RESULTS

Total Peak Area. HPLC chromatograms of the resolved methanol extractables from the apical leaves of the control (nonelicited) and treated (elicited) plants are shown in Figure 1. The total peak area was only significantly larger (t test, $P \leq 0.05$) in the elicited plants (199,500 $\pm$ 28,600, mean $\pm$ SE) as compared to the nonelicited plants (162,400 $\pm$ 5900) at 48 hr.

Peak Identification. The major allelochemical, daidzein, cochromatographed within peak group 7. When daidzein was added to the plant extractables, peak group 7 significantly increased from an area of 53,183 $\pm$ 3712 to 70,340 $\pm$ 1819 (mean $\pm$ SE, $P \leq 0.05$, t test). Peak 14 was previously identified as the allelochemical coumestrol (Burden and Norris, 1992).

Individual Peak-Group Areas. The resolved area for several individual peak groups was significantly larger in the elicited as compared to the nonelicited plants (Table 2). The area was significantly ($P \leq 0.05$) larger in five (17, 20, 21, 22, and 23) peak groups from elicited plants at 24 hr and in four (2, 4, 20, and 21) peak groups at 72 hr. The quantitatively largest peak group, 7, which contains the proven major allelochemical daidzein, was significantly ($P \leq 0.05$) larger in elicited plants at 48 hr, and peak group 9 was significantly ($P \leq 0.05$) larger in the elicited plants at 96 hr. Peak group 16 was the only group significantly larger in nonelicited plants than in elicited plants at 72 hr.

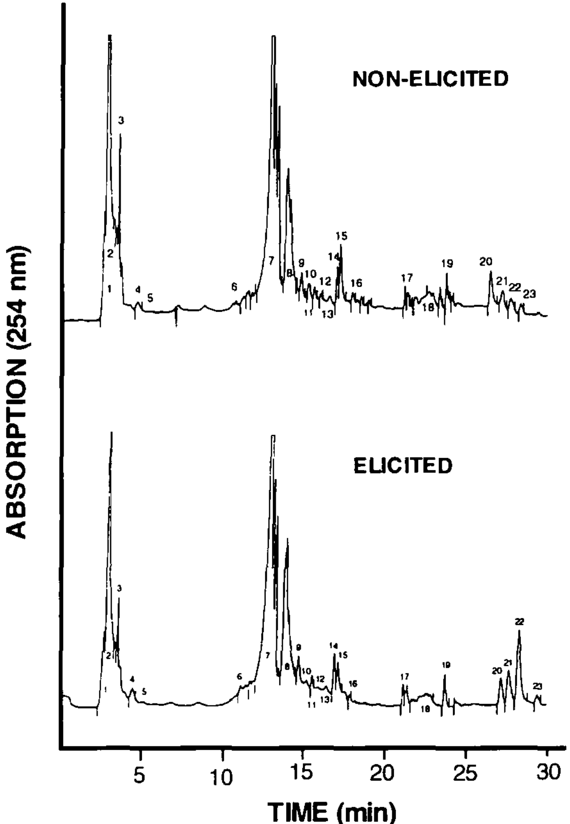

FIG. 1. HPLC chromatograms of the methanol extractables from the apical leaves of elicited and nonelicited Davis *G. max*. A given number refers to the same peak group in both elicited and nonelicited plants.

DISCUSSION

The findings from this study with Davis *G. max* support the interpretation that an increase in HPLC-resolvable secondary aromatic allelochemicals is important for explaining an altered chemical defense in iodoacetic acid-elicited (stressed) plants. Peak group 7, a major contributor to the increase, involves the major antifeedant and antibiotic, daidzein (Keen and Taylor, 1975; Sharma and Norris, 1991). The retention time of peak group 9 was between that of daidzein and coumestrol (14), which suggests that it may be genistein (Porter et al., 1985; Graham, 1991). Genistein is a known allelochemical effective

TABLE 2. PEAK GROUPS AND POSTELICITATION TIMES WITH SIGNIFICANT DIFFERENCES BETWEEN ELICITED AND NONELICITED PLANTS BASED ON RESOLVABLE PEAK AREA ATTRIBUTABLE TO INDICATED PEAK GROUP

	Postelicitation time (hr)			
Peak group[a]	24	48	72	96
2			E	
4			E	
7		E		
9				E
16			NE	
17	E[b]			
20	E		E	
21	E		E	
22	E			
23	E			
Total	5	1	5	2

[a]Only peak groups that were significantly different between the elicited and nonelicited plants are listed ($P \leq 0.05$ General Linear Model, estimate function).
[b]E indicates that the percentage of the total peak area was significantly greater in the elicited plants. NE indicates the same for the nonelicited plants. The absence of E or NE indicates that there was no significant difference between elicted and nonelicited plants.

against some herbivores; thus, it may contribute to plant resistance (Sutherland et al., 1980). Peak groups 20–23 are relatively nonpolar compounds, and seemingly deserve further study as chemicals potentially involved in *G. max* resistance to herbivores. Peak group 14, coumestrol, is a known antixenosic and antibiotic to *E. varivestis* (Burden and Norris, 1992), but its production was not significantly altered by elicitation.

Methanol extractables, as compared to less polar solvent extractables, from *G. max* leaves contain significantly more antiherbivore activity (Chiang et al., 1986; Caballero et al., 1986; Sharma and Norris, 1991). Such extractables include the flavonoids daidzein, glyceollins, and coumestrol, which have antifeedant and antibiotic effects against *T. ni* larvae (Sharma and Norris, 1991). Several of the above flavonoids were resolved in the present study. The overall effects of such allelochemicals may involve synergism (Gunasena et al., 1988; Felton et al., 1989).

The larger numbers of peak groups with significantly larger peak areas in the elicited plants occurred at 24 and 72 hr postelicitation. Twenty-four to 72 hr is also the time span of reported increased production of the enzymes involved in herbivory-stressed *G. max* biosynthesis of aromatic allelochemicals (Chiang et al., 1987). Such enzymes include phenylalanine ammonia lyase (PAL) and

tyrosine ammonia lyase (TAL). In another study, the production of mRNAs for PAL and for chalcone synthase, another key enzyme in flavonoid biosynthesis, was initially detected at 40 hr after elicitation and reached a maximum at approximately 70 hr (Templeton and Lamb, 1988).

The 24- to 72-hr time span after elicitation also correlates with increased antiherbivory in *G. max* tissues that were stressed (elicited). Increased *G. max* antiherbivory to *E. varivestis* was observed at 24 and 48 hr after stress. In other studies, larval *T. ni* antiherbivory was increased at 72 hr by several elicitors, e.g., the sulfhydryl reagents iodoacetic acid and *N*-ethylmaleimide, and was accompanied by significant increases in the allelochemicals daidzein, coumestrol, and glyceollins (Neupane and Norris, 1990, 1991a,b, 1992). Thus, such elicitory sulfhydryl reagents, as environmental stresses, alter the pertinent sulfhydryl/disulfide-dependent redox chemistry in receptor proteins in the plasma membrane around *G. max* cells, and this results in the induction of key enzymes responsible for the production of antixenosic and antibiotic compounds.

In summary, our findings show that iodoacetic acid, as an environmental stress analogous to herbivory (Neupane and Norris, 1990), alters *G. max* biosynthesis of aromatic compounds involved in soybean-inducible resistance to insect herbivory.

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TOLUENE AND WEASEL (2-PROPYLTHIETANE) ODORS SUPPRESS FEEDING IN THE RAT

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Abstract—The odors of toluene and 2-propylthietane have been shown to elicit fast wave bursts of 15–30 Hz in the olfactory bulb and dentate gyrus of rats. The odors of cadaverine, butyric acid, and caproic acid were found to be ineffective by comparison. The present study investigated feeding in rats offered a choice between food pellets treated with one of the above mentioned odors or untreated pellets. Unscented pellets were also presented in a control condition. The results indicate that 2-propylthietane or toluene scented food is avoided; cadaverine scented food is preferred; but caproic acid and butyric acid scents have no effect. Toluene and 2-propylthietane may activate central pathways involved in predator detection/avoidance, while cadaverine may activate pathways involved in approach and feeding behavior.

Key Words—Predator odor, feeding, toluene, 2-propylthietane, cadaverine, butyric acid, caproic acid, weasel, rat, olfaction.

INTRODUCTION

Rats exposed to components of weasel (*Mustela erminea*) odor (2-propylthietane), red fox (*Vulpus vulpus*) odor (trimethylthiazoline), or to toluene vapors exhibit fast wave oscillations (15–30 Hz) in both the olfactory bulb and the hilus of the dentate gyrus (Heale et al., 1994). Cadaverine, caproic acid, and butyric acid (components of the odors of rotting flesh, goat and rancid butter respectively), as well as a number of other odors that are strong and offensive to humans do not elicit fast waves in the rat dentate gyrus, although they may

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sometimes elicit an olfactory bulb response (Heale et al., 1994). It has been proposed that the dentate fast wave response may be associated with predator detection mechanisms (Heale et al., 1994) and that toluene may mimic the effects of naturally occurring predator odors.

Work with voles, rats, mountain beavers, and pocket gophers indicates that predator odors elicit avoidance and a decrease in consumption of scented food (Berdoy and MacDonald, 1991; Calder and Gorman, 1991; Epple et al., 1993; Sullivan et al., 1988, 1990a,b; Vernet-Maury et al., 1992). Conversely, it has been shown that food pellets scented with cadaverine are both hoarded and eaten at a greater frequency by rats than similar untreated food pellets (Montoya et al., 1981), while food scented with butyric acid or nonpredator urine was eaten indiscriminantly by mountain beavers (Epple et al., 1993). The effects of toluene odor on feeding do not appear to have been studied in any mammalian species, and the effects of 2-propylthietane, butyric acid, and caproic acid have not been assessed in rats. The present study will assess the effect of these compounds as well as cadaverine on feeding in rats.

METHODS AND MATERIALS

Subjects. The subjects consisted of 18 male Long Evans rats weighing between 250 and 300 g at experimental onset. When not being tested, they were individually housed in wire mesh cages with access to ad libitum food and water and a 12-hr on/off light cycle.

Apparatus. The test apparatus consisted of a plywood alley coated with a waterproof varnish and measuring 125 cm length × 23 cm width × 20.4 cm height. A rat's living cage (26 × 18 × 26 cm) was placed at one end of the alley and a hole permitted access to it. The cage was covered with an opaque material and an attached bottle allowed continuous access to water. Nine rats were tested simultaneously in nine separate alleys.

Procedure. All rats received a 23-hr habituation period in individual alleys before testing. Trials were always begun in the afternoon and terminated 23 hr later, after which cages and alleys were cleaned with hot water. The food objects used were Purina rat food pellets, each approximately 2 cm long and weighing 5 g. In the odor tests, 15 normal Purina rat chow pellets and five pellets treated with an odorous compound were randomly placed 60–125 cm away from the rat living cage. Pellets were treated by applying an odorous liquid to the upper surface with a Q-tip (a wooden stick with cotton wrapped tightly around one end). This method is not quantitative; one can only distinguish treated from untreated pellets. Treated pellets were marked by filing the under surface so that they could be identified by the experimenter.

In scoring the results, half eaten or missing pellets were considered eaten.

As the rats were not continually watched, it was often not possible to determine whether eaten pellets had been hoarded or merely eaten in the alley. The measurements taken were the percentage of pellets eaten out of the total available of each type (i.e., five treated or 15 untreated) and the total amount of all available food consumed out of 20 during each treatment condition. This procedure is similar to one previously used by Montoya et al. (1981). A control condition provided data on baseline levels of consumption for rats offered 20 untreated pellets (five marked by filing the under surface and 15 unmarked). Testing was conducted using five different odorous compounds: cadaverine, toluene, butyric acid, caproic acid, and 2-propylthietane, which were tested in this order for one group ($N = 9$) and in the reverse order for the second group ($N = 9$). The odors of all five liquids are mildly or markedly unpleasant for humans. Cadaverine is a component of the odor of rotting flesh, toluene is an aromatic organic solvent produced from the distillation of tar oil, caproic acid is a component of goat odor, butyric acid is a component of the odor of rancid butter (Morrison and Boyd, 1979), and 2-propylthietane is the main component of weasel scent (Crump, 1980). Since all these compounds are liquids, no additional solvents were needed. Once rats were tested with one odor, they were returned to the rat colony room and not tested again for at least 24 hr.

Statistical Analysis. Results were reported in terms of mean percentages and standard error values of the number of food pellets eaten. Statistical analysis was conducted using SPSSPC computer software (Norusis, 1986). To determine if there was a difference between the percentage of untreated and treated pellets eaten, within-design multivariate analysis of variance (MANOVA) tests were performed, along with Tukey post hoc tests, on scores of each treatment condition. To determine if the order of odor presentation had an effect, an analysis of variance (ANOVA) was used to compare the consumption scores of each group of nine rats. To assess the differences between the total amount of available food consumed during each odor and control condition, within-subject MANOVA tests were performed.

RESULTS AND DISCUSSION

Table 1 shows the percentage of total available pellets consumed during each odor test condition. There was a significant difference from control values on this measure during 2-propylthietane [$F\ (1, 17) = 20.3$; $P < 0.0001$], toluene [$F\ (1, 17) = 22.5$; $P < 0.0001$] and cadaverine [$F\ (1, 17) = 5.11$; $P < 0.05$] test sessions. Both 2-propylthietane and toluene resulted in a significant decrease in the overall amount of available food consumed relative to control values, while cadaverine resulted in a significant increase in the total amount of available food consumed compared to controls. There was no significant ($P >$

TABLE 1. PERCENTAGE OF ODOR-TREATED AND UNTREATED FOOD PELLETS EATEN BY RATS IN 23-HOUR TEST SESSIONS

	Odor trials					
Pellets eaten (%)	Control	Weasel	Toluene	Cadaver	Butyric acid	Caproic acid
Untreated	35.8 ±1.5	29.9 ±1.7	31.7 ±1.9	32.7 ±1.9	30.7 ±2.3	35.4 ±2.5
Treated	35.6 ±4.1	16.6b,e ±4.9	15.5b,e ±3.4	58.9b,d ±5.4	37.8 ±7.0	26.7 ±6.9
Total	35.6 ±1.2	26.4[a] ±1.1	28.3[a] ±1.3	40.8c ±1.9	32.5 ±1.1	33.3 ±1.3

[a]Data are presented as means ± standard errors; significantly different from the respective control value: a, $P < 0.001$; b, $P < 0.01$; c, $P < 0.05$; significantly different from untreated pellet consumption: d, $P < 0.01$; e, $P < 0.05$. All rats were tested under all conditions; odor condition was counterbalanced. $N = 18$.

0.05) change in the total amount of available food consumed during caproic acid and butyric acid test sessions relative to controls.

Table 1 also displays the mean percentage of odor-treated and untreated pellets eaten by rats during each test condition. The percentage of treated versus untreated pellets consumed differed significantly [$F(5, 85) = 5.01$; $P < 0.0001$] within odor test trials. Tukey post hoc tests showed this to be true for treatment conditions involving toluene ($P < 0.05$), 2-propylthietane ($P < 0.05$), and cadaverine ($P < 0.01$). There were fewer treated than untreated pellets consumed during toluene and 2-propylthietane test sessions, while more treated pellets were consumed relative to untreated pellets during cadaverine test sessions. The percentage of untreated and treated pellets consumed was not different for caproic acid, butyric acid, or control test sessions (Tukey, $P > 0.05$).

The percentage of odor-treated pellets eaten during each odor condition was also compared to the percentage of marked pellets eaten during control trials. Both 2-propylthietane and toluene compounds elicited decreased consumption of odor-treated pellets relative to control values (Tukey, $P < 0.01$). Cadaverine elicited a significant increase in consumption of treated pellets relative to controls (Tukey, $P < 0.01$). The consumption of treated pellets during caproic acid and butyric acid test conditions did not differ from control values (Tukey, $P > 0.05$). There was no significant difference in the percentage of untreated pellets consumed relative to control values for any of the odors tested (Tukey, $P > 0.05$). Furthermore, comparisons between the two groups of nine rats showed that the order of odor testing did not affect the percentage of pellets

consumed ($P > 0.05$). This demonstrates that any odor that may have lingered in the apparatus after one test did not influence the results of a subsequent test.

A principal finding of this study was that both 2-propylthietane and toluene depress feeding in rats, affecting mainly the consumption of scented food pellets but not of nearby unscented food pellets. This is consistent with previous work showing that food tainted with predator odor is avoided by a variety of prey species (Calder and Gorman, 1991; Epple et al., 1993; Sullivan et al., 1988, 1990a,b; Vernet-Maury et al., 1984, 1992). Furthermore, the fact that toluene had the same behavioral effect as 2-propylthietane, together with the observation that both compounds elicit fast wave oscillatory bursts in the dentate gyrus (Heale et al., 1994), suggests that the odors of both compounds activate similar brain mechanisms. Toluene may activate predator-detection circuitry that evolved in rats as a result of predation by weasels and foxes (King, 1989; Weldon, 1990). It remains to be determined how toluene and predator odors elicit similar electrophysiological and behavioral responses. Both may act upon the same set of olfactory receptors or, alternatively, different receptors but a common central pathway. The avoidance reaction of the rats to an artificial stimulus such as toluene may be analogous to the pecking reaction elicited in newly hatched herring gull chicks by a thin red stick with three white rings painted near the end (Tinbergen, 1953).

It is worth noting that a number of aversive sensory stimuli (ammonia vapor, tail clamping, tastes of acetic acid and quinine, loud noises) do not elicit dentate gyrus fast waves (Heale et al., 1994; Heale and Vanderwolf, 1994; Vanderwolf, 1992). Therefore, the effect is not simply related to aversion in a general sense but appears to be specific to noxious olfactory stimuli. Cadaverine, butyric acid, and caproic acid, while appearing noxious to humans are apparently not aversive to rats. These odors also do not elicit fast waves in the rat dentate gyrus (Heale et al., 1994). In fact, cadaverine was found to increase feeding in rats, in confirmation of a previous finding by Montoya et al. (1981). This may relate to the fact that rats are often scavengers and may be naturally attracted to the odor of decaying flesh (Cahalane, 1961). Finally, novelty-induced reductions in feeding (Garbe et al., 1993) can be ruled out in the present study since all odors were novel to the rats tested, yet not all reduced feeding.

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EFFECTS OF HOST PLANT, *Gossypium hirsutum* L., ON SEXUAL ATTRACTION OF CABBAGE LOOPER MOTHS, *Trichoplusia ni* (HÜBNER) (LEPIDOPTERA: NOCTUIDAE)

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Abstract—Unmated female or male cabbage looper moths, *Trichoplusia ni* (Hübner), were attracted more often in a flight tunnel to a cage with moths of the opposite sex and a bouquet of cotton foliage. Increased sexual attractiveness of females with plants may be a result of stimulation of pheromone release in response to plant odor, since more males were attracted when odor of cotton foliage was passed over females than when odor of females was passed over cotton foliage before venting into the flight tunnel. Increased sexual attractiveness of males with plants is due in part to host odor enhancement of female attraction to male pheromone, since more females were attracted to synthetic male pheromone (a blend of enantiomers of linalool and isomers of cresol) and a cotton leaf extract than were attracted to male pheromone alone. A short synthesis procedure was developed for (*S*)-(+)-linalool, the major component of the male sex pheromone, isolated from hair pencils, used in these tests.

Key Words—Cabbage looper, *Trichoplusia ni*, Lepidoptera, Noctuidae, pheromone, kairomone, attractants, host finding, (*S*)-(+)-linalool.

INTRODUCTION

Few species of Lepidoptera are known to possess mate-finding behavior involving host-plant influences on sexual attractiveness. Males of several species of

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nymphalid butterflies and arctiid moths use plant compounds as pheromone precursors (Conner et al., 1981; Eisner and Meinwald, 1987; Schneider et al., 1981), and in a few other species of moths, host-plant chemicals stimulate pheromone biosynthesis and influence pheromonal calling (see review by McNeil and Delisle, 1989a). Hendrikse and Vos-Bunnemeyer (1987) demonstrated host-plant stimulation of pheromone release in females of two *Yponomeuta* species and presented data suggesting that male attraction to females of these two species is enhanced by the presence of host odor. Females of the sunflower moth, *Homeosoma electellum* (Hulst) are stimulated to release sex pheromone by the presence of host pollen (McNeil and Delisle, 1989b). Raina (1988) demonstrated stimulation of pheromone synthesis and calling in female *Helicoverpa phloxiphaga* (Grote and Robinson) by the presence of a host plant (Texas paintbrush, *Castilleja indivisa* Engelmann) and later demonstrated a similar effect in female *Helicoverpa zea* (Boddie) exposed to ears of corn, *Zea mays* L. (Raina et al., 1992).

The cabbage looper, *Trichoplusia ni* (Hübner), possesses both female and male sex attractant pheromones comprised of different multiple component chemical blends (Bjostad et al., 1984; Landolt and Health, 1990). Although there are no published indications of host-plant effects on female sexual attractiveness in this species, it has been demonstrated that males are more attractive to females when in the presence of host-plant foliage (Landolt and Heath, 1990). Furthermore, males released increased quantities of sex pheromone [(*S*)-(+)-linalool, *p*-cresol, and *m*-cresol] when in the presence of the female major pheromone component (*Z*)-7-dodecenyl acetate and when in the presence of a combination of female pheromone and cabbage odor, but not when in the presence of cabbage odor alone (Landolt and Heath, 1990; Heath et al., 1992). It is not clear why males with host plants were more attractive to females.

We report here results of a series of experiments that show host-plant odor effects on the sexual attractiveness and sexual responsiveness of male and female cabbage loopers, using cut foliage and solvent washings of cotton, *Gossypium hirsutum* L, a principal host of *T. ni* over part of its range (Vail et al., 1989). The main objective was to determine if host-plant chemicals stimulate pheromone release in either sex of the cabbage looper and if host-plant odors enhance sexual attraction to the pheromones of male or female cabbage looper moths.

We also report the details of a new, short, and high-yielding synthesis of (*S*)-(+)-linalool (D-linalool), the principal component of the male sex pheromone of the cabbage looper (Landolt and Heath 1990; Heath et al., 1992), used in one of these experiments. A multistep and low-yielding synthesis of (*S*)-(+)-linalool was recently reported (Ohwa et al., 1986), and several syntheses of enantiomerically enriched (*S*)-(+)-linalool have been sketchily reported in communications (Hanson and Sharpless, 1986; Gao et al., 1987). A new synthetic

method was thus devised to produce more efficiently enantiomerically pure (*S*)-(+)-linalool for sex attraction tests.

METHODS AND MATERIALS

Cabbage loopers were reared on a pinto bean diet using methods described by Guy et al. (1985). Pupae were sorted by sex and placed in separate screened cages (30 × 30 × 30 cm) for adult emergence. Pupae were moved to new cages daily to provide emerged adults of known age groups. Males and females were held in separate environmental chambers. Chamber conditions were 24 ± 0.5°C, 70 ± 5% relative humidity, and a 14L:10D photoperiod, with lights off at 0730 hr and lights on at 1730 hr EST. Males and unmated females used in bioassays were four to six days old. Mated females were obtained by setting up cages (45 × 45 × 45 cm) of 25 two-day-old females and 30 three- to six-day-old males at the end of the scotophase, as described in Landolt (1993). Males and females were separated 24 hr later and females were tested the following scotophase when 4 days old. Cages were supplied with cups of a 3:1 mixture of honey and sucrose, applied as a 20% solution on sterilized cotton balls.

Cotton plants were grown from seed (Germaine 510 variety) planted in pots of heat-sterilized commercial potting soil in a greenhouse. Plants were used in bioassays when 30–35 cm tall, 4–6 weeks old, and before onset of flower buds. Plants were fertilized with Peters Professional Plant Food (20-20-20) (Grace Sierra Hort. Products, Milpitas, California) every 10 days.

The flight tunnel used for all bioassays was a Plexiglas box (1 × 1 × 2 m), housed inside an environmental chamber, with air from inside the chamber (at ambient conditions) pulled through the tunnel and vented to the outside. Airspeed inside the tunnel was 0.2 m/sec. Laminar flow of air inside the tunnel and a symmetrical plume geometry were verified visually using a smoke plume generated by a ventilation smoke tube kit (Mine Safety Appliances Co., Pittsburgh, Pennsylvania). Observations were made of moth behavior in the flight tunnel with the aid of an overhead fluorescent lamp with a red filter and with light from a red incandescent lamp reflected off the chamber walls. Moths and plants were placed in the flight tunnel chamber 1–1.5 hr before the onset of experiments. Chamber conditions were 23 ± 1°C and 70 ± 10% relative humidity.

Moths were tested individually for all attraction bioassays. Each moth was placed in a 20-ml polystyrene vial with a screened bottom and open top, which was hung horizontally near the center of the downwind end of the tunnel. The activities of the moth were observed for 2 min following release, and it was scored for zigzagging upwind flights downwind of the odor source and for

contact with the odor source following such flights. The combination of such upwind flights leading to contact with the cage or dispenser is hereafter referred to as "attraction to contact." Moths were used once and discarded.

Experimental Protocol

Four sets of experiments were conducted to evaluate the effects of cotton foliage and cotton chemicals on cabbage looper sexual attractiveness. These were: (1) an assessment of the temporal pattern through the scotophase of attraction of either sex to the opposite sex held with cotton foliage, (2) a direct comparison of the attractiveness of moths, cotton foliage, and moths on cotton foliage (conducted for both sexes), (3) determination of the effects of cotton volatiles (in an airstream) on the attractiveness of either males or females, and (4) determination of the effects of a solvent extract of cotton foliage on the attractiveness of sex pheromone extracted from pheromone glands.

Temporal Pattern of Attractiveness of Moths and Cotton. Previous experiments demonstrated a poor correlation between the periodicity of cabbage looper activities outdoors versus inside an environmentally controlled chamber (Lenczewski and Landolt, 1991). Thus, we documented attraction of male and female cabbage looper moths to the opposite sex with and without a bouquet of cotton foliage hourly during the scotophase. The temporal patterns of response obtained were used to select the times for subsequent experiments. This experiment was conducted for unmated female attraction to males and for male attraction to unmated females.

During each hour of the scotophase, five unmated female moths were tested, one at a time, for attraction to a cage of 15 male moths placed at the upwind end of the flight tunnel. On alternate days, a bouquet of cotton foliage (two cut plants placed in a flask of water) was included in the cage of males. This was conducted over five days for males without the foliage and over five days for males with the foliage, with each day's test considered a replicate.

The same experimental design was used to identify the pattern of male attraction to females with and without cotton foliage. However, only five females were placed in the cage. This test was also replicated over five days without the cotton foliage and over five days with the cotton foliage.

Attractiveness of Moths versus Cotton Foliage and Combination of Moths and Foliage. Results of the preceding experiment indicated that moths (either sex) in a cage with cotton foliage were more attractive to the opposite sex than were moths without cotton foliage. A more direct comparison of the attractiveness of these treatments was conducted to determine if indeed moths on cotton are more attractive than moths not on plants.

The comparison of moths, cotton foliage, and moths with cotton foliage was first conducted using unmated female cabbage looper attraction to males. For this experiment, a cage of 15 male moths was placed at the upwind end of the flight tunnel and 10 females were tested for attraction to the cage of males. The cage of males was then replaced with a cage containing a bouquet of cotton foliage, and 10 more females were tested for attraction to the cage of cotton foliage. The original 15 males were then placed in the cage of cotton foliage and a third set of 10 females was tested for attraction responses to the cage containing both cotton foliage and male cabbage loopers. This comparison was replicated five times. The experiment was conducted during the second and third hours of the scotophase. A second experiment was subsequently conducted using the same design but with mated females tested for attraction to cages of 15 males, cotton foliage, or a combination of the two. This also was replicated five times over five days. A third experiment was conducted testing for attraction of males to five unmated females, cotton foliage or a combination of the two. This third experiment was conducted during the third and fourth hours of the scotophase.

Mean response percentages for different treatments within each treatment comparison were evaluated for significant differences using Student's t test.

Effects of Cotton Volatiles on Attractiveness of Cabbage Loopers. Because increased attraction to a combination of moths and plant foliage might be due to plant kairomonal stimulation of pheromone release or synergism of sexual attraction by the presence of plant kairomones, an experiment was conducted to test for evidence of greater pheromone release in the presence of cotton foliage volatiles.

Moths were tested for attraction in response to an airstream passed through: (1) a chamber holding moths of the opposite sex, (2) a chamber holding cut cotton foliage, (3) two chambers in series with the first holding moths of the opposite sex followed by cotton foliage, and (4) two chambers in series with the first holding cotton foliage followed by moths of the opposite sex. Two 4.6-liter glass jars served as holding chambers for moths and plant foliage. Airflow of 1 liter/min was provided by a small aquarium pump. Air was purified by passage through a 10-cm × 1-cm column of activated charcoal and humidified by passage through a gas diffusion bottle before entering the chambers holding moths or cotton foliage. Airflow exiting the chambers entered the flight tunnel near the center of the upwind end through a 0.6-cm-ID vertical stainless steel pipe with a 90° elbow fitting to direct the effluent airstream downwind. Flexible Teflon tubing (0.6 cm ID) was used to connect chambers to the steel pipe and to each other.

This two-chamber airflow design was used to test both unmated female attraction to males and male attraction to unmated females. In the first of these experiments, six males were placed in one chamber and cut cotton foliage was

placed in the second chamber. A series of five females was tested for attraction to the airflow passed over the chamber of males, followed by a series of five females tested for attraction responses to the airflow passed over the cotton foliage. The two chambers were then connected in series, with the metered, purified, and humidified air first passing through the chamber of males, then the chamber of cotton foliage, before being vented into the flight tunnel. Five females were tested to the airstream over this combination before the chambers were reversed and another five females were tested to the airstream from over first, the cotton foliage and then the males. This test was replicated five times over five days ($N = 125$). The same experimental design was used to test for attraction of males to five females and cotton foliage. This test was also replicated five times over five days ($N = 125$).

Percentage response rates for different treatments were subjected to ANOVA and significant differences among means were determined using Duncan's new multiple-range test (Steel and Torrie, 1960).

Effect of Cotton Leaf Extract on Attractiveness of Cabbage Looper Pheromones. To determine if increased attraction to combinations of moths and plant foliage is due in part to a greater attractiveness of the combined blends of plant odorants and pheromones (possible synergism), combinations of dosages of cotton leaf extract and pheromone were tested in the flight tunnel bioassay.

Six release rates of male pheromone were evaluated: 0, 7.5, 15, 30, 60, and 150 ng of pheromone per hour (estimated to be 0, 0.25, 0.5, 1.0, 2.0, and 5.0 male equivalents). The pheromone blend consisted of a 97.5 : 2.5 ratio of (*S*)-(+)- to (*R*)-(−)-linalool and a 1 : 1 ratio of para- to metacresol. The cresols were obtained from Aldrich Chemical Co. (Milwaukee, Wisconsin). The 97.54% (*S*)-(+)-linalool was synthesized as described below. The linalools and cresols were formulated in capillary tubes, with release rates (and a ratio of 9 : 1 of linalools to cresols) obtained with predetermined tubing inner diameters and air column heights (Heath et al., 1992). On five different days, five females were tested for attraction to pheromone at each of the six release rates, beginning with the lowest release rate. On another five days, the same series was evaluated, but with the addition of a 0.4-gram-equivalent dosage of a hexane extract of cotton leaves applied to a 5.5-cm-diam. filter paper. The treated filter paper was hung with a paper clip from a ring stand ca. 0.5 cm behind the capillary formulation of male pheromone. The filter paper was replaced with a freshly treated one with each change in male pheromone release rate. Series of male pheromone release rates were evaluated with and without cotton leaf extract on alternate days. Mean percentage response rates for female attraction to male pheromone with and without the cotton extract were compared by Student's t test for significant differences for each male pheromone dosage.

Six dosages of female pheromone were evaluated with and without cotton leaf extract, using the same design as the previous experiment. An extract was

made of female pheromone glands by cutting the abdominal tips of 30 females during the sixth hour of the scotophase and soaking them in 300 μl hexane for 30 min. This extract was tested in the flight-tunnel bioassay at dosages of 0, 0.01, 0.03, 0.1, 0.3, and 1.0 female equivalents, each in a solution of 100 μl hexane applied to a 5.5-cm filter paper disk. The dosage series was conducted either with a 0.4-gram-equivalent dosage of cotton leaf extract or without, on alternate days. Five moths were tested for responses to each different application on a filter paper so that 30 moths were tested per series (6 dosages × 5 moths tested per dosage). Each series was replicated five times, on five different days, providing a total of 125 moths tested per treatment. The cotton leaf extract applied to a filter paper was placed directly behind the female cabbage looper pheromone extract on filter paper when the two were tested simultaneously in the flight tunnel.

Analysis and Synthesis of (S)-(+)-*Linalool*

Enantiomerically enriched (*S*)-(+)-linalool was synthesized in three steps from geraniol, in 61% yield overall (Scheme 1). Thus, geraniol **1** was regio- and stereoselectively epoxidized with the catalytic modification of the Sharpless epoxidation (Hansen and Sharpless, 1986; Gao et al., 1987), using (+)-diethyl tartrate to form the chiral catalyst. (2*S*, 3*S*)-Epoxygeraniol **2** was obtained in 89% yield. Epoxyalcohol **2** was converted to (2*R*, 3*S*)-epoxybromide **3** in 84% yield by treatment with CBr_4 and triphenylphosphine. Bromide **3** was then cleanly converted in 82% yield to (*S*)-(+)-linalool **4** by treatment with butyllithium (Nicolau et al., 1984).

Proton NMR spectra were recorded on a GE-300 NMR at 300 MHz. GC-FTIR spectra were recorded with a Nicolet 20SXC spectrometer. Unit resolution

OH

1

O H OH

(2S,3S)-2

O Br

(2R,3S)-3

OH

(3S)-4

SCHEME 1.

mass spectra were taken with a Hewlett-Packard 5970 mass selective detector interfaced to a H.-P. 5890 GC. An Ultra-2 column was used (20 m × 0.2 mm ID, H.-P.). Flash chromatography was run with 230–400 mesh silica gel (Aldrich Chemical Co.).

The four-step procedure used for synthesizing (*S*)-(+)-linalool (Scheme 1) follows. A dry 500-ml three-neck flask was charged with 240 ml CH_2Cl_2 (dried over a 3 Å molecular sieve) and 6 g powdered 3 Å molecular sieve. The slurry was cooled to −20°C, and 2.67 ml of titanium(IV) isopropoxide (9 mmol), 2.18 ml (+)-diethyl tartrate (12.6 mmol), and 49.8 ml 3 M *t*-butylhydroperoxide (149 mmol) in isooctane were added sequentially with stirring. The mixture was stirred 30 min at −20°C, then cooled to −30°C, and geraniol 1 (11.55 g, 75 mmol) was added dropwise. The mixture was maintained at < −25°C until the reaction was complete (~45 min). The mixture was then warmed to 0°C, 60 ml ice water was added, followed by stirring for 40 min, addition of aqueous NaOH (10 ml of 30% solution saturated with NaCl), and further stirring for 30 min. The organic phase was separated, washed with brine, dried, and concentrated. The slightly milky residue was flash chromatographed on silica (30% EtOAc in hexane), yielding (2*S*, 3*S*)-2-hydroxymethyl-3-(4-methyl-3-pentenyl)-oxirane **2** (11.3 g, 89%), $[\alpha]_{25}^{D}$ = −5.3° (lit. −5.3°; Hanson and Sharpless, 1986, Gao et al, 1987). NMR, IR, and mass spectra matched those previously reported (Gao et al., 1987).

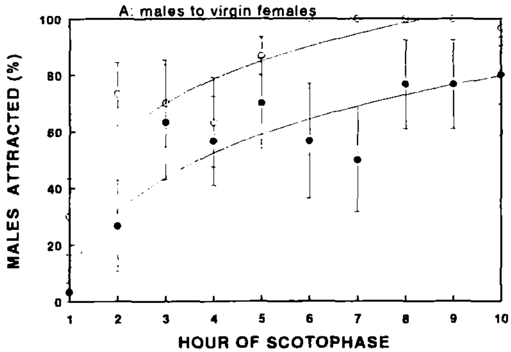
Triphenylphosphine (15.7 g, 60 mmol) was added in portions to a stirred ice-cold solution of (2*S*, 3*S*)-**2** (9.35 g, 55 mmol) and CBr_4 (19.9 g, 60 mmol) in 125 ml CH_2C_{12}. The mixture was warmed to room temperature and concentrated on a rotary evaporator. The resulting brown oil was triturated with hexane (200 ml, 2 × 100 ml), and the combined hexane solutions were stored overnight at −20°C to precipitate most of the triphenylphosphine oxide. The mixture was filtered and concentrated, and the residue was flash chromatographed (5% ether in hexane), yielding 10.82 g of (2*R*, 3*S*)-2-bromomethyl-3-(4-methyl-3-pentenyl)-oxirane **3**. (84%), $[\alpha]_{25}^{D}$ = +20.5°. Spectra were identical to those previously reported (Gash et al., 1989).

Butyl lithium (40 ml of a 2.5 M hexanes solution, 100 mmol) was added dropwise to a cooled (−25°C) solution of epoxygeranyl bromide **3** (9.32 g, 40 mmol) in dry THF (250 ml). The solution was stirred for 30 min, then warmed to 0°C, and quenched with saturated aqueous NH_4Cl, and the mixture was extracted three times with hexane. The hexane extracts were washed with brine, dried (anhydrous Na_2SO_4), and concentrated. The residue was flash chromatographed in two portions (column 5 × 24 cm), eluting with 12% EtOAc in hexane, yielding 5.1 g (83%) of (3*S*)-3, 7-Dimethyl-1,6-octadien-3-ol [(*S*)-(+)-Linalool]. The purified product was Kugelrohr distilled (oven temp 110°C, 12 mm Hg) to remove traces of silica gel. $[\alpha]_{25}^{D}$ = +18.2° (c = 0.43, $CHCl_3$). Mass, NMR, and IR spectra were identical to those of racemic linalool (Fluka

Chemical Co.). The enantiomeric composition of the final product used in bioassays, determined by capillary gas chromatographic analysis, using methods of Heath et al. (1992), was 97.5% (*S*)-(+)- and 2.5% (*R*)-(−)-linalool.

RESULTS

Unmated female cabbage loopers placed in a cage in the flight tunnel generally were more attractive to males later in the scotophase (Figure 1A). At each hour of the scotophase, the percentages of males attracted to and contacting



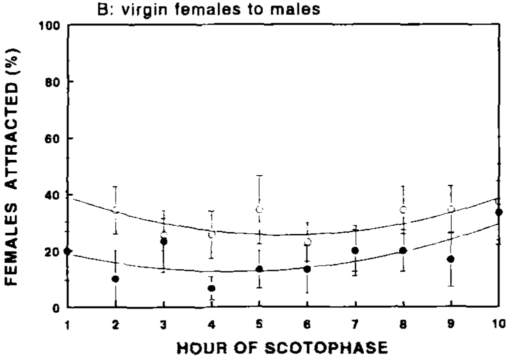
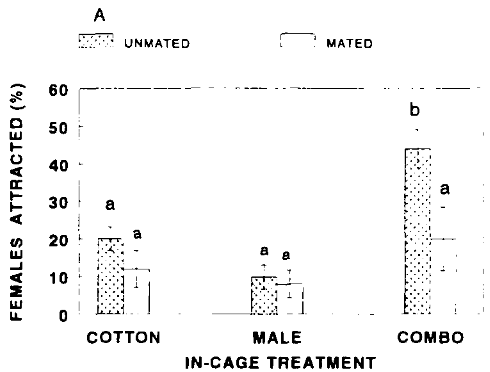


FIG. 1. Percentages ($\overline{X} \pm$ SEM) of moths tested exhibiting attraction to and contact with a cage containing moths of the opposite sex, either with (open circles) or without (solid circles) a bouquet of cotton foliage in the cage and at different hours of the scotophase. (A): Males were tested to a cage of five virgin females, and (B) virgin females were tested to a cage of 15 males.

the cage were higher when the cage contained both females and a bouquet of cotton foliage. The temporal pattern of female attraction to males was undefined (Figure 1B). The percentages of females attracted to males in cages with cotton also were higher than the percentages of females attracted to and contacting cages of males without cotton (Figure 1B).

Significantly greater percentages of unmated females were attracted to and contacted a cage containing 15 males and a bouquet of cotton foliage than a cage with 15 males alone ($T = 4.54$, $df = 4$, $P = 0.01$) or a cage with cotton



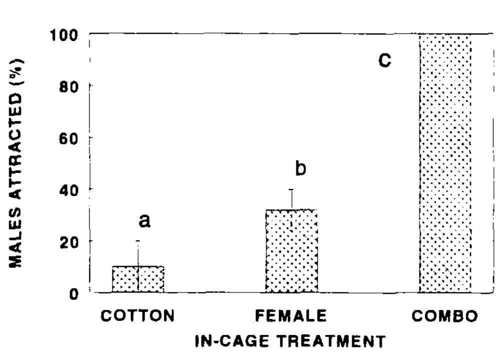


FIG. 2. Percentages ($\bar{X} \pm$ SEM) of moths tested exhibiting attraction and contact with cages containing the opposite sex, a bouquet of cotton foliage, or a combination of moths of the opposite sex and a bouquet of cotton foliage. Part A is for unmated and mated female attraction to males and part B is for male attraction to unmated females. Percentages within a set labeled with the same letter are not significantly different at $P = 0.05$ by Student's t test.

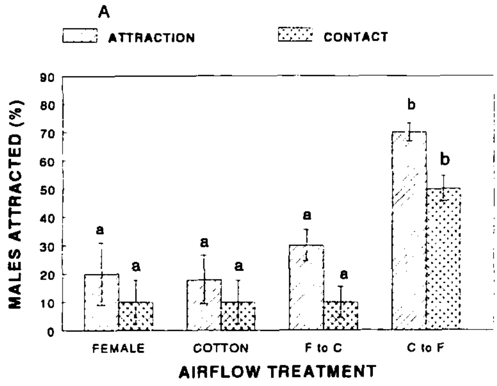
foliage alone ($T = 4.71$, $df = 4$, $P = 0.009$) (Figure 2A). Percentage of unmated females attracted to and contacting the cage of males (10%) was not significantly different than the percentages attracted to and contacting the cage of cotton foliage (20%) ($T = 2.23$, $df = 4$, $P = 0.09$). There were no significant differences among percentages of mated females attracted to males, cotton foliage, or both males and cotton foliage together in a cage (Figure 2A). Male cabbage loopers were attracted significantly more often to females placed in a cage with cotton (100%) compared to females alone (32%) ($T = 8.5$, $df = 4$, $P = 0.001$) or cotton foliage alone (10%) ($T = 9$, $df = 4$, $P > 0.001$) (Figure 2B).

Male cabbage loopers were attracted to and contacted the effluent pipe in the flight tunnel significantly more often when the vented airflow had passed first over cotton foliage and then over the chamber of five females (50%), compared to airflow over females only (10%), cotton foliage only (10%), or over first females and then cotton foliage (10%) (Figure 3A). There were no other significant differences among those four treatments. Female cabbage loopers did not respond to the airflow passed first over cotton foliage and then over a chamber of six males (Figure 3B), and there were no significant differences among the mean percentages of females attracted in response to airflow passed over males, over cotton foliage, or over both males and cotton foliage (Figure 3B).

Female cabbage loopers were attracted significantly more to synthetic male cabbage looper pheromone in combination with cotton leaf extract at 0.4 gram equivalents, compared to male pheromone alone, at all release rates tested (Figure 4A). Males, however, were not attracted more to the combination of cotton leaf extract and female cabbage looper abdominal tip extract, compared to the female extract alone (Figure 4B). At any given female pheromone dosage, mean percentages of males attracted were nearly identical between treatments with and without cotton leaf extract (Figure 4B).

DISCUSSION

The cabbage looper is apparently unusual among the Lepidoptera in possessing both a female-produced sex attractant (Berger, 1966) and a male-produced sex attractant (Landolt and Heath, 1989), although this may occur in certain species of Arctiidae, such as *Estigmene acrea* (Drury) (Willis and Birch 1982). It now appears that sexual attraction in both sexes of *T. ni* is somehow influenced or enhanced by host-plant odors. Although reported for other insect groups, such as some Coleoptera and Diptera (i.e., Dickens et al., 1990; Landolt et al., 1992), host odor effects on sexual behavior have not been commonly reported in butterflies and moths (McNeil and Delisle, 1989a).



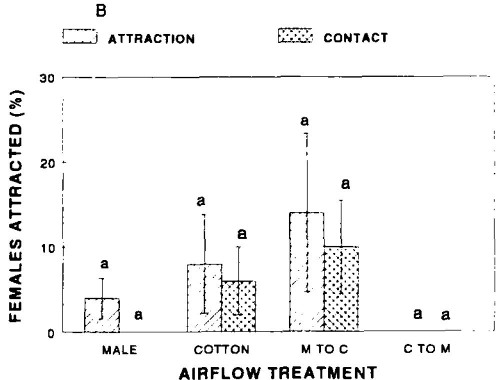
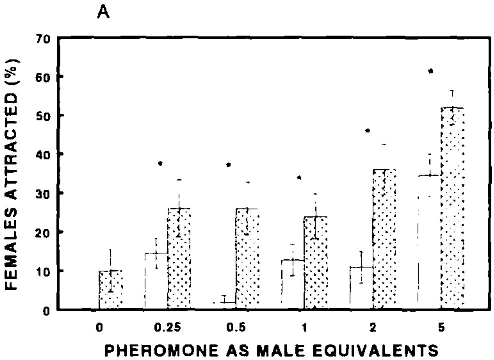


FIG. 3. Percentages ($\overline{X} \pm$ SEM) of moths tested exhibiting attraction (slashed bars) leading to contact (cross-hatched bars) with the effluent pipe opening in the flight tunnel, when the airflow vented into the tunnel had passed over moths, cotton foliage, a combination of first moths and then cotton foliage, and a combination of first cotton foliage and then moths. Part A shows male cabbage looper attraction to females and cotton. Part B shows female attraction to males and cotton. Percentages within a category with the same letter are not significantly different at $P \leq 0.05$ by Duncan's new multiple-range test.

Previously, it was demonstrated that male cabbage looper moths were more attractive to unmated females when in the presence of cabbage plants and cabbage odor (Landolt and Heath, 1990). However, it was not determined if such increased attractiveness was due to the stimulation of release of male pheromone by host chemicals or was due to an additive or synergistic effect of the combination of male pheromone and host odor on female attraction responses.



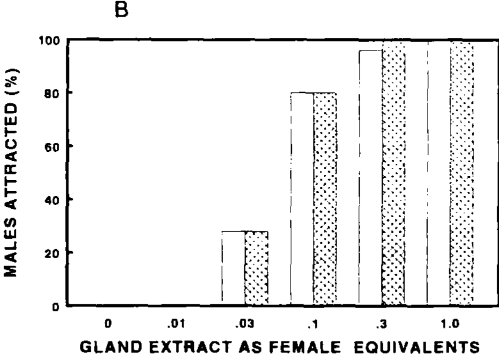


FIG. 4. (A) Percentages ($\bar{X} \pm$ SEM) of female cabbage loopers that were attracted to and contacted a capillary formulation releasing different amounts of a three-component male pheromone blend, either without (open bars) or with a 0.4-gram-equivalent dosage of cotton leaf extract on a filter paper (cross-hatched bars). Means for a given pheromone dosage followed by the same letter are not significantly different by Student's t test at $P \leq 0.05$. (B) Mean percentages ($\pm$SEM) of male cabbage loopers that were attracted to a filter paper treated with different dosages of female abdominal tip extract, either without cotton leaf extract (open bars), or with another filter paper treated with 0.4-gram-equivalent dosage of cotton leaf extract (cross-hatched bars).

We did not find evidence, in studies reported here, of stimulation of male pheromone release by host odor, using cotton. That is, we did not observe greater female attraction to airflow passed over males when the males were exposed to host-plant odor (cotton foliage volatiles), compared to airflow over males not so exposed and combined with airflow over cotton. It is possible that such effects may occur with other host-plant species or may involve nonvolatile

host chemicals that did not pass from one chamber to another in our experimental design. Despite the lack of evidence here, we cannot rule out the possibility that males may call from or call in response to host plants. Female response to male pheromone was increased by the simultaneous presentation of cotton leaf extract with the pheromone. This indicates that the increased attractiveness of males on plants is due in part to host odor enhancement of sex attraction responses of females.

We also observed that female cabbage looper moths were more attractive to males when in the presence of cotton foliage. We did not demonstrate any additive or synergistic effect of cotton foliage extract on male attraction responses to female pheromone (pheromone gland extract). An airstream carried first over females and then over cotton foliage was not more attractive than females alone or cotton foliage alone, also indicating an absence of an additive or synergistic effect of cotton odor on male response to female pheromone. Subsequent results demonstrated that females were more attractive to males, however, when cotton odors were passed over them. The increased attraction of males to females on plant foliage may then be due to the stimulation of pheromone release by host odor, rather than to host odor enhancement of sexual attraction responses of males.

It is not apparent from these studies what advantage may be gained by female cabbage loopers calling in response to host odors or females orienting preferentially to male pheromone in the presence of host odor. McNeil and Delisle (1989b) showed that pollen stimulated calling by female *H. electellum* and indicated that this may be part of a strategy by the moth to maximize the likelihood of mating and ovipositing when at a suitable host plant. Similarly, the stimulation of pheromone biosynthesis and release in female *H. zea* by host odor may be a way of synchronizing sexual and reproductive behavior to prevent oviposition in the absence of suitable host material (Raina et al., 1992). Thus, a female at a good oviposition site may be at a competitive disadvantage if unmated, with a resultant greater urgency to find or attract a mate. An alternative explanation, forwarded by Hendrikse and Vos-Bunnemyer (1987) for species of *Yponomeuta*, is that host odor effects on female calling may aid in maintaining reproductive isolation among sibling species that use different species of plants as larval hosts. There is little direct evidence for or against these hypotheses for the cabbage looper at this time.

These findings on *T. ni* have direct applications for the study of moth sex pheromones and for the development of lures to trap moths in the field. Host odor effects on female moth calling, such as that known for *H. electellum* (McNeil and Delisle, 1989b), *H. zea* (Raina et al., 1992), and *T. ni*, may alter release rates and release rate patterns of pheromones from calling moths, and quantification of pheromone production and release made in a laboratory environment may then be misleading. Furthermore, host odor enhancement of female

attraction to male pheromone, such as that shown here for *T. ni*, may provide opportunities for increasing the power of sex attractant lures through the addition of appropriate host kairomones.

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RISK-SENSITIVE HABITAT USE BY BROOK STICKLEBACK (*Culaea inconstans*) IN AREAS ASSOCIATED WITH MINNOW ALARM PHEROMONE

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Abstract—Brook stickleback (*Culaea inconstans*) share habitat and predators with cyprinid species, and they exploit the alarm pheromone of fathead minnows (*Pimephales promelas*) to avoid areas of high predation risk. In this field experiment, we measured the retention and duration of area avoidance by brook stickleback from areas marked with alarm pheromone of fathead minnows. Area avoidance was greatest during the first 2 hr after the source of the alarm pheromone was removed ($P < 0.05$), but after 4 hr, area use was not significantly different from premarking levels. This study shows that brook stickleback: (1) use the alarm pheromone of fathead minnows to avoid high risk areas, (2) continue to avoid locations associated with predation risk after the source of the pheromone has gone, and (3) avoid risky areas temporarily, and resume use of risky areas after 2–4 hr. This behavioral response by stickleback to minnow alarm pheromone could serve to minimize risk of predation.

Key Words—Predation risk, area avoidance, brook stickleback, *Culaea inconstans*, fathead minnow, *Pimephales promelas*, alarm pheromone, Schreckstoff.

INTRODUCTION

Fishes of the superorder Ostariophysi produce an alarm pheromone ("Schreckstoff") in specialized epidermal club cells. The alarm pheromone can only be released when these cells are ruptured (for review see Smith, 1992). This occurs

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when the skin is damaged during a predator attack (Pfeiffer, 1963). Nearby individuals that detect the pheromone respond with a fright reaction composed of species-specific antipredator behaviors (Frisch, 1941) and thereby reduce their probability of being captured by the predator (Mathis and Smith, 1993a). Fathead minnows avoid areas in the stream marked with cyprinid alarm pheromone (Mathis and Smith, 1992).

Brook stickleback have an alarm response to conspecific skin extract (Mathis and Smith, 1993b; Chivers and Smith, 1994b), even though they are nonostariophysan fishes (Gasterosteiformes: Gasterosteidae) and lack the specialized epidermal cells which contain alarm pheromone in cyprinid fishes (Pfeiffer, 1960).

Cross-species responses to alarm signals occur in closely related (Pfeiffer, 1963; Smith, 1982; Smith et al., 1991) and distantly related (Mathis and Smith, 1993b; Chivers and Smith, 1994b) fish species. In many areas brook stickleback and fathead minnows occupy the same habitat and are vulnerable to the same predators, especially fish-eating birds (Scott and Crossman, 1973). Stickleback exploit the alarm system of minnows and thus reduce their own risk of predation. Brook stickleback avoid areas in a stream where the alarm pheromone of fathead minnows is present and wild-caught stickleback respond to this pheromone in the laboratory with a fright response (Mathis and Smith, 1993b). However, to date, field studies of this phenomenon (e.g. Mathis and Smith 1992, 1993b) have not monitored the retention and duration of antipredator behavior or area avoidance after exposure to minnow alarm pheromone.

Sensitivity to the alarm pheromone of cyprinids has obvious survival benefits. These benefits can accrue to the cyprinids producing the pheromone and other species, such as brook stickleback, that use this system to recognize and avoid predators and/or areas associated with high predation risk (Smith, 1992; Mathis and Smith, 1993a).

Minnows learn to recognize predators when the alarm pheromone is associated with the sight of a predator (Chivers and Smith, 1994c) or the odor of a predator (Göz, 1941; Magurran, 1989; Suboski et al., 1990; Chivers and Smith, 1993, 1994a). When a sample of stream water is presented simultaneously with alarm pheromone, fathead minnows (*Pimephales promelas*) can later distinguish between water from that water sample and water from another sample taken 15 m away in the same stream (Chivers and Smith, 1994d). Taken together, these experiments indicate that minnows can learn to recognize predators and risky habitats after a single pairing of the predator- or habitat-specific stimuli with alarm pheromone.

A number of studies have documented permanent habitat shifts by prey species caused by the presence of predators (e.g., Seghers, 1974; Werner et al., 1983; Power et al., 1985; see Lima and Dill, 1990 for review). However, predation risk may vary seasonally, daily, or from minute to minute, depending on the ecological context of the predator–prey interaction. Over time, all loca-

tions in a stream may eventually be associated with the release of cyprinid alarm pheromone caused by predation events. Permanent avoidance of all stimuli associated with predation would incur unacceptably high costs in terms of loss of forage areas, foraging time, refuge, and spawning habitat (Lima and Dill, 1990; Milinski, 1993). Therefore, for the alarm system to be useful as a mechanism of predator avoidance, individuals should behave in a way that reflects a tradeoff between the short-term benefits and costs of area avoidance. This may take the form of temporary avoidance of areas marked with alarm pheromone, followed by a gradual resumption of use of these areas as the threat of the predator's return decreases, i.e., when the predator has either moved to other areas or become satiated (Milinski, 1993).

In this field experiment we tested the retention and duration of area avoidance by brook stickleback to see if brook stickleback behavior would reflect this trade-off. We tested this by temporarily marking stream locations with alarm pheromone from the skin of fathead minnows (simulating a predation event). We predicted that brook stickleback would avoid marked areas because of a high risk of imminent predation, then gradually resume use of these areas.

METHODS AND MATERIALS

Preparation of Skin Extract. Ten adult fathead minnows (mean standard length $\pm$ SE = 54.7 $\pm$ 1.2 mm) were killed by a blow to the head. We then removed the skin from both sides of each minnow. We collected a total of 32.8 cm^2 of skin. We placed the skin in 100 ml of glass-distilled water and homogenized the sample using a polytron homogenizer. We filtered the homogenate through glass wool and then diluted the supernatant with 300 ml of glass-distilled water for a total volume of 400 ml. Fourteen blocks of synthetic sponge (5 $\times$ 4 $\times$ 2 cm) were soaked in this solution and frozen at $-20°C$ for later use. Each sponge was weighted with a black rubber stopper attached to a piece of stainless steel wire. As a control, 14 additional sponge blocks were soaked with glass-distilled water and frozen at $-20°C$. Frozen skin extracts retain the capacity to elicit a fright response from stickleback (Mathis and Smith, 1993a).

Study Site and Sampling Schedule. Oscar Creek is a small body of water located 75 km northwest of Saskatoon, Saskatchewan (52°46'N, 107°07'W). At the study site, the creek ranges from about 1–10 m in width and contains populations of brook stickleback and fathead minnows. Current speed varied with basin morphology. Traps were placed in approximately 1-m-deep water, where the rate of flow was visibly present but relatively slow.

On October 7, 1993, 28 minnow traps (Gee's improved minnow traps: cylinders of 5 $\times$ 5 mm wire mesh, measuring 43 cm long and 22 cm in diameter, with inverted cone entrances at each end), were placed along the edge of Oscar

Creek about 5–10 m apart. To ensure that trap location did not change from sample to sample, we attached each trap to a stake with cord. Each trapping period (one prestimulus and four poststimulus samples) lasted 2 hr. The sampling schedule was as follows. A prestimulus sample was collected from all trap locations. We counted the number of brook stickleback caught in each trap and then immediately released them at the same location. Next, we removed each trap from the stream and replaced it with a block of sponge. Traps were removed to prevent brook stickleback from associating the alarm pheromone with the traps. We attached each sponge to the appropriate stake on shore to ensure that sponges were in the same location previously occupied by each trap. Traps were set in pairs to maintain equal duration of trapping effort between treatments (alarm pheromone or distilled water control). Treatment was randomly assigned such that there was one trap per treatment within each consecutive pair of traps. Fourteen trap locations were marked with a block of sponge soaked in alarm pheromone, and the other 14 locations were marked with a block of sponge soaked in distilled water. Sponges soaked in distilled water allowed us to control for any effects of repeated trapping (i.e., either increased avoidance or habituation).

After 1 hr, we removed the sponges and immediately replaced the traps that had been used at each location for the prestimulus sample. This was the first poststimulus sample. After 2 hr we emptied the traps, counted and released the fish at the point of capture, and then reset the traps for another 2 hr. This procedure was repeated 2 hr later. These samples were the second and third poststimulus samples, respectively. The following day, 24 hr after the sponges were removed from the water, we collected a final 2-hr sample. This was the fourth poststimulus sample.

Statistical Analyses. For each trap location, we calculated the difference between the number of brook stickleback caught in each poststimulus sample and the prestimulus sample. The differences were ranked among the 28 traps, and a statistical comparison was made between locations marked with alarm pheromone and locations marked with distilled water using a Wilcoxon–Mann-Whitney test (Siegel and Castellan, 1988). This test was performed for each poststimulus sample to determine the statistical significance and duration of area avoidance by brook stickleback.

RESULTS

Brook stickleback avoided areas that had previously been marked with cyprinid alarm pheromone. The strength of this avoidance decreased over time (Table 1; Figure 1). There was a steady return to prestimulus levels in the number of brook stickleback caught in traps at locations previously marked with

TABLE 1. MEAN (±SE) NUMBER OF BROOK STICKLEBACK CAUGHT PER TRAP BEFORE (PRE) THE ADDITION OF STIMULUS SPONGES, AND 2, 4, 6, AND 24 HOURS AFTER (POST) THEIR REMOVAL

Sample	Alarm pheromone (N = 14)	Control (N = 14)
Pre-Stim	6.21 ± 1.75	4.00 ± 1.32
Post 2 hr	3.14 ± 1.04	4.79 ± 1.29
Post 4 hr	4.21 ± 1.30	4.71 ± 1.45
Post 6 hr	5.21 ± 1.01	5.71 ± 1.16
Post 24 hr	2.50 ± 0.97	1.36 ± 0.68

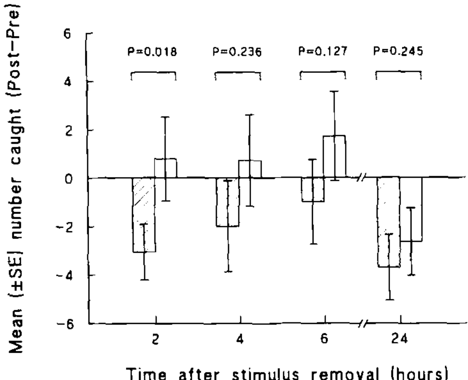

FIG. 1. The mean (± SE) difference in the number of brook stickleback caught per trap before stimulus sponges were placed in the stream and 2, 4, 6, and 24 hr after stimulus removal. Hatched bars, locations marked with cyprinid alarm pheromone; open bars, locations marked with distilled water. Means and SE's are used to illustrate trends in the data only. The data were analyzed using nonparametric statistics (see text).

alarm pheromone for samples taken 2, 4, and 6 hr after stimulus removal. Two hours after the removal of the sponges (first poststimulus sample), the number of brook stickleback caught relative to prestimulus catches was significantly less at trap locations marked with alarm pheromone than trap locations marked with distilled water (W_x = 130, P = 0.018, one-tailed). Stickleback avoidance of these areas was not statistically significant for any of the subsequent poststimulus samples ($P > 0.127$). The number of brook stickleback caught in traps at control locations increased slightly, but not significantly, for the remainder of the day (Table 1). The number of brook stickleback caught in the fourth poststimulus sample, 24 hr after the removal of the sponges, were equally low relative to the prestimulus sample for both treatments groups (Table 1; Figure 1).

DISCUSSION

These data demonstrate that brook stickleback avoided areas marked by cyprinid alarm pheromone after the stimulus had been removed. This behavior is consistent with brook stickleback recognizing cyprinid alarm pheromone as a signal that the marked area carried a high risk of predation (Mathis and Smith, 1993b). A shift in area use by brook stickleback away from locations associated with minnow alarm pheromone may have caused the slight increase in the number of brook stickleback caught in traps at control locations (Table 1). Alternatively, the increase in stickleback catches in control traps may reflect a diurnal pattern.

The duration of avoidance suggests that brook stickleback significantly avoided high-risk areas for 2–4 hr after the source of the stimulus was gone. There is an indication (based on small sample sizes) that the number of brook stickleback caught in traps marked with alarm pheromone continued to converge with prestimulus numbers for the 4- and 6-hr poststimulus samples. This pattern was not evident in locations marked with control sponges. We do not know whether any alarm pheromone remained in the sponges after 1-hr exposure in the stream. It is possible that some alarm pheromone lingered in the area for a short time after the removal of the sponge. However, there was sufficient current to dilute and disperse residual alarm pheromone within a short time relative to the duration of the first 2-hr trapping effort.

Catches after 24 hr were low for both treatment groups. This could have resulted if stickleback were less active on the second day than on the first for reasons not related to the experiment. These low catches do not represent area avoidance, because alarm and control traps caught equally low numbers of brook stickleback.

Risk-sensitive foraging is an active field of research. Numerous studies indicate that the trade-off between predation risk and foraging benefits is a dynamic process (Milinski and Heller, 1978; Milinski, 1985) where foraging individuals frequently sample their surroundings to assess predation risk and foraging potential (e.g., Clark and Mangel, 1984) and facultatively adjust their behavior in response to changes in these two opposing variables (Lima and Dill, 1990). For example, juvenile coho salmon reduce foraging rates after detection of chemical stimuli from predation events (Martel and Dill, 1993). After the disappearance of a predation threat, juvenile Atlantic salmon (*Salmo salar*) gradually resume foraging activity over a 2-hr period (Metcalfe et al., 1987). Elective shoal size of European minnows did not return to normal (undisturbed) levels one day after exposure to a northern pike (*Esox lucius*), a predator of minnows (Pitcher et al., 1983).

Food benefit may be independent of predation risk (Cerri and Fraser, 1983). However, in European minnows (*Phoxinus phoxinus*), avoidance of open areas

vulnerable to aerial attack by a model kingfisher continued for at least 6 hr when food levels were held constant, but returned to normal within 6 hr when food reward in open areas was high (Pitcher et al., 1988). Furthermore, the rate at which parasitized three-spine stickleback resumed use of foraging patches following attacks by a model heron increased with food availability (Godin and Sproul, 1988).

Oscar Creek is a small body of water with no large piscivorous fish (i.e., esocids, centrarchids, percids). Important predators of small fish, such as minnows and brook stickleback, in this type of habitat are fish-eating birds (Scott and Crossman, 1973; Krause, 1994). Great blue herons (*Ardea herodias*) frequent the study site where these data were collected. Herons are sit-and-wait predators that remain in the same location for long periods of time. Kingfishers swoop down from high above the water surface to capture prey, and often initiate diving attacks from a favored perch. If predators tend to remain in the same area while fishing, then stickleback can assess the expected probability of encounter with a predator as unacceptably high in areas where cyprinid alarm pheromone is detected (Lima and Dill, 1990). When this occurs, avoidance of an area for two or more hours, followed by a gradual, cautious resumption of use of the area should be a useful tactic for brook stickleback that should reduce predation risk from aerial predators. Additional field experiments are needed to test the universality of this phenomenon in other species sympatric with cyprinids and in other habitats with different species of predators.

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CUTICULAR HYDROCARBONS WHEREBY *Messor barbarus* ANT WORKERS PUTATIVELY DISCRIMINATE BETWEEN MONOGYNOUS AND POLYGYNOUS COLONIES. ARE WORKERS LABELED BY QUEENS?

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Abstract—The results of laboratory experiments carried out with both monogynous and artificially polygynous *Messor barbarus* ant colonies (which under natural conditions are always monogynous) have shown that the workers belonging to monogynous colonies were able to discriminate between intruders from other monogynous colonies and those from polygynous (di- and trigynous) ones. What mechanisms are involved in this discriminatory ability? Since differences in the relative proportions of the hydrocarbons they carry are known to convey complex messages that are used for recognition purposes, it was proposed here to investigate whether there existed any differences in the proportions of the various hydrocarbons carried by the diverse categories of intruders tested in our experiments. It emerged that one set of hydrocarbons, which were usually present in rather small proportions and included all the families that constitute this species' chemical signature (*n*-alkanes, mono-, di-, and trimethylalkanes), was characteristically associated with workers from monogynous colonies. Another set of hydrocarbons, which included some of the above components, mostly in larger relative proportions, can be said to have characterized the digynous and trigynous colonies.

Key Words—Chemical communication, nestmate recognition, role of the

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queen, colony closure, aggressive behavior, pheromone, cuticular hydrocarbons, ant, *Messor barbarus*, Hymenoptera, Formicidae.

INTRODUCTION

It has been established in several studies on social insects, particularly ants, that the queen plays a fundamental role in the social organization and the cohesion of the group, via the pheromonal influence she exerts over the other members. The cohesion of a social group also depends partly on the recognition mechanisms at work between the various members of the group. Nestmate recognition is mediated in ants by chemical cues and, more specifically in some cases, by the relative proportions of particular cuticular hydrocarbons (dimethylalkanes in the case of *Camponotus vagus*; Bonavita-Cougourdan et al., 1987, 1990).

Numerous behavioral studies have been carried out on the endogenous versus exogenous source of the signals involved in recognition processes, but few chemical studies have focused on the question as to whether and to what extent the queen may be responsible for a colony odor.

Hölldobler and Michener (1980) have suggested that it is in monogynous rather than polygynous colonies that the cues whereby the members of a colony recognize each other are mainly generated by the queen.

In the ant *Leptothorax lichtensteini*, the chemical signature changes with time as regards the proportions of some of the hydrocarbons. The presence of the queen seems to accelerate the change over one year (Provost et al., 1993). Carlin and Hölldobler (1983, 1986, 1987, 1988) have shown that in various monogynous *Camponotus* species, the main source of the colony odor is the queen. Bennett (1989) has made comparisons between the recognition systems of two species of *Formica*, one of which is monogynous and the other polygynous. This author observed that to identify each other, the workers of the monogynous species used the cues produced by their nestmates rather than those produced by the queen. In species where the colonies are polygynous, on the contrary, it is the cues produced by the queen that are the most important. The results of the experiments carried out by Brian (1986) on three *Myrmica* species suggest that the queen produces discriminators that are transferred to the workers.

Vienne et al. (1992) have reported that in the artificial mixed colonies they set up, the *Manica* queens affected both the behavior and the odor carried by the *Myrmica* workers, whereas *Myrmica* queens had less effect on *Manica* workers. The preliminary results obtained suggest that the *Manica* queen may have stimulated the production of her own species' hydrocarbons in the allospecific workers.

With a view to elucidating the role of the queen in colony closure and nestmate recognition processes, several studies have been carried out where

colonies have been split into two groups, only one of which has a queen, before being reunited. Haskins and Haskins (1950) observed, for instance, that in two *Myrmecia* species, the workers in the two groups no longer recognized each other after being separated for four months and showed mutually aggressive behavior. Similar results were obtained with *Leptothorax lichtensteini* (Provost, 1987, 1989), but in this case, the role played by the queen was even more obvious: when the queen was moved regularly from the one group to the other during the four-month period of separation, the workers mingled together again with no aggression when reunited. In *Camponotus lateralis* (Provost, 1980), peaceful encounters between workers from two different colonies were possible only when both colonies had been previously deprived of their queen.

There exist, however, a number of cases where the main source of a colony's odor is not the queen. In *Pseudomyrmex ferruginea*, some North American species of *Leptothorax*, monogynous colonies of *Solenopsis invicta*, and *Rhytidoponera metallica*, inherited worker discriminators and cues mediated by the environment contribute more to establishing the colony odor than those emanating from the queen (Mintzer, 1982; Stuart, 1987, 1988; Obin and Vander Meer, 1988, 1989; Morel et al., 1990; Crosland, 1989, 1990). The differential levels of aggressive behavior displayed by queenright and queenless colonies were the only indication that the queen was involved in the inter- and intracolony recognition processes at work in *Cataglyphis cursor* (Berton and Lenoir, 1986).

Berton et al. (1991) have suggested that in *Cataglyphis cursor* ant colonies, the queen may produce pheromones of two kinds: those serving to attract the workers and those enabling them to recognize the queen. The attractive substances released by the queen seem to be identical to those constituting the colony odor (Berton, 1989). However, the queen alone is not responsible for the colony odor (Berton et al., 1992). Keller and Passera (1989) in their study on the number of queens as a nestmate recognition factor in *Iridomyrmex humilis* established that the attractiveness of a queen to her workers depends on queen recognition processes. The fact that the workers from polygynous colonies were less strongly attracted to their nestmate queens than workers from monogynous colonies was explained by the authors as follows: since a group of less closely related individuals (workers and/or queens) will produce a mixture of pheromones of various origins, the colony odor will be less distinct in polygynous than in monogynous colonies.

It is generally recognized that polygynous colonies are more open than monogynous ones (Hölldobler and Wilson, 1977). This has been generally confirmed in the case of many species, although some exceptions have been found to exist (see for example Stuart, 1991). The inherited recognition signals are assumed to be more varied in polygynous colonies, which contain a larger range of genotypes than monogynous ones. Discrimination on the basis of genetic labels is therefore likely to be less reliable in polygynous than in monogynous

colonies (Crozier and Dix, 1979; Hölldobler and Michener, 1980; Keller and Passera, 1989).

If the queens in a polygynous colony all have different chemical signatures, the workers may well become more tolerant of unfamiliar odors, and are not necessarily less capable of discrimination.

In nature, the colonies of the fire ant *Solenopsis invicta* can be either monogynous or polygynous. Morel et al. (1990) have carried out a comparative study on the nestmate recognition processes at work in populations of the two kinds. Workers from polygynous colonies displayed significantly less aggressive behavior than those from monogynous colonies when exchanges were effected between the two kinds of colonies. The results of these authors' recognition bioassays showed that there existed no significant difference between the responses of monogynous residents to monogynous and polygynous intruders. Nestmate recognition in the polygynous colonies seems to have been based on discriminators emanating from the workers of various maternal lineages rather than on the multiple discriminators provided by the queens. Vander Meer et al. (1990) have hypothesized that the behavior observed in the workers from polygynous colonies may have been unaggressive either because these workers encountered such a wide variety of environmental and inherited cues that they were unable to build up an efficient template, or because the template they had built up overlapped so much with the labels emanating from workers belonging to other colonies that little if any discrimination was possible.

Discrimination has been found to take place between the monogynous and polygynous forms of the above species on the basis of the cuticular hydrocarbons (Greenberg et al., 1990). The data by Yamaoka (1990) and Yamaoka and Kubo (1990) suggest, on the other hand, that the common cuticular hydrocarbon profile among *Formica* workers depends to a large extent on the queen: workers experimentally deprived of their queen showed considerable differences in their main cuticular hydrocarbon levels. The longer the colonies had been queenless, the greater these differences became.

In the study we carried out on *Messor barbarus* (Provost and Cerdan, 1990), involving exchanges of workers between experimentally established polygynous colonies and monogynous colonies, the polygynous colonies were found to be significantly more open than the monogynous ones. Unlike the workers from polygynous colonies, those from monogynous ones seemed to be able to discriminate between intruders from monogynous and polygynous colonies: they accepted the presence of the latter significantly more frequently than that of the former. It looked as if the two categories of intruders carried different information about their origins, and as if this information was detected and decoded by the workers they encountered.

The aim of the present study was to investigate whether any correlation

exists in this experimental model between the cuticular hydrocarbon profiles of the foragers and the number of queens present at a *Messor barbarus* colony.

METHODS AND MATERIALS

Material. Female foundresses of the granivorous monogynous ant *Messor barbarus* were collected in the Crau plain (Rhône Delta) at three stations several kilometers apart, in March 1986. Six monogynous colonies were founded by a single queen, and six polygynous colonies (four digynous and two trigynous) were formed experimentally by placing together either two or three queens from other colonies, using the methods described by Cerdan and Delye (1987, 1988). These colonies were then raised at the laboratory as described in a previous study (Provost and Cerdan, 1990). In artificial polygynous colonies, the queens have shown no aggressive behavior towards each other and had no individual territory. The state of the queens' ovaries has shown that they all participate in developing the colony.

Behavioral and chemical analyses were carried out in 1990 on these 12 colonies when they had become submature (not yet able to produce sexuate individuals): each of them then contained between 200 and 1500 workers and one, two, or three queens. In order to obtain homogeneous cuticular hydrocarbon samples, these were collected only from medium-sized adult workers engaged in foraging activities outside the nest.

Chromatographic Analyses. Chromatographic analysis was carried out on 10 individuals from each of the 12 colonies. Each ant was killed by freezing. The cuticular wax was extracted by placing each ant in 100 μl of hexane for 10 min in a Wheaton tube closed with a teflon-faced rubber septum and cap. The extracts were concentrated by evaporation under a stream of nitrogen. A small volume (2 μl) was analyzed on a Delsi 330 gas chromatograph with a flame ionization detector (FID), a split-splitless injector, and a Chrompack nonpolar CP-SIL 5 WCOT column (25 m $\times$ 0.22 mm ID, film thickness 0.12 mm). The carrier gas was helium at a pressure of 1 bar. The injections were performed in the splitless mode for 15 sec, with the injector temperature at 250°C; the oven was programmed from 150°C to 300°C at 3°C/min. The peaks were integrated with an Enica 21 integrator.

Some 47 compounds were analyzed. Any substances amounting to less than 10^{-3} of the total amount in one chromatogram were not used in the calculations. The peak areas given by the integrator were corrected depending on the chain length by a factor K ($K = 0.042X + 0.11$, where X is the number of carbon atoms per molecule of the substance), according to the formula by Bagnères et al. (1991) and Bagnères and Morgan (1990). This procedure was applied to correct for any variations in the sensitivity of the flame ionization detector depending on the chain length.

Chemical Analyses. The hydrocarbons were identified by gas chromatography–mass spectrometry (GC-MS), performed on a Hewlett Packard 5890 GC coupled to a 5970 B Mass Selective Detector (quadrupole MS using 70-eV electron impact ionization). The system and data analysis were controlled with a Hewlett Packard series 300 computer with HP 59970C Chemstation.

The chromatography was carried out with an active capillary column equivalent to OV-1 (Sac Chromatography, Letchworth, U.K.) (12 m × 0.2 mm × 0.33 mm) linked through a 10-m × 0.2-mm deactivated fixed capillary column (Sac Chromatography) to the MS source. Solid samples of cuticle were injected in the split/splitless mode for 30 sec using the method of Bagnères and Morgan (1990). The oven was programmed from 150°C to 300°C at 3°C/min and then maintained at that temperature. A standard alkane mixture from C_{18} to C_{30} in hexane solution was chromatographed under the same conditions in the split mode to calculate the equivalent chain length (ECL). The identification of cuticular compounds was based upon their retention times and mass spectra and compared with the data in the literature (e.g., those published by de Renobales et al., 1991; Lockey, 1988; Lockey and Oraha, 1990; Grunshaw et al., 1990; Page et al., 1990).

Statistical Analyses. The proportions of each of the 47 peaks were calculated with the Lotus 1.2.3. computer program. The differences in the proportions of the various cuticular hydrocarbons recorded between monogynous and polygynous colonies were assessed using multivariate analyses: principal component analysis; discriminant analysis (Statgraphics program, version 5); factorial correspondence analysis based on contingency tables consisting of 188 rows and three columns, where each variable (the proportion of one substance) was divided into four classes (4 × 47) depending on its value, in such a way that all the classes contained approximately the same number of individuals, and taking the three categories of colony (mono-, di-, and trigynous) and then accounting for individuals in supplementary columns (Biomeco); and, lastly, a stepwise discriminant analysis (SAS program).

In what follows, workers from mono-, di-, and trigynous colonies will be referred to as mono-, di-, and trigynous individuals for the sake of simplicity.

RESULTS

Chemical Determination. The cuticular compounds of *Messor barbarus* consisted of series of saturated alkanes from C_{23} to C_{30} (Figure 1). An earlier, incomplete determination was carried out (Provost et al., 1992), which was completed in the present study using the same material. Forty-eight hydrocarbons were determined (Table 1) and found to be the usual internally branched mono- and dimethylalkanes on the basis of their corresponding *n*-alkane. A

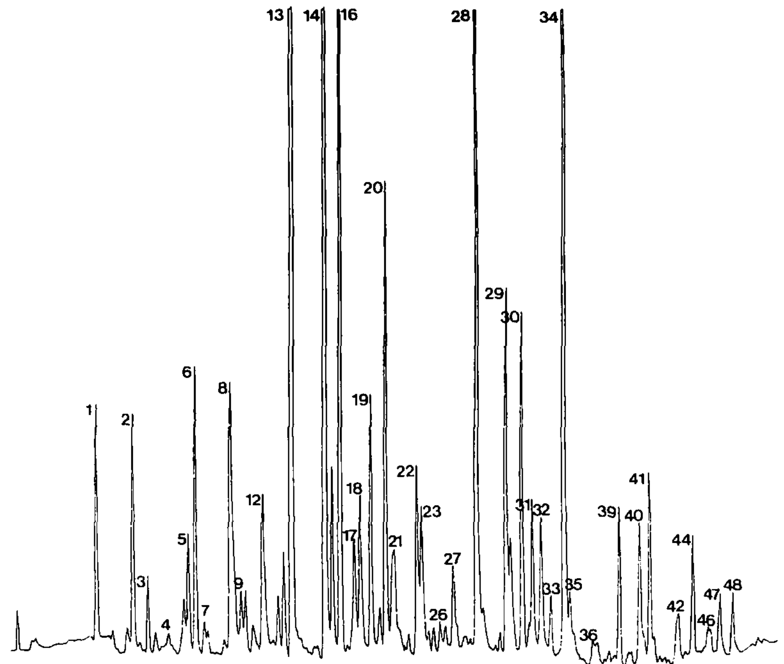

FIG. 1. Gas chromatogram of the cuticular hydrocarbon profile of one worker of the ant *Messor barbarus*.

series of trimethylalkanes (4,10,14 and 4,12,16) with a C_{23} to C_{29} backbone was detected, which is not common in insects. The equivalent chain lengths (ECL) of the latter compounds in C_{23}, C_{25}, C_{27} were around 10 units after the even *n*-alkanes from C_{24}, C_{26}, C_{28}, respectively, and that in C_{29} was similar to the retention time of *n*-C_{30}.

8,12,16-Trimethyloctacosane has previously been described by Lockey (1991) but under our chromatographic conditions, the ECL was 28.75 (as opposed to 28.61).

6-Methyloctacosane, which was often present in trace amounts only, was not included in the statistical analyses.

Comparisons between Cuticular Hydrocarbon Profiles of Monogynous and Polygynous Colonies. The three categories of colony could be distinguished from the results of the factorial correspondence analysis carried out on the contingency table of the proportions of the cuticular hydrocarbons recorded in 120 individuals (60 of which were monogynous, 40 digynous, and 20 trigynous) and on the set of 47 hydrocarbons divided into four classes (Figure 2A). Taking

TABLE 1. DETERMINATION OF CUTICULAR HYDROCARBONS OF ANT *Messor barbarus* USING LINKED GAS CHROMATOGRAPHY–MASS SPECTROMETRY

Peak	ECL	Hydrocarbon	Diagnostic ion EI/MS	MW
a1	23.00	*n*-Tricosane	324	324
m2	23.36	9- and 11-Methyltricosane	140/1, 168/9, 196/7, 224/5	338
m3	23.51	5-Methyltricosane	84/5, 280/1	338
m4	23.74	3-Methyltricosane	56/7, 308/9	338
d5	23.88	5,9-Dimethyltricosane	84/5, 154/5, 224/5, 294/5	352
a6	24.00	*n*-Tetracosane	338	338
t7	24.10	4,10,14- and 4,12,16-Trimethyltricosane	70/1, 126/7, 154/5, 168/9, 196/7, 224/5, 238/9, 266/7, 322/3	366
m8	24.35	10-, +11-, and 12-Methyltetracosane	154/5, 168/9, 182/3, 196/7, 210/1, 224/5	352
m9	24.46	6-Methyltetracosane	98/9, 280/1	352
m10	24.52	5-Methyltetracosane	84/5, 294/5	352
m11	24.60	4-Methyltetracosane	70/1, 308/9	352
m12	24.69	3-Methyltetracosane	56/7, 322/3	352
a13	25.00	*n*-Pentacosane	352	352
m14	25.36	11- and 13-Methylpentacosane	168/9, 196/7, 224/5	366
m15	25.42	7-Methylpentacosane	112/3, 280/1	366
m16	25.52	5-Methylpentacosane	84/5, 308/9	366
d17	25.68	11,15-Dimethylpentacosane	168/9, 238/9	380
m18	25.74	3-Methylpentacosane	56/7, 338/9	366
d19	25.86	5,9-Dimethylpentacosane	84/5, 154/5, 252/3, 322/3	380
a20	26.00	*n*-Hexacosane	366	366
t21	26.10	4,10,14- and 4,12,16-Trimethylpentacosane	70/1, 154/5, 168/9, 182/3, 196/7, 224/5, 238/9, 252/3, 266/7, 350/1	394
m22	26.35	11-, +12-Methylhexacosane	168/9, 182/3, 224/5, 238/9	380
m23	26.39	8-Methylhexacosane	126/7, 280/1	380
m24	26.45	6-Methylhexacosane	98/9, 308/9	380
m25	26.51	5-Methylhexacosane	84/5, 322/3	380
m26	26.63	4-Methylhexacosane	70/1, 336/7	380
d27	26.69	6,10-Dimethylhexacosane	98/9, 168/9, 252/3, 322/3	394
a28	27.00	*n*-Heptacosane	380	380
m29	27.35	11- and 13-Methylheptacosane	168/9, 196/7, 224/5, 252/3	394
m30	27.50	5-Methylpentacosane	84/5, 336/7	394
d31	27.62	11,15-Dimethylheptacosane	168/9, 196/7, 238/9, 266/7	408
m32	27.73	3-Methylheptacosane	56/7, 364/5	394
d33	27.81	5,9- and 5,11-Dimethylheptacosane	84/5, 154/5, 182/3, 252/3, 280/1, 350/1	408
a34	28.00	*n*-Octacosane	394	394
t35	28.09	4,10,14- and 4,12,16-Trimethylheptacosane	70/1, 168/9, 182/3, 196/7, 210/1, 238/9, 252/3, 266/7, 280/1, 378/9	422

TABLE 1. CONTINUED

Peak	ECL	Hydrocarbon	Diagnostic ion EI/MS	MW
m36	28.32	12- and 14-Methyloctacosane	182/3, 210/1, 224/5, 252/3	408
m37	28.43	8-Methyloctacosane	126/7, 308/9	408
m38	28.57	6-Methyloctacosane	98/9, 336/7	408
m39	28.66	4-Methyloctacosane	70/1, 364/5	408
t40	28.75	8,12,16-Trimethyloctacosane	126/7, 196/7, 266/7, 336/7	436
a41	29.00	*n*-Nonacosane	408	408
m42	29.35	11-, 13-, and 15-Methylnonacosane	168/9, 196/7, 224/5, 252/3, 280/1	422
m43	29.40	7-Methylnonacosane	112/3, 336/7	422
m44	29.55	5-Methylnonacosane	84/5, 364/5	422
d45	29.65	11,15-Dimethylnonacosane	168/9, 224/5, 238/9, 294/5	436
m46	29.76	3-Methylnonacosane	56/7, 392/3	422
d47	29.81	5,11-Dimethylnonacosane	84/5, 182/3, 280/1, 378/9	436
at48	30.00	*n*-Triacontane	422	422
		4,10,14-, and 4,12,16-Trimethylnonacosane	70/1, 168/9, 196/7, 210/1, 238/9, 266/7, 280/1, 308/9, 406/7	450

the centers of gravity marked with circles (1, 2, and 3 indicate mono-, di-, and trigynous colonies, respectively), the horizontal axis (axis 1), which accounts for 55.95% of the total inertia, separates the monogynous colonies (negative pole) from the polygynous ones (positive pole), whereas the vertical axis (axis 2), which accounts for 44.05% of the inertia, separates the digynous colonies (negative pole) from the trigynous ones (positive pole).

It should be noted that the trigynous colony E and the monogynous G make this distinction less clear-cut than it might otherwise have been: nine of 10 of the individuals from trigynous colony E had cuticular profiles that were quite similar to those of the monogynous colonies, and seven of the 10 individuals from monogynous colony G had hydrocarbon profiles resembling those of the digynous colonies.

The intracolony variability of the cuticular hydrocarbon profiles was of roughly the same order in both monogynous and polygynous colonies.

Figure 2B and Table 2 give the cuticular hydrocarbons most characteristic of the three categories of colony. Monogynous workers (the negative end of axis 1) were associated with one *n*-alkane (*n*-heptacosane), 11 monomethylalkanes, four dimethylalkanes, and the 4,10,14- and 4,12,16-trimethylpentacosane mixture, all present in smaller proportions apart from 5-methylhexacosane, *n*-heptacosane, and especially 3-methylnonacosane.

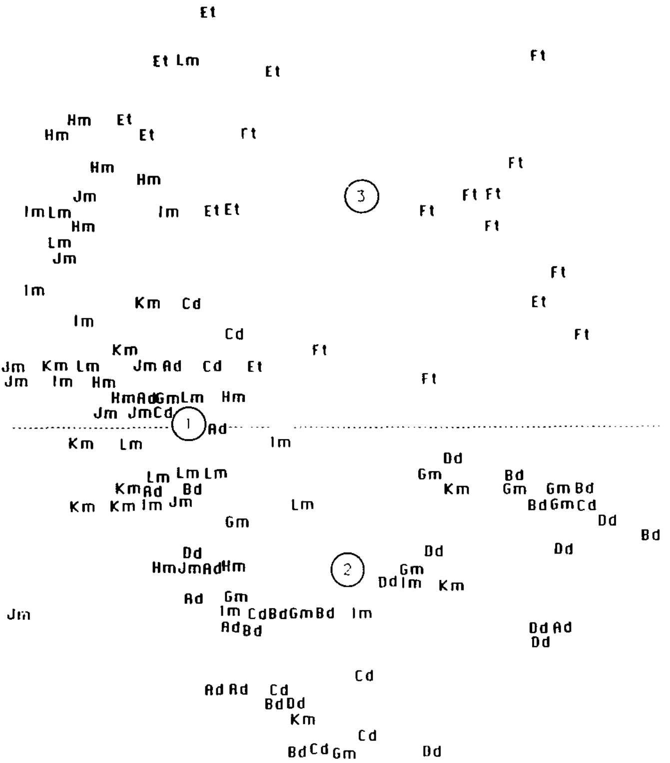

FIG. 2. Results of a factorial correspondence analysis performed on all 120 individuals and all 47 hydrocarbons. (A) Plot of individuals on factorial mapping 1–2 (horizontal axis = axis 1, vertical axis = axis 2). Axis 1 accounts for 55.95% of the inertia, and axis 2, for 44.05%. Numbers 1, 2, 3, indicate the centers of gravity of the three categories of colony: 1 = monogynous, 2 = digynous, 3 = trigynous. Each individual is denoted by a capital letter (A, B, . . . , L) indicating the colony from which it originated, along with a lowercase letter (m, d, t) depending on whether the colony was mono-, di-, or trigynous. (B) Plot of variables on factorial mapping 1–2. Chemical families are indicated by a letter, as follows: a = *n*-alkane, m = monomethylalkane, d = dimethylalkane, t = trimethylalkane, at = *n*-alkane and trimethylalkane mixture. Each hydrocarbon was divided into four classes, depending on its proportional level (low to high, 1 to 4): the right-hand figure in each group. The first figure or figures are the number of the peak on the chromatogram in Figure 1.

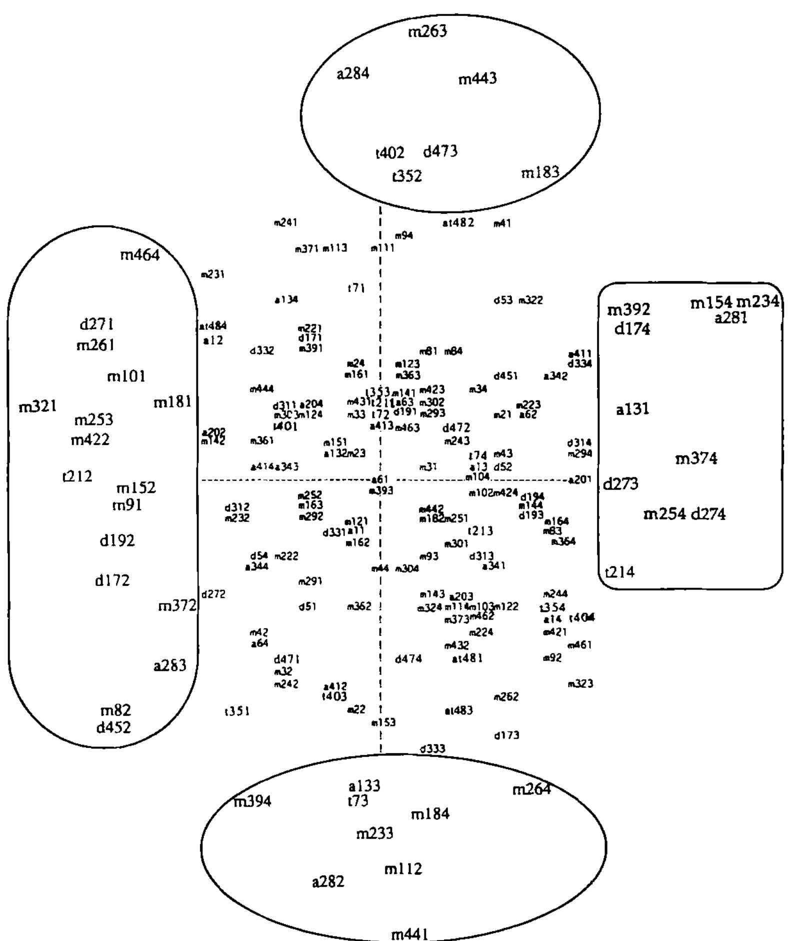

FIG. 2. Continued.

The polygynous workers (at the positive end of axis 1) were associated with two *n*-alkanes and one monomethylalkane present in smaller amounts, four other monomethylalkanes, three dimethylalkanes, and one trimethylalkane present in larger relative amounts. In addition to these substances characterizing the workers from polygynous colonies, the digynous workers (negative part of axis 2) were also characterized by four monomethylalkanes and one trimethylalkane, and the trigynous workers (positive end of axis 3) had three monomethylalkanes and one dimethylalkane, which were both present in relatively higher propor-

TABLE 2. CUTICULAR HYDROCARBONS CHARACTERIZING THREE CATEGORIES OF WORKERS BY RELATIVELY LOW (+) TO HIGH (++++) HYDROCARBON PROPORTIONS[a]

Cuticular hydrocarbons	Monogynous workers	Digynous workers	Trigynous workers
n-Pentacosane		+++	+
n-Heptacosane	+++	++	+/++++
10-, 11-, and 12-Methyltetracosane	++		
6-Methyltetracosane	+		
5-Methyltetracosane	+		
4-Methyltetracosane		++	
7-Methylpentacosane	++	+++	++++
3-Methylpentacosane	+	++++	+++
8-Methylhexacosane		+++	++++
5-Methylhexacosane	+++	++++	++++
4-Methylhexacosane	+	++++	+++
3-Methylheptacosane	+		
8-Methyloctacosane	++	++++	++++
4-Methyloctacosane		++++	++
11-, 13-, and 15-Methylnonacosane	++		
5-Methylnonacosane		+	+++
3-Methylnonacosane	++++		
11,15-Dimethylpentacosane	++	+++	++++
5,9-Dimethylpentacosane	++		
6,10-Dimethylhexacosane	+	++++	++++
11,15-Dimethylnonacosane	++		
5,11-Dimethylnonacosane			+++
4,10,14- and 4,12,16-Trimethyltricosane		+++	
4,10,14- and 4,12,16-Trimethylpentacosane	++	++++	++++
4,10,14- and 4,12,16-Trimethylheptacosane			++
8,12,16-Trimethyloctacosane			++

[a] When no + is indicated, this means that the proportions of the substance varied among workers of the category under consideration.

tions, and two trimethylalkanes present in relatively lower proportions. All in all, 26 of the 47 cuticular hydrocarbons detected characterized one or the other of the three categories of workers tested because of their proportions. Seventeen hydrocarbons, 14 of which were present in smaller proportions, characterized the individuals from monogynous colonies. Fifteen hydrocarbons, 12 of which were present in higher proportions, characterized those from digynous colonies. Sixteen substances, 11 of which were present in higher proportions, characterized those from trigynous colonies. Among the nine hydrocarbons that were common to all three categories of individuals, eight were present in lower pro-

portions in the monogynous individuals and in higher proportions in the polygynous individuals.

A stepwise discriminant analysis (SAS software) was carried out on all the individuals, and the results were used to determine the discriminant character of the various hydrocarbons. The distribution of the six main substances is given in Table 3. The substance that yielded the greatest differences between the three categories of workers was 6,10-dimethylhexacosane; it was present in smaller proportions in the monogynous workers and in larger proportions in the di- and trigynous workers. Next came 5-methylnonacosane, which separated the digynous workers (which carried smaller amounts) from the trigynous ones (which carried larger amounts); in the monogynous workers, the relative levels of this substance were variable. Then came 8-methylhexacosane, which was present in variable proportions among the monogynous workers, but in larger proportions in the digynous workers and in even larger proportions in the trigynous workers. The subsequent items on the list were *n*-heptacosane, 7-methylpentacosane, and the 4,10,14- and 4,12,16-trimethylpentacosane mixture. These six main substances had already emerged as discriminant variables in the factorial correspondence analysis. Upon calculating the correlation ratio, i.e., the intragroup to intergroup variance ratio, three of these substances were found to be important and were classified in the same order as that resulting from the stepwise discriminant analysis.

A discriminant analysis was also performed on all the individuals, and the 22 substances designated by the stepwise discriminant analysis as being the main components distinguishing between the three categories of workers correctly classified (jack-knife method) 103 of the 120 individuals, i.e. 85%. A similar discriminant analysis on the whole set of cuticular hydrocarbons accurately classified (jack-knife method) 100 of the 120 individuals, i.e. 91%.

Upon performing a principal component analysis (Figure 3A) omitting the two colonies E (trigynous) and G (monogynous), which behaved like a monog-

TABLE 3. CLASSIFICATION OF MOST DISCRIMINANT CUTICULAR HYDROCARBONS OBTAINED BY PERFORMING STEPWISE DISCRIMINANT ANALYSIS (SAS SOFTWARE)

Cuticular hydrocarbons	Monogynous workers	Digynous workers	Trigynous workers
6,10-Dimethylhexacosane	+	++++	++++
5-Methylnonacosane		+	+++
8-Methylhexacosane		+++	++++
n-Heptacosane	+++	++	+
7-Methylpentacosane	++	+++	++++
4,10,14- and 4,12,16-Trimethylpentacosane	++	++++	++++

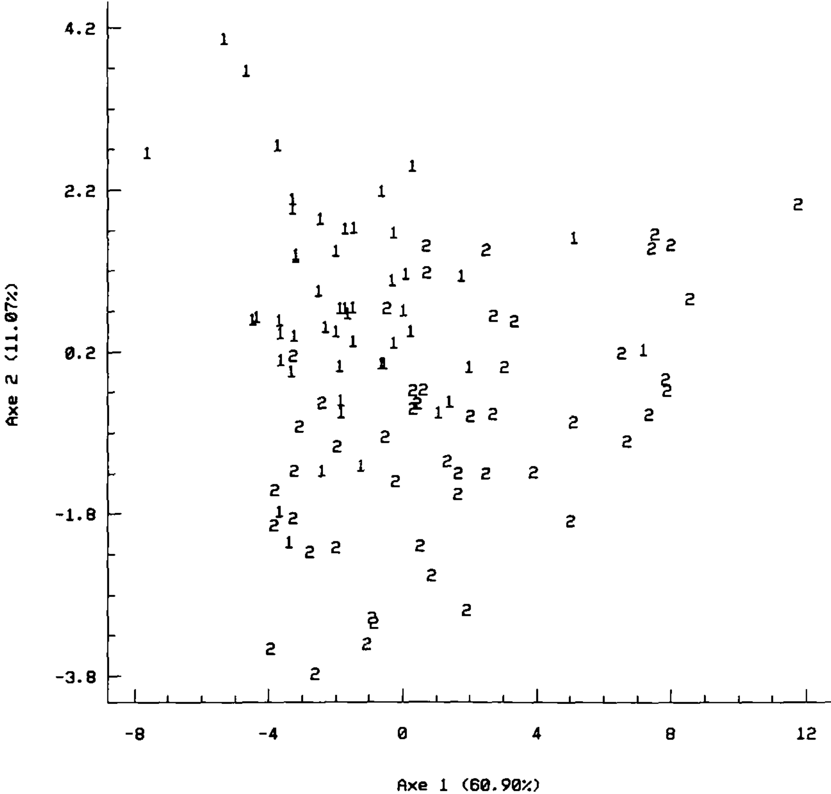

FIG. 3. Principal components analysis on 100 individuals and 22 cuticular hydrocarbons on which the most distinct mean values were obtained by the two categories of colony (monogynous and polygynous). (A) Plot of individuals on the factorial mapping 1–2 (horizontal axis = axis 1, vertical axis = axis 2), accounting for 72% of the total variation. 1 = individual from a monogynous colony; 2 = individual from a polygynous colony. (B) Correlation circle of the variables. The letter indicates the type of hydrocarbon involved: a = *n*-alkane, m = monomethylalkane, *d* = dimethylalkane, *t* = trimethylalkane. The number is that of the peak on the chromatogram in Figure 1.

ynous and a digynous colony, respectively, and selecting only variables on which the most distinct mean values were obtained by the two groups (50 monogynous and 50 polygynous) of individuals with which we are dealing in this part of the study, it was observed that the two groups tended to diverge along an axis that was a linear combination of the first two axes of inertia, amounting to 72% of the total variation. This separation occurred in the case

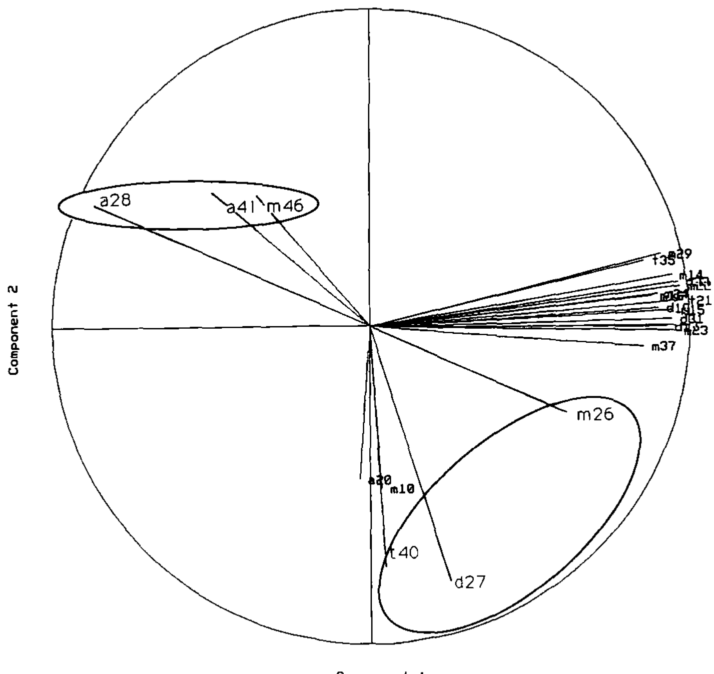

FIG. 3. Continued.

of a series of six substances (Figure 3B), three of which (*n*-heptacosane, *n*-nonacosane, and 3-methylnonacosane) characterized monogynous workers, while the other three (4-methylhexacosane, 6,10-dimethylhexacosane, and 8,12,16-trimethyloctacosane) characterized the polygynous individuals, again because of their higher proportions. Here, in the less stringent case of the distinction between two groups alone (monogynous and polygynous), two hydrocarbons (*n*-heptacosane and 6,10-dimethylhexacosane) sufficed to accurately classify 73 of 100 individuals.

DISCUSSION

The gas chromatography data obtained on the cuticular hydrocarbons of *Messor barbarus* foragers from monogynous and artificially polygynous (di- and trigynous) colonies showed the existence of differences in the proportions of some of the hydrocarbons, depending on the type of colony from which the workers originated. Among the cuticular hydrocarbon families constituting the

chemical signature of this ant species (*n*-alkanes, mono-, di-, and trimethylalkanes), 17 were found to characterize the monogynous colonies, usually because they were present in lower proportions. Another series of 15 hydrocarbons, some of which were the same as the above, characterized the individuals from digynous colonies, 12 of them because of their higher proportions. Lastly, 16 hydrocarbons characterized trigynous individuals, 11 of which were present in larger proportions. These differences involved more than half of all the hydrocarbons present on the workers' cuticles. The chemical signatures of workers from mono-, di-, and trigynous colonies can therefore be said to be different.

In a previous study (Provost and Cerdan, 1990), we observed the existence of a correlation between the number of queens in a colony and its degree of closure. Here we further establish the existence of a correlation between the presence of one or several queens and the cuticular hydrocarbon profiles of the foragers at their colonies. These data suggest that the structure of the various profiles is determined at least partly by the queens. We are not yet able to say, however, whether the queen intervenes by triggering changes in some of the cuticular hydrocarbons produced by the workers or whether she herself transmits particular substances to the workers with which she is in contact.

It should be pointed out that, beyond these quantitative differences, no qualitative differences between the cuticular profiles of the three categories of workers were observed in this study.

When making comparisons between odors from monogynous versus polygynous colonies, previous authors (such as Keller and Passera, 1989) have often suggested that the odor of polygynous colonies may be "less distinct" than that of monogynous colonies because the genetic make-up of the individuals in polygynous colonies is more variable. This may also explain why these individuals have only weak, if any, discriminatory capacities and therefore tend to be less aggressive. Vander Meer et al., (1990) have suggested that the lack of aggressive behavior observed between workers from polygynous colonies exposed to a wide variety of environmental and genetic cues might be attributable to their having a less restrictive template, rather than a strictly uniform cuticular profile.

In the present study, in addition to the lack of qualitative differences observed, a similar level of interindividual variability was found to exist in all three categories of colony, contrary to what might have been expected to occur in view of the greater genetic variability of the individuals belonging to polygynous colonies. This relative uniformity may have resulted from our sample of workers being too small or from the number of queens being too low for the genetic makeup of the individuals to be very variable. The initial group of foundress females also may have been genetically related, since they originated from the same population. Under these experimental conditions, the chemical signature of the polygynous workers, while differing from that of the monogy-

nous workers, may have been equally narrow. The polygynous workers were nevertheless less aggressive than the monogynous ones during the experimental exchanges (Provost and Cerdan, 1990). It is therefore possible that differences in the proportions of some of the cuticular hydrocarbons might constitute only part of the information used by these ants to distinguish between the members of monogynous and polygynous colonies.

In conclusion, although the differences observed in the present study between the chemical signatures of monogynous and polygynous colonies seem likely to be the cues used by monogynous workers to distinguish between workers of the two types, no explanation is available so far for the fact that these same workers were more aggressive to other monogynous than towards polygynous individuals.

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COMPARATIVE ANALYSIS OF ALLELOPATHIC EFFECTS PRODUCED BY FOUR FORESTRY SPECIES DURING DECOMPOSITION PROCESS IN THEIR SOILS IN GALICIA (NW SPAIN)

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Abstract—The development of toxicity produced by vegetable litter of four forest species (*Quercus robur* L., *Pinus radiata* D.Don., *Eucalyptus globulus* Labill, and *Acacia melanoxylon* R.Br.) was studied during the decomposition process in each of the soils where the species were found. The toxicity of the extracts was measured by the effects produced on germination and growth of *Lactuca sativa* L. var. Great Lakes seeds. The phenolic composition of the leaves of the four species was also studied using high-performance liquid chromatographic analysis (HPLC). It was verified that toxicity was clearly reflected in the first stages of leaf decomposition in *E. globulus* and *A. melanoxylon*, due to phytotoxic compounds liberated by their litter. At the end of half a year of decomposition, inhibition due to the vegetable material was not observed, but the soils associated with these two species appeared to be responsible for the toxic effects. On the other hand, the phenolic profiles are quite different among the four species, and greater complexity in the two toxic species (*E. globulus* and *A. melanoxylon*) was observed.

Key Words—Allelopathy, decomposition, litter, *Quercus robur* L., *Pinus radiata* D.Don., *Eucalyptus globulus* Labill, *Acacia melanoxylon* R.Br., phenolics.

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INTRODUCTION

Many metabolic products that may be involved in plant–plant chemical interactions are released from plants primarily through leachates from living aboveground parts and later by litter fall. These play a significant role in the plants' distribution.

Forest litter has been recognized as a possible cause for differences in germination and growth of plant species beneath trees of various species (Kuiters, 1989; Souto et al., 1992). Basanta et al., (1989), and Rigueiro and Silva-Pando (1983), who investigated the structure and diversity in the undergrowth of oak woods and other arboreal communities, concluded that there were great differences among them. In Galicia (NW of Spain), *Quercus robur* is an autochthonous species, and it is dominant in climax forests, whereas *Pinus radiata*, *Eucalyptus globulus*, and *Acacia melanoxylon* are foreign species, introduced for commercial purposes. The exotic character of these three species could play an important role in their toxic capacity (Rabotnov, 1974). Leachates also influence the number and behavior of soil microorganisms that affect the soil-forming processes, soil fertility and susceptibility, and immunity of plant species to pests (Gigon and Ryser, 1986).

Phenolic compounds have been considered responsible for allelopathic effects by various authors (Rice, 1984; Inderjit and Dakshini, 1991; Blum et al., 1991). Some authors state that phenolic compounds are present in the majority of the soils associated with forestry ecosystems (Kuiters and Denneman, 1987). Kögel and Zech (1985) found that the phenolic acid composition in the soil humus layer is determined to a high degree by the phenolic substances originating from leaf litter. Therefore, the identification of these types of compounds, particularly phenolics of low molecular weight and flavonoids, was attempted in the leaves of the species studied.

In this work, the allelopathic potential of four forestry species was investigated, and it was attempted to relate this effect to the low vegetative diversity in the undergrowth of some of these species. The study also attempted to see if interactive effects existed between soil leaf litter and microbiotic activity in each soil. Finally, the phenolic profiles of the leaves of the four species were compared before investigating the compounds that impart toxicity in the soils during decomposition of the litter.

METHODS AND MATERIALS

The plots studied were an autochthonous oak wood (*Quercus robur*), an eucalyptus crop (*Eucalyptus globulus*), an acacia crop (*Acacia melanoxylon*), and a pine crop (*Pinus radiata*), which, in the NW of the Iberian Peninsula where the study was carried out, are nonindigenous species.

The stands were situated within the coordinates UTM 535,420–4,748, 792 and 535,970–7,748,702 and between 250 and 290 m above mean sea level. The slope varied between 15% and 17%. The distance between them was 300 m, and they were found in an area where the parent material is granite with mica.

In January 1990 recently fallen leaves were collected and taken to the laboratory, homogenized, and air-dried until the tests were carried out. Dry weight was determined by drying in an oven at 100°C until a constant weight for three aliquots of each sample was obtained. This allowed calculation of the fresh weight equivalent to each dry weight.

Decomposition in Natural Soils. The equivalent of 10 g dry weight in fresh weight was put into nylon net bags with 2-mm-diameter pores (McCauley, 1975; Gloaguen and Touffet, 1980; Woods and Raison, 1982; Kelman and Lang, 1982). In this way, 72 bags were filled with plant material of each especies.

Bags were distributed in each stand (autochthonous oak wood, eucalyptus crop, acacia crop, and pine crop) as follows: six bags of each species were buried in the surface soil (first 15 cm) in three different places chosen at random. In total 288 bags were buried.

The litter bags were removed at 1, 7, 15, 30, 180, and 365 days after their burial. At the end of each period, a bag from each of the three points in the plot was removed at random, taken to the laboratory and homogenized so that the decomposing vegetable material could be put into distilled water for 24 hr in the dark at room temperature, keeping a 1 : 1 ratio of weight to volume (grams dry weight per milliliter).

Two other solutions were prepared from the initial extract by adding distilled water to form concentrations of 1 : 2 and 1 : 10 dilution. Measurements of pH and conductivity were taken and the extracts bioassayed with *Lactuca sativa* var. Great Lakes. Three Petri dishes (9 cm diameter) were prepared for treatment with Whatman 3MM paper sown with 50 seeds of the receptor species in each dish.

The dishes were watered with 4 ml of the corresponding solution and kept in an oven at 28°C and constant humidity. After 60 hr, the dishes were taken to a 4°C cold chamber for at least 4 hr in order to stop the growth of the seedlings, after which the percentage germination and length of the emergent radicles were calculated.

The effects produced on the germination and growth of *L. sativa* by distilled water and by macerated decomposing leaves of *Q. robur* buried in their own wood, (1, 7, 15, 30, 180, and 365 days) were taken as controls. There were no significant differences between them, so the last one was taken as a real control. This arboreal formation was considered as a climax stage in the studied area, including also the associated soil flora. The data represented in this work

are a function of the values obtained with oak in an oak woods and are assumed to be 100%.

The data were analyzed for variation using two-way ANOVA as well as an LSD multiple class test. By means of linear regression and correlation, the effects of pH and conductivity on germination and radicle growth were investigated in order to rule this out as the cause of the inhibitions observed. All the statistical analyses were carried out by the statistical program SPSS/PC+.

Extraction, Purification, and Identification of Phenolic constituents. The phenolic composition of leaves of four species was studied. Ten grams of plant material were powdered and shaken with 300 ml of methanol–water (80:20) for 24 hr at room temperature. The extracts were filtered, and the methanol was evaporated under reduced pressure at 28°C. The aqueous solution was extracted with 30 ml of diethilic ether three times, then evaporated to dryness in a vacuum evaporator and finally redisolved in methanol.

The samples were subjected to HPLC analysis. To identify flavonoids, a Hewlett Packard chromatograph equipped with a UV-Diodo Array detector was used. Identification of the compounds was done by using a reverse-phase Hypersil ODS C-18 (4 × 200) column with a 5-μm particle size. Extracts were analyzed using two mobile phases: A, methanol–phosphoric acid (999:1); and B, water–phosphoric acid (999:1). Linear gradients starting at 20% A and ending at 100% A were used over the first 40 min with an additional 5 min at 100% A. The flow rate of the mobile phase was 1 ml/min, and the eluate was analyzed at 250–400 nm.

To identify phenolics of low molecular weight, a Unicam chromatograph equipped with a UV-Diodo Array detector was used. Analyses were made by using a reverse-phase Waters Nova-Pak C-18 (3.9 × 300) column with a 4-μm particle size. Extracts were analyzed using two mobile phases: A, water–acetic acid (98:2); and B, water–methanol–acetic acid (68:30:2). Linear gradients starting at 100% A and ending at 20% A were used over the first 59 min, with an additional 6 min at 20% A. The flow rate of the mobile phase was 0.8 ml/min, and the eluate was analyzed at 210–400 nm.

Identification in both cases were based on retention times and UV spectra of authentic standards.

RESULTS AND DISCUSSION

Effects of Soil Vegetable Material. During the first 30 days of decomposition, the leaves from oak did not show any toxicity in any of the woods where they were buried (Figure 1). On the other hand, the eucalyptus macerates showed strong inhibitory effects both on radicle growth and germination of the seeds,

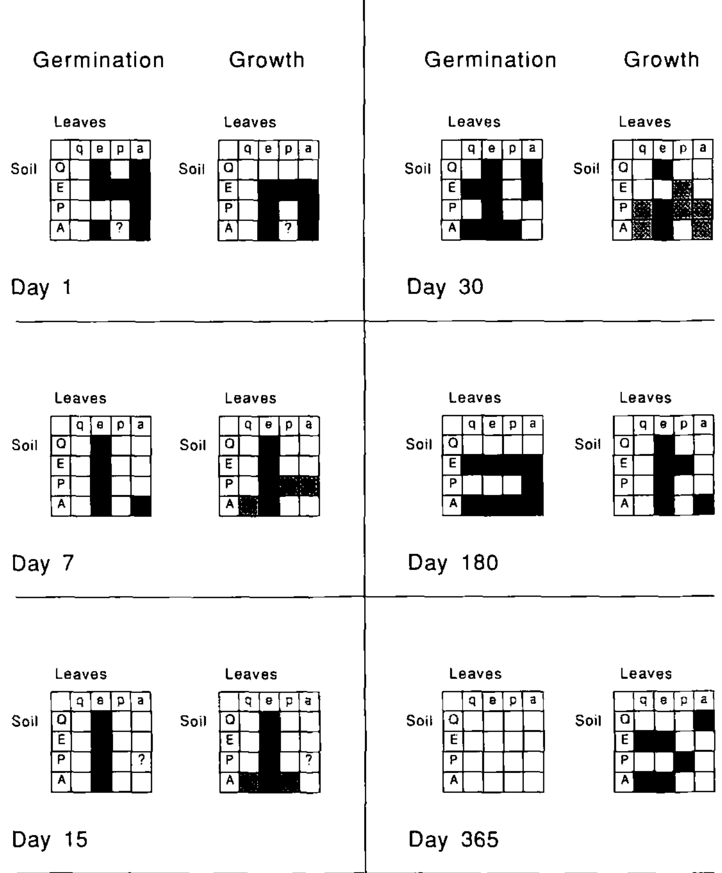

FIG. 1. *Concentration 1.* Evolution of phytotoxicity produced by leaves of four species in decomposition in four different soils, measured as inhibition of germination and growth of the radicles of *Lactuca sativa* seeds. On the horizontal axis, the kind of vegetable matter is represented; on vertical axis the soils where the residues are decomposing are shown. The black squares indicate inhibition with respect to the control (residues of *Quercus robur* decomposing in its own wood) and the grey squares indicate stimulatory effects with respect to the same control, with significant differences at the level of 5% in both cases. **q, e, p,** and **a** indicate leaves of *Quercus robur*, *Eucalyptus globulus*, *Pinus radiata*, and *Acacia melanoxylon*, respectively. **Q, E, P,** and **A** indicate soils where the decomposition process is that of *Quercus robur* wood and *Eucalyptus globulus*, *Pinus radiata*, and *Acacia melanoxylon* crops respectively.

which was independent of the soil where they were buried. This effect continued until 180 days in the case of radicle growth inhibition. Leaf litter from acacia induced strong inhibition (more than 80%) on growth and germination at the beginning of its decomposition.

After 30 days, strong inhibition due specifically to leaf litter was not found but was seen to be more a function of soil type where the decomposition takes place. Thus, even materials from oak and pine, which apparently do not produce toxic effects on their own, are capable of inhibiting germination when they decompose in eucalyptus stands (the case for oak) or in acacia stands (the case for both oak and pine).

At 180 days, the tendency observed 30 days after the start of decomposition was confirmed; that is, all the leaves from all the species show inhibition of germination when they decompose in either eucalyptus or acacia.

At 365 days after the start of decomposition, the inhibitory effects on germination disappeared; however, the toxicity on growth still remained. This seems to indicate that toxicity on germination precedes the inhibition on radicle growth, which is a similar result to that found by Reigosa et al. (1984). Ecologically, it would be more effective for toxic species to show initial inhibition on germination and later on emergent plant growth of plants that have escaped the first control inhibiting their development.

We thus see that eucalyptus and acacia are potentially toxic species. Although the acacia only shows release of toxins at the start of decomposition, it adds vegetable matter to the soil throughout the year. Thus, the toxic effect could be continuous and very important during the germination period of undergrowth species.

Once the starting vegetable material stops being the main cause of toxicity, the soils that induce toxicity are those associated with eucalyptus and acacia stands.

Interactive Effects. At the beginning of the decomposition process and 30 days after the start of the experiment, the interaction between the starting material and different microbiological activity is statistically significant (Table 1). We should also take note of the interaction that can be seen at 365 days only on radicle growth. These analyses indicate that the process of liberation of allelopathic compounds during decomposition does not just depend on the decomposing material and the microbiological activity of the soil, but also on the combination of both effects.

Effects of Concentrations 2 and 3. The results for concentration 2 (Figure 2 and Table 2) maintain the same pattern of variation as the more concentrated treatment, although the inhibitory effects are reduced slightly with respect to concentration 1. An obvious toxicity at the beginning of decomposition of acacia is again seen, which is the same as for the first 30 days in eucalyptus. In the same way, at 180 days the toxicity is basically due to the effect of the soil,

TABLE 1. TWO-WAY ANOVA FOR INHIBITORY EFFECTS OF MACERATED LEAVES DECOMPOSING IN FOUR DIFFERENT SOILS, CONCENTRATION 1 : 1[a]

	Germination			Growth		
Day	Leaves	Soil	Interaction	Leaves	Soil	Interaction
1	***	***	***	***	**	**
7	***	NS	*	***	NS	*
15	***	NS	NS	***	NS	**
30	***	***	***	***	***	***
180	*	***	NS	***	***	NS
365	*	NS	NS	NS	**	***

[a] $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; NS, no significant differences.

where decomposition continues but toxic effects, after a year, are practically nonexistent.

At concentration 3 (Figure 3 and Table 3) a clear predominance of neutral effects is seen. However, despite the strong dilution of the macerate, there is an evident initial difference due to the starting material, where acacia and eucalyptus are clearly toxic. During the decomposition process, this effect is diluted, showing toxicity caused by the soil where the decomposition takes place at the end. As in the more concentrated dilutions toxicity is seen in solutions associated with the eucalyptus and acacia stands.

Phenolic Composition. The phenolic contents in the samples analyzed are shown in semiquantitative mode in Table 4. Large differences between species were observed. Thus, more complex profiles are seen in samples of *E. globulus* and *A. melanoxylon* than in *P. radiata* and *Q. robur*. In the extracts of eucalyptus, gallic, vanillic, ferulic, 3,4,5-trimethoxybenzoic, and ellagic acids; 4-hydroxybenzaldehyde; 4-hydroxyphenethyl alcohol; 4-hydroxy-3-methoxybenzyl alcohol; quercitrin; and quercetin were identified. Due to UV spectra, ellagitannins, flavonols and some flavanones also were observed, although it was not possible to determine their structures. Vanillic and ferulic acids such as vanillin, 4-hydroxy-3-methoxybenzyl alcohol, quercetin-3-glycoside (possibly rutin), quercitrin, luteolin, and apigenin were identified in acacia. Flavonols, flavones, and flavanones were also detected by their UV spectra. Vanillic, *p*-coumaric, ferulic, and ellagic acids; 4-hydroxy-3-methoxybenzyl alcohol; vanillin, quercitrin, and toxifolin were also identified in pine. Flavone was furthermore detected in its spectrum. In oak extracts, 3,4-dihydroxybenzoic, vanillic, and ellagic acids were detected as were 4-hydroxybenzaldehyde, quercetin, and kaempferol.

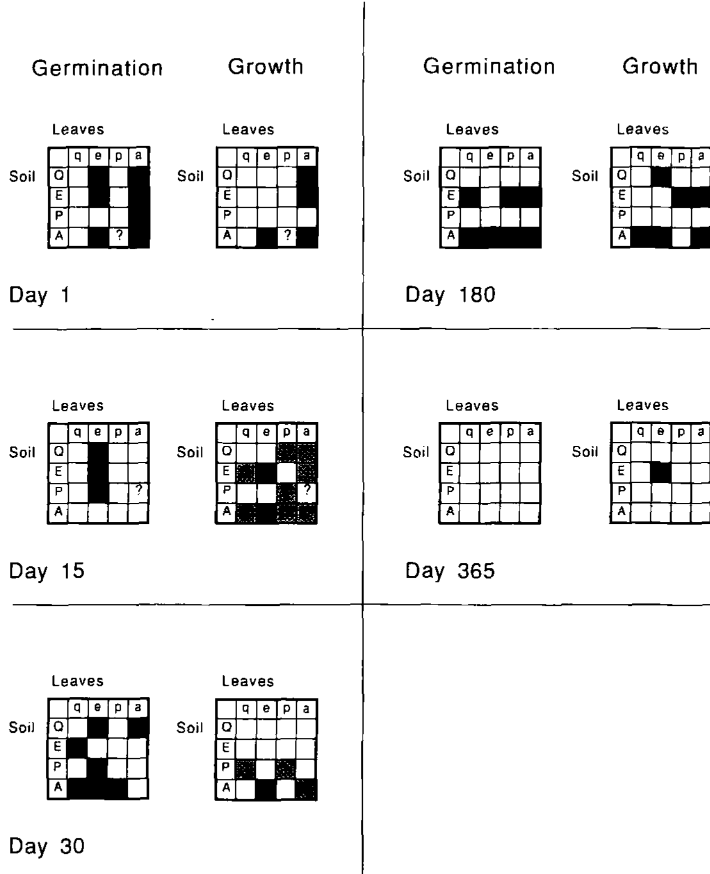

FIG. 2. *Concentration 2.* Evolution of phytotoxicity produced by leaves of four species in decomposition in four different soils, measured as inhibition on the germination and growth of the radicles of *Lactuca sativa* seeds. On horizontal axis, the kind of vegetable matter is represented; on vertical axis, the soils where the residues are decomposing are shown. The black squares indicate inhibition with respect to the control (residues of *Quercus robur* decomposing in its own wood) and the grey squares indicate stimulatory effects with respect to the same control, with significant differences at the level of 5% in both cases. Letter symbols are as in Figure 1.

TABLE 2. TWO-WAY ANOVA FOR INHIBITORY EFFECTS OF MACERATED LEAVES DECOMPOSING IN FOUR DIFFERENT SOILS, CONCENTRATION 1:2[a]

	Germination			Growth		
Day	Leaves	Soil	Interaction	Leaves	Soil	Interaction
1	***	***	***	***	*	**
15	***	NS	NS	***	*	***
30	***	***	***	***	**	**
180	NS	***	NS	NS	**	**
365	NS	NS	NS	NS	*	*

[a] $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; NS, no significant differences.

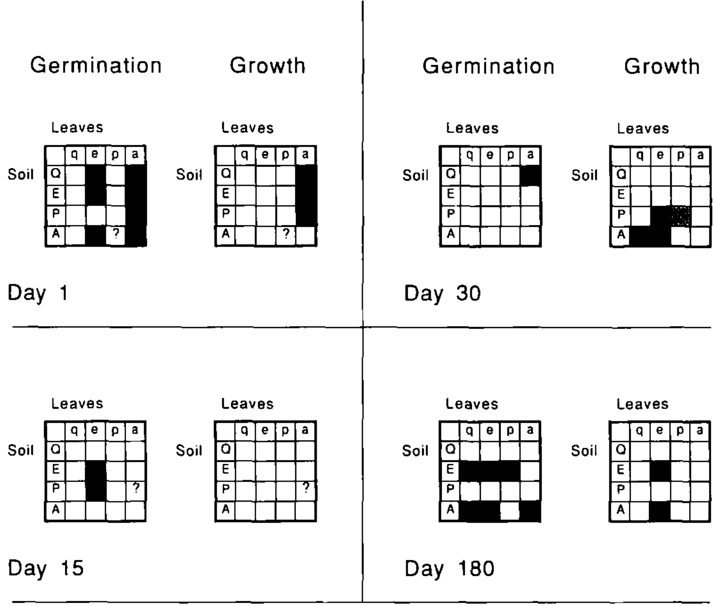

FIG. 3. *Concentration 3.* Evolution of phytotoxicity produced by leaves of four species in decomposition in four different soils, measured as inhibition on the germination and growth of the radicles of *Lactuca sativa* seeds. On horizontal axis, the kind of vegetable matter is represented; on vertical axis, the soils where the residues are decomposing are shown. The black squares indicate inhibition with respect to the control (residues of *Quercus robur* decomposing in its own wood) and the grey squares indicate stimulatory effects with respect to the same control, with significant differences at the level of 5% in both cases. Letter symbols are as in Figure 1.

TABLE 3. TWO-WAY ANOVA FOR INHIBITORY EFFECTS OF MACERATED LEAVES DECOMPOSING IN FOUR DIFFERENT SOILS, CONCENTRATION 1 : 10[a]

	Germination			Growth		
Day	Leaves	Soil	Interaction	Leaves	Soil	Interaction
1	***	NS	**	***	NS	NS
15	NS	NS	NS	NS	NS	NS
30	***	***	***	NS	NS	***
180	NS	***	NS	NS	***	NS

[a] $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; NS, no significant differences.

TABLE 4. PHENOLIC COMPOSITION OF LEAVES OF FOUR FOREST SPECIES: *Quercus robur, Eucalyptus globulus, Pinus radiata*, and *Acacia melanoxylon*[a]

	Compound	Oak	Eucalyptus	Pine	Acacia
1	Gallic acid	−	+ + + + +	−	−
2	3,4,-Dihydroxybenzoic acid	+	−	−	−
3	Vanillic acid	+	+	+	+ +
4	4-hydroxybenzaldehyde	+	+	−	−
5	4-hydroxyphenethyl alcohol	−	+ +	−	−
6	4-hydroxy-3-methoxybenzyl alcohol	−	+	+ + + +	+ + + + +
7	p-coumaric acid	−	−	+	−
8	Vanillin	−	−	+	+
9	Taxifolin	−	−	+ +	−
10	Ferulic acid	−	+	+	+
11	3,4,5,-Trimethoxybenzoic acid	−	+ +	−	−
12	Quercetin 3-Glycoside	−	+	+	+ + +
13	Ellagic acid	+ + + +	+ + + + +	+ +	−
14	Quercitrin	−	+ + +	+ +	+ +
15	Ellagitannins	+ +	+ + + +	−	−
16	Quercetin	+ + +	+ + +	−	−
17	Kaempferol	+ + +	−	−	−
18	Luteolin	−	−	−	+ + + + +
19	Apigenin	−	−	−	+ + + + +

[a] Semiquantitative values based on the relative intensity of the chromatographic peaks.

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INADVERTENT INTRODUCTION OF SQUALENE, CHOLESTEROL, AND OTHER SKIN PRODUCTS INTO A SAMPLE

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Abstract—Recent developments in analytical techniques permit the chemical ecologist to achieve identification of naturally occurring compounds with relatively small amounts of the products of interest. However, the microanalytical techniques employed frequently require the handling of sample vials and other transferral instruments such as syringes and micropipets, where the analyst's hands come into close contact with the sample. Here we show how inadvertent contamination of a sample with skin lipids can occur simply by catching a 1-ml sample vial by the neck rather than the base or by activating a syringe by holding the plunger extension between the fingers rather than taking it by the head. Squalene, cholesterol, and, to a lesser extent, hydrocarbons and fatty acids from fingers are easily introduced into the sample in this manner. These findings are particularly relevant for a parasitology laboratory such as ours, investigating the function of vertebrate-derived products in hematophagous arthropods.

Key Words—Squalene, cholesterol, skin lipids, contamination, sample contamination.

INTRODUCTION

Modern methods in analytical chemistry permit the chemical ecologist to work with very small amounts of extracts of biological origin for purposes of identification of naturally occurring products. The work is normally carried out with a minimum of solvent, as it is well known that the latter may contain some pollutants such as phthalates which, on concentration of an extract, may become so predominant as to obscure the product(s) of interest during analysis. Added

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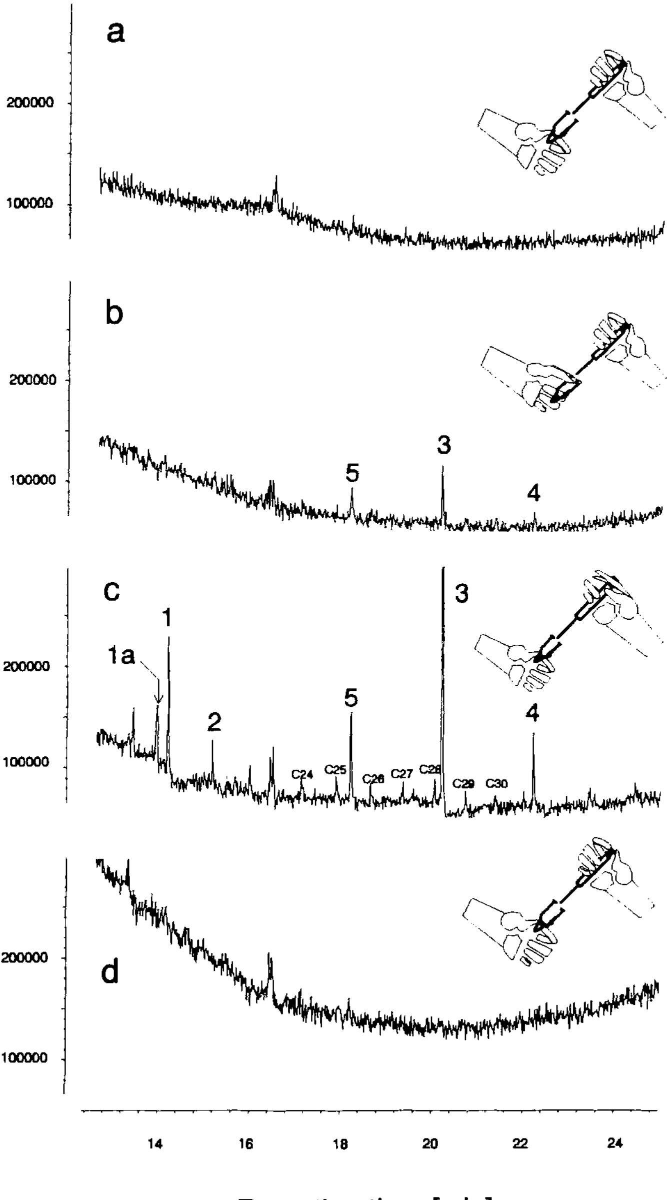
to this is the obligation of keeping the use of solvents to a minimum in order to avoid environmental contamination and to reduce costs of elimination to a minimum. However, micro-methods require handling of syringes, micropipets, small sample vials, and other substrates. In the process, the investigator's fingers will frequently come near the solvent via the containers holding it. Here we show how some commonly occurring skin products from fingers can inadvertently find their way into a sample.

METHODS AND MATERIALS

Contamination. Contamination by fingers was investigated by manipulation of a small volume of solvent in a sample vial with a syringe in three different ways (Figure 1), and in addition, by purposely touching a piece of filter paper that was subsequently extracted and analyzed. One milliliter of chloroform (Merck, analytical grade) was introduced via a glass–PTFE dispenser (10 ml Repipet Dispenser, Labindustries, California), permanently mounted on the $CHCl_3$ bottle, into a 1.1-ml tapered borosilicate screw-top vial (Chromacol, U.K.) held as specified below (a–d) and concentrated to dryness under a nitrogen stream. The Pasteur pipet, from which the nitrogen stream issued, was connected via stainless steel tubing and connections with PTFE ferrules to a fat-free metal bellows manometer on the nitrogen cylinder. The chloroform was remarkably pure, for even when it was concentrated 10,000 times, no trace of any of the products was detected. Then, 100 μl of chloroform were added with a 500-μl syringe (Hamilton 750 series microliter syringe, Bonaduz, Switzerland) to rinse down the walls of the vial and the solvent was again concentrated to dryness. The position of the fingers on the vial and syringe was varied systematically during four different manipulations to wash down the walls of the vial (Figure 1): (a), the vial was held by the bottom with the left hand, and the syringe plunger was activated by holding the head between the index finger and thumb

FIG. 1. GC-MS analysis of a MSTFA-derivatized chloroform sample held in a 1.1-ml tapered vial and manipulated with a 500-μl syringe. Dioctyl phthalate (5), squalene (3), and cholesterol (4) are introduced into the sample by holding the vial by the neck (b) rather than at the base (a). Activation of the syringe by holding the plunger extension between the index and middle fingers (c) leads to even more significant contamination of the sample with the skin products squalene and cholesterol, in addition to palmitoleic acid (1a), palmitic acid (1), stearyl alcohol (2), and the alkane series C_{24}–C_{30}. Activation of the plunger by the head and holding the vial by the base avoids this inadvertent contamination of the sample (a and d). For details of the derivatization and analytical procedures see Methods and Materials. (b) Peak 3 represents ca. 1 ng, and (c) 10 ng.


Abundance
a
200000
100000
b
200000
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5
3
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c
1
1a
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4
C24
C25
C26
C27
C28
C29
C30
d
200000
100000
14
16
18
20
22
24
Retention time [min]

of the other hand (the remainder of the fingers being employed to hold the barrel of the syringe); (b) the same as in "a", but holding the vial by the neck; (c) the vial was held as in "a", but the syringe plunger was activated by holding it between the index and middle fingers just under the head, i.e., on the sleeve, designed for this purpose, 10 mm long on the plunger extension just under the head, which does not enter the barrel; (d) manipulations as in "a" (control).

The same sample vial was used in the four manipulations but washed between manipulations with $CHCl_3$ and dried by heating in an oven at 110°C. The syringe was rigorously washed between successive manipulations by removing the plunger and washing it down with chloroform-soaked tissue and by evacuating several milliliters of chloroform through the syringe using a vacuum applied to the barrel. Each time, the dried-out content of the vial was redissolved in 10 μl $CHCl_3$ using the 25-μl syringe employed for derivatization (below). This syringe, and the 10-μl one used for injection of the sample for GC-MS analysis, were always activated holding the plunger by head.

The consequences of handling a piece of filter paper with the fingers was investigated by first washing a filter paper disk held by forceps (Schleicher & Schuell, 4.5-cm diam.) in some 10 ml of $CHCl_3$, and then drying at 110°C in the oven. Two small pieces of the disk (ca. 4 × 7 mm) were cut with $CHCl_3$-cleaned scissors. One piece was held for about 3 sec between the index and thumb (which had been unwashed for 1 hr), then introduced with forceps into the sample vial described above and extracted for 10 min in 200 μl $CHCl_3$ by manipulating the syringe and vial as in "a" above. The piece of filter paper was then removed with forceps and the extract concentrated under nitrogen to dryness. Then 10 μl of $CHCl_3$ was added with the 25-μl syringe to rinse down the walls of the vial and to redissolve the extract for derivatization. The second piece of filter paper was treated as the first but without holding it between the fingers, thus serving as control for the extraction and derivatization procedures.

Derivatization. Ten microliters of *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA, Fluka puriss) were added with a 25-μl syringe (Hamilton) to each of the six redissolved samples described above and the mixture held for 1 hr at room temperature under nitrogen. In this manner, all products with alcohol and acid functions are derivatized as trimethylsilyl esters. These less polar derivatives were more efficiently resolved by capillary gas chromatography (below). The sample was then brought to dryness under nitrogen and collected from the tapered vial in 2 μl $CHCl_3$ with a 10-μl Hamilton syringe for GC-MS analysis.

Gas Chromatography-Mass Spectrometry (GC-MS). Derivatized samples were analyzed by GC-MS (Hewlett Packard 5890 series chromatograph linked to a HP 5971A mass selective detector), by injecting the 2 μl of derivatized sample on-column onto a DB-5HT nonpolar high-resolution fused-silica capillary column (15 m, 0.25 mm ID 0.10-μm film thickness, J & W Scientific,

California) equipped with a precolumn (1 m deactivated fused-silica) and connected via a 1-m deactivated fused-silica capillary (0.25 mm ID) to the MS (ionization chamber temperature 300°C; ionization energy 70 eV). The detector, operating in the El mode, scanned for masses of 30 to 650. Helium was used as carrier gas under constant flow (velocity 40 cm/sec at 60°C). The components of the sample were identified by comparing the mass spectra of unknowns with those of standards in the computer-based library of the GC-MS associated HP chemstation and by comparison of retention times of unknowns with those of standards, derivatized and underivatized, injected under the same conditions as the derivatized samples.

RESULTS

Once the fingers were brought into contact with the mouth of the 1.1-ml vial containing the 1-ml solvent sample, both squalene and cholesterol were systematically found in the analysis of the concentrate, as well as increased levels of dioctyl phthalate (Figure 1b). Grasping the vial by the base while activating the plunger of the 500-μl syringe by holding the sleeve on the plunger extension led to the greatest contamination of the sample (Figure 1c). Some 10 times more squalene and cholesterol was found in the concentrate in this case. In addition, other products were also detected in the concentrate such as palmitic acid, palmitoleic acid, stearic alcohol, dioctyl phthalate, the saturated hydrocarbon series C_{24}–C_{30}, and wax esters, all at levels some 50% that of cholesterol. By contrast, simply holding the vial by the base and the syringe plunger by the head avoided all such pollution of the concentrate (Figure 1a and d).

Proof that the source of these commonly occurring chemicals is the fingers of the analyst was obtained by investigating the extract of the piece of filter paper that had been purposely handled. Here, palmitic acid, squalene, and cholesterol again predominated in the multicomponent extract (Figures 2a and 3). This extract also contained the alkane series C_{20}–C_{34}, stearyl alcohol, the fatty acids palmitoleic, stearic and oleic acids, the wax esters palmityl and stearyl palmitate, palmityl, stearyl, arachidyl, behenyl, and lignoceryl palmitoleate, along with some steroids and other unidentified compounds, all at levels some 5–30% that of cholesterol. Overall, direct contact with the fingers yielded quantities of the products some 10 times higher than those obtained by manipulating a solvent sample with fingers on the plunger sleeve of the syringe (''b'' above). By contrast, the extract of the untouched piece of filter paper (control) contained only a trace of the saturated C_{16} acid (Figure 2b).

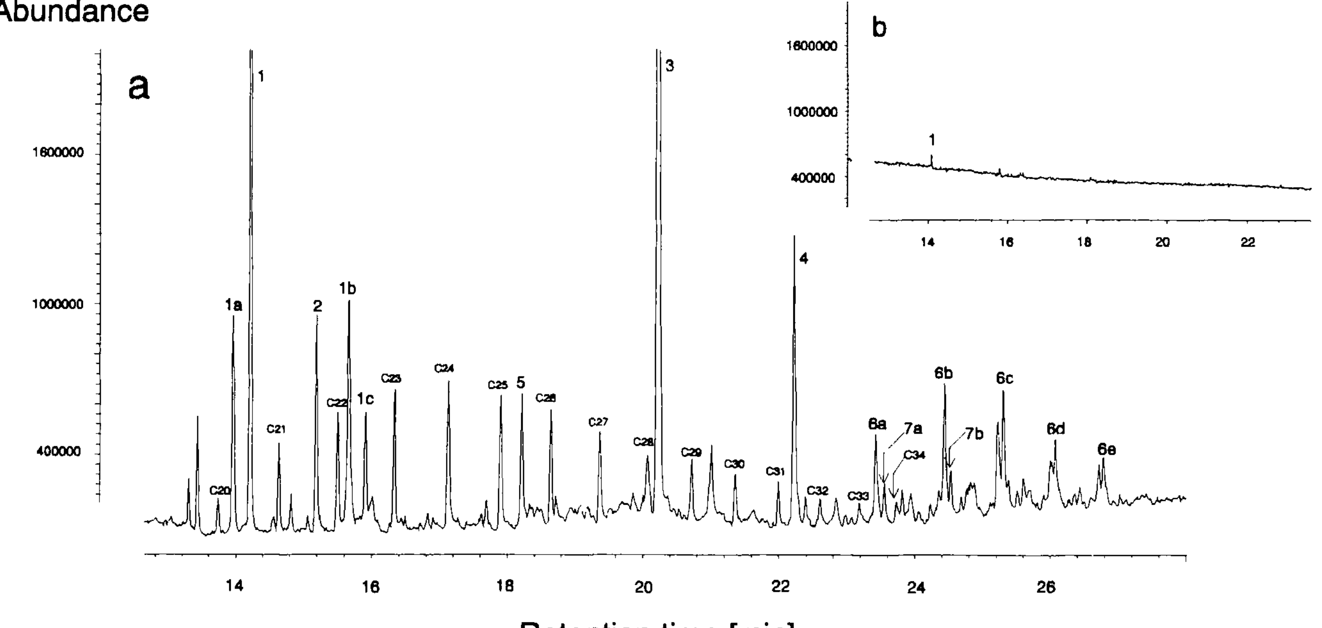

FIG. 2. GC-MS analysis of a MSTFA-derivatized chloroform extract of two 28-mm^2 pieces of filter paper. One was held between index and thumb for 3 sec (a) and the other left untouched (b). The major skin components that impregnated the filter paper in "a" are squalene (3), palmitic acid (1), and cholesterol (4). Other skin products detected by the mass selective detector include palmitoleic acid (1a), oleic acid (1b), stearic acid (1c), stearyl alcohol (2), the alkane series C_{20}–C_{34}, dioctyl phthalate (5), and the wax esters palmityl palmitate (7a), stearyl palmitate (7b), palmityl palmitoleate (6a), stearyl palmitoleate (6b), arachidyl palmitoleate (6c), behenyl palmitoleate (6d), and lignoceryl palmitoleate (6e). For details of the derivatization and analytical procedures see Methods and Materials. Peak 3 represents ca. 100 ng.

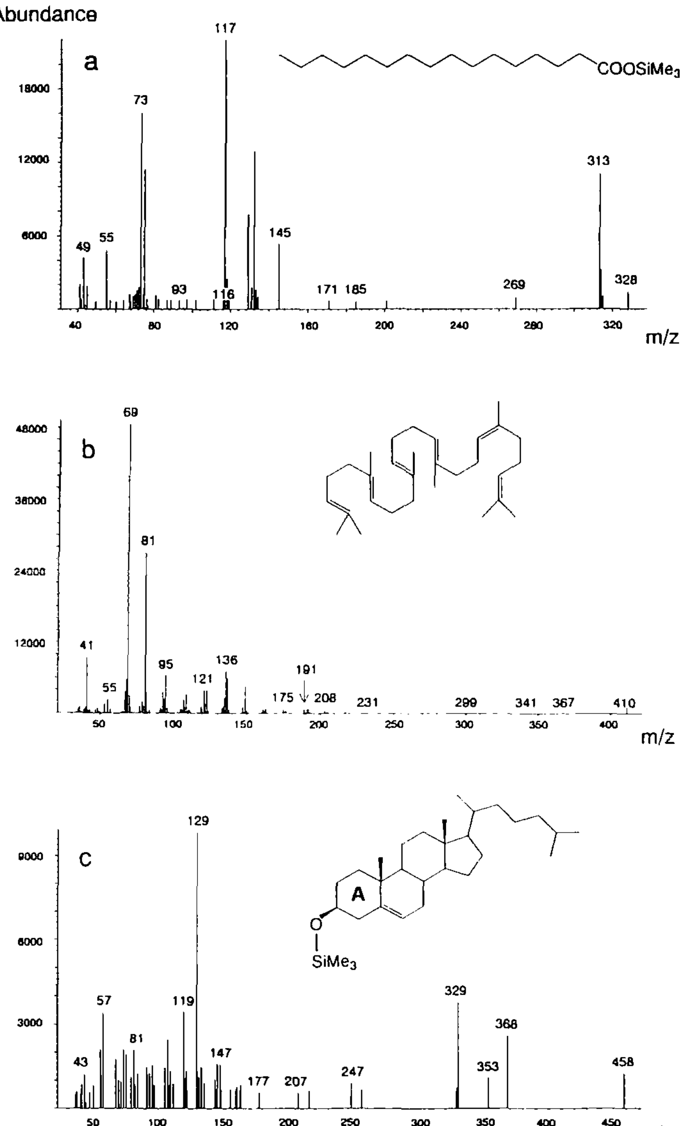

FIG. 3. Mass spectra of the major constituents of the MSTFA-derivatized chloroform samples. (a) Trimethyl silyl derivative of palmitic acid with $M^{+\cdot}$ 328, intense key fragment ion *m/z* 313 ($M^{+\cdot} - 15$) and base peak *m/z* 117 of $(CO_2SiMe_3)^+$; (b) squalene with a weak $M^{+\cdot}$ 410 and key fragment base peak *m/z* 69 of $(C_5H_9)^+$; and (c) the trimethyl silyl derivative of cholesterol with $M^{+\cdot}$ 458, the key fragment *m/z* 368 ($M^{+\cdot} - 90$) due to loss of $(OSiMe_3)^+$, *m/z* 329 ($M^{+\cdot} - 129$) and base peak *m/z* 129 of $(C_3H_4OSiMe_3)^+$ after cleavage of the A ring.

DISCUSSION

The possibility that a sample could be contaminated by material from the analyst's fingers was brought to our attention by the presence of squalene, and other products of vertebrate origin, in quite diverse samples. All of the products detected in the samples analyzed in this study are well-known constituents of skin (Biedermann and Grob, 1991; Downing et al., 1981, 1987; Liu et al., 1976). Squalene (2,6,10,15,19,23-hexamethyl-2,6,10,14,18,22-tetracosahexaene) is well known to analytical chemists as a contaminant in handled samples. It is a precursor of cholesterol synthesis in vertebrates. However, one would never have thought that levels up to 1 ng of this product could be transferred to the pure solvent sample simply by placing one's fingers at the mouth of the vial, at some 9 mm from the solvent surface. Admitting that the solvent underwent a ca. 1000-fold concentration step, levels of squalene at over 10 ng/μl in the concentrated sample where the plunger was held by the sleeve are such as to be visible in most routine analyses. Evidently, evaporation and recondensation of chloroform within the vial (as with the volatiles along the walls of a glass of brandy) suffices to recover the skin materials, but what happens by holding the syringe plunger by the sleeve is even more dramatic. Here the solvent creeps up within the barrel on repeated movements of the plunger of the 500-μl syringe, and this solvent evidently comes into close enough contact with the sleeve to pick up skin products every time the plunger is pushed home. Mixing of this solvent around the plunger with that in the barrel of the syringe is apparently sufficient to contaminate the sample being manipulated.

The levels of squalene on the fingers are indeed very high, as indicated by the amounts of about 100 ng recovered from the piece of filter paper that was purposely handled for just 3 sec. The same experiment made with fingers that had been unwashed for several hours showed an amount of more than 1 μg. Levels of 60 μg were recovered by Biedermann and Grob (1991) from one finger tip extracted with hexane. These authors demonstrate solvent contamination with the same type of products described here via inadvertent touching of glassware. The high levels of squalene detected are not very surprising. A maximum of about 475 μg/g dry weight has been measured in human skin, but the amounts of squalene contained in the blood or secreted by the skin of humans will vary enormously with diet (Liu et al., 1976). Wearing latex gloves will hinder the inadvertent introduction of skin products, but the consequence is the introduction of phthalates from the latex into the sample. Other substances reported from the skin surface of humans include branched fatty acids, phospholipids, cerebrosides, ceramides, sterol esters, and triglycerides (Downing et al., 1987; Melnik et al., 1989).

Inadvertent pollution of a sample is a bothersome occurrence for any analyst. However, the contamination described here is particularly acute for labo-

ratories investigating the origin and function of natural products of mammalian origin. In this laboratory, for example, we investigate the role of semiochemicals for hematophagous arthropods. These organisms do not synthesize products such as squalene and cholesterol, but may sequester these products or their derivatives for a number of essential functions. For example, cholesterol esters are employed as pheromones in ticks (Hamilton et al., 1989). Host-derived arachidonic acid (20:4) is concentrated in the salivary glands of ticks, presumably as a precursor of prostaglandins, which are employed by ticks to overcome hemostasis, inflammatory responses, and host immunity (Shipley et al., 1993; Bowman et al., 1994). Ascribing particular amounts of products associated with human skin to the parasite, or to one of its organs, can at most only be tentative if the possibility of inadvertent contamination of the sample by the investigator has not been eliminated, i.e., with an adequate control.

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Book Review

Host-Plant Selection by Phytophagous Insects. E.A. Bernays and R.F. Chapman. New York: Chapman & Hall, 1994. 312 pp.

The field of insect–plant relationships has long been one of the most prominent components of chemical ecology and is of interest to a great diversity of scientists, ranging from agricultural entomologists to evolutionary ecologists. The importance of plant defenses against insects has long been recognized in the development of resistant crop varieties, and insects may have played a major role in the evolution of these defenses in plants. Many reviews and books on the subject of insect–plant relationships have been published throughout the years. However, this book is the first to deal exclusively with the topic of host plant selection, which is the most critical event mediating the development of host ranges and patterns of host use by insects.

The book is eloquently written, well organized, and easy to read. The language is simple enough and the terminology is adequately explained so that readers in a wide range of disciplines should find it both informative and interesting. It should be useful to students at all levels as well as to teachers and researchers who need a general view of the subject as well as background information and reference material for further reading.

Bernays and Chapman have emphasized the central role of behavior in host-plant selection. They have drawn on their extensive experience with grasshoppers, along with examples from a wide range of phytophagous insects, to illustrate the factors involved. The first chapter describes the degree of specificity that results in observed patterns of host-plant use by phytophagous insects. This includes specialization on different plant parts and logically leads to questions about the mechanisms by which insects find the appropriate plant (or plant part) that are addressed in subsequent chapters. The second chapter provides an excellent review of plant chemistry, which is the key to many interactions with insects. The importance of volatiles, surface waxes, nutrients, and internal secondary compounds is highlighted. The variability in plant chemistry is emphasized and the nutritional and environmental factors contributing to this variation are discussed. The next chapter describes the sensory systems involved in the perception of behaviorally important chemicals by insects. Clear illustrations are used to show how the senses of smell, taste, touch, and sight work to provide the information leading to host selection behavior. Chapter 4 then deals with the actual behavior involved in host finding and the subsequent acceptance of a

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specific plant. The behavioral events, including orientation, oviposition, and feeding are described in some detail, with numerous examples illustrating the key role of specific chemicals as well as physical characteristics of the plant. A section devoted to the behavioral sequences that occur in the field was particularly appropriate, as most of our knowledge in this area is based on laboratory experiments. In Chapter 5, the impact of ecology and physiology on behavior is discussed. The effects of habitat and other plants, interactions with other insects or organisms, and abiotic factors are considered. The physiological state of the insects is also discussed as it contributes to variation in motivation and subsequent behavior of individuals. The effects of insect experience, including habituation, sensitization, learning, and induction of food preferences are outlined in Chapter 6, and some of the neural phenomena are summarized. The next chapter then examines the effects of genetic variation, both within populations and between populations, and possible mechanisms of behavioral variability are proposed. The final chapter addresses the evolution of host range. The roles of insect characteristics, resource availability, plant phenology and chemistry, and interactions among insects are presented.

The authors are to be commended for their generous use of diagrams, graphs, and tables to support their text. These are generally very easily absorbed by the reader and serve a useful purpose in clarifying many of the examples. The authors have chosen not to cite references in the text so that the flow of language would not be interrupted. At the end of each chapter, a list of selected references is given, along with suggested reading, which is conveniently organized according to subject matter. However, on several occasions, I was curious about the sources of information mentioned in support of certain arguments and was somewhat frustrated by the fact that I could not identify the original references. I would have preferred a numbering system that presents the reader with a minimal level of distraction in return for a complete listing of references. However, this is a personal view, and ease of reading for students must be a major consideration.

The book is very well conceived and the information is logically presented. It is remarkably free of errors and flows well. Bernays and Chapman have excelled in their own research over the years, and this compilation of facts and ideas provides an opportunity for all students and practitioners in the field of plant–insect interactions to benefit from their experience. For plant scientists and entomologists interested in plant resistance mechanisms or the evolution of plant–insect associations, this book will be a valuable source of information. As the only text that is entirely devoted to the subject of host selection by phytophagous insects, it is highly recommended as quality reading material and a very useful reference.

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(Z,Z)-5,27-TRITRIACONTADIENE: MAJOR SEX PHEROMONE OF *Drosophila pallidosa* (DIPTERA; DROSOPHILIDAE)

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Abstract—A crude cuticular extract from both sexes of 3660 fruit flies (*Drosophila pallidosa*) was subjected to SiO_2 and $AgNO_3/SiO_2$ column chromatography, accompanied by bioassay for the sex pheromone activity After three chromatographic steps, the active fraction was obtained. The main component of the active fraction was determined to be (*Z,Z*)-5,27-tritriacontadiene [(*Z,Z*)-5,27-$C_{33:2}$], on the basis of gas-liquid chromatographic analysis, chemical derivatization and gas chromatography-mass spectrometry. Synthetic (*Z,Z*)-5,27-$C_{33:2}$ at 5 female equivalents (FE) elicited a clear courtship response with a high courtship index among *D. pallidosa* males. Therefore it was concluded that (*Z,Z*)-5,27-$C_{33:2}$ was a major sex pheromone component in this species

Key Words—*Drosophila pallidosa*, Diptera, Drosophilidae, sex pheromone, cuticular hydrocarbon, (*Z,Z*)-5,27-tritriacontadiene, courtship behavior, fruit fly

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INTRODUCTION

Drosophila pallidosa is a species belonging to the *D. ananassae* complex, in which a total of 10 species are included (Tobari, 1993). *D. ananassae* is widely distributed over tropical and subtropical areas. In contrast, *D. pallidosa* has restricted habitats in Fiji, Tonga, and Samoa, where *D. ananassae* can also be found (Futch, 1973; Tobari, 1993). Three color forms of the abdomen are known in *D. ananassae*: light, dark, and cosmopolitan (Futch, 1966). *D. pallidosa* has been described as a new species from the light form of *D. ananassae* (Bock and Wheeler, 1972). F_1 hybrids were obtainable from both species and were fertile in the laboratory (Futch, 1966, 1973). Male *D. pallidosa* actively courts female *D. ananassae* and even tries to copulate (Doi et al., unpublished). These facts suggest that chemicals on female *D. ananassae* play a role in eliciting courtship behavior among male *D. pallidosa*, and there must be some pheromone exchanges between both sexes in these species.

Sex pheromones have been demonstrated in three species of *Drosophila* (Diptera: Drosophilidae); *D. melanogaster*, *D. similans*, and *D. virilis*. Compounds related to these species are (*Z*,*Z*)-7,11-heptacosadiene, (*Z*)-7-tricosene, and (*Z*)-11-pentacosene, respectively (Jallon, 1974; Oguma et al., 1992a). In *D. melanogaster*, however, behavioral responses of male to the natural pheromone and synthetic compounds were surveyed and studied, revealing that synthetic (*Z*,*Z*)-7,11-heptacosadiene and (*Z*)-7-pentacosene, compared to natural forms, gave dose–response curves that suggested the presence of unknown synergistic substance(s) in the natural pheromone blend (Antony et al., 1985). Several alkenes and alkadienes including 7-pentacosene and 7,11-heptacosadiene, which were possible sex pheromones, were studied in the *D. melanogaster* species subgroup (Cobb and Jallon, 1990). In *D. virilis*, (*Z*,*Z*)-5,13- and (*Z*,*Z*)-5,15-pentacosadiene and (*Z*,*Z*)-7,15-heptacosadiene elicited a courtship response in males at higher dose than the natural amount in the female fly. The mixtures of these alkadienes with (*Z*)-11-pentacosene have reproduced all elements of courtship behavior such as orientation, following, wing display, and licking (Oguma et al., 1992b).

In this paper, we report the isolation and identification of the sex pheromone in female *D. pallidosa*.

METHODS AND MATERIALS

Flies and Rearing. The *Drosophila pallidosa* isofemale strain was established as a laboratory stock (NAN4) in 1981 at Lautoka, Fiji. Flies were cultured in glass bottles (180 ml) containing standard *Drosophila* medium (sucrose–dried yeast–corn meal) and under a regime of 25 ± 1°C, 55% relative humidity, and

a 14-hr light (0700–2100 hr)–10-hr dark light cycle. Virgin flies were collected within 12 hr of emergence under light CO_2 anesthesia.

Male flies were aged individually in glass vials (3 cm ID, 10 cm height) for five to seven days with two transfers of the food vial containing standard *Drosophila* medium. Their courting activities were checked by exposing each male to a freshly freeze-killed virgin female (5 to 7 day old). If the courting behavior lasted more than 20 sec within a observation period (5 min), it was judged as active and used for the bioassay.

Bioassay. The bioassay system was based on that of in our previous paper (Oguma et al., 1992a) with small modifications. Freeze-killed *D. melanogaster* males (almost no response from *D. pallidosa* males, see Results and Discussion) were washed with hexane three times and coated with a given amount of the candidate hexane solution using a glass capillary containing natural or synthetic hydrocarbons (hereafter the washed *D. melanogaster* male for bioassay termed a decoy). After evaporation of the solvent, the decoy was placed in a glass assay chamber (15 mm ID × 3 mm deep) covered with a slide glass plate and a male was introduced without anesthetizing. Courtship behaviors were observed for 5 min and evaluated by four typical courtship elements selected for assay: orientation, wing vibration with both wings, circling around the decoy, and attempted copulation (grasping the decoy and bending the abdomen strongly). Continuing a courtship element for more than 2 sec was recorded and evaluated both qualitatively and quantitatively. The kinds, frequency of occurrence, and the time spent for courtship elements were recorded individually. The percentage of total time occupied by courtship elements (seconds) during the observation time (5 min) was calculated as the courtship index (CI).

For pheromone purification, the assay method was simplified as follows: 10 female equivalent (FE) of sample was applied to a decoy and, if it elicited any kind of courtship elements among test males, it was designated as active. All assays were done within 6 hr after the beginning of a light period and under the same conditions as rearing.

Column Chromatography. Three chromatographic steps were carried out to purify the pheromone. SiO_2 column (Wako-gel C-200, 370 mg, 5 mm ID × 3 cm height) was developed by a series of hexane–ether mixtures in a stepwise manner (100:0, 97:3, 95:5, 90:10, 0:100, by volume). A silver nitrate-impregnated silica gel ($AgNO_3$–SiO_2) column (10% w/w of $AgNO_3$ impregnated Wako-gel C-200, 615 mg, 5 mm ID × 11.0 cm height) was eluted stepwise by the same series of hexane–ether mixtures (each 6 ml). An $AgNO_3/SiO_2$ column of the same size was developed with 10% benzene–hexane (24 ml) and then benzene (4 ml). Eluates from each column were fractionated every 2 ml.

Analysis. A gas–liquid chromatograph (GLC) model 380FS (GL Science) with an FFAP chemical bonded fused silica capillary column (0.25 mm ID × 25 long, df = 0.25 μm, Quadrex) was operated at splitless mode under a

temperature programmed condition (160–250°C, 2.5°C/min). For quantification, docosane and tritriacontane were used as internal standards. For estimating hydrocarbon content in a single fly, a 7-day-old fly was soaked in 10 μl of hexane for 5 min. One microliter of this microextract was injected into a cooled on-column capillary GLC system (model OCI-3, GL Science) with an FFAP capillary column and with temperature programmed condition (60–250°C). Peak areas were compared with known quantities of internal standards. A Fourier transform infrared absorption spectrum was measured by a FT/IR-5MP (Jasco) equipped with a PC9801VX personal computer (NEC). Electron impact mass spectra (EI-MS) were obtained using a M80-b (Hitachi) gas chromatograph–mass spectrometer (GC-MS) at 70 eV equipped with an Al-clad methyl silicone capillary column (0.25 mm ID × 25 m, *df* = 0.25 μm, Quadrex). The double-bond locations of the purified pheromone compound were determined by detecting diagnostic ions in the EI-MS of dimethyl disulfide (DMDS) adducts (Carlson et al., 1989).

Chemicals. Docosane and tritriacontane for GLC standards were both purchased from Nacalai Tseque (Kyoto, Japan). The strategy of long-chain alkadiene synthesis was coupling between two monoene units.

(*Z*,*Z*-5,27-Tritriacontadiene) [(*Z*,*Z*)-5,27-$C_{33:2}$] was prepared by a coupling reaction between (*Z*)-11-hexadecenyl magnesium bromide in THF and (*Z*)-11-heptadecenyl bromide, using Li_2CuCl_4 as catalyst. (*Z*)-11-Hexadecenyl magnesium bromide was obtained by reacting magnesium with (*Z*)-11-hexadecenyl bromide, which was prepared from (*Z*)-11-hexadecenyl acetate by hydrolysis and subsequent bromination. (*Z*)-11-Heptadecenyl bromide was prepared by a Wittig reaction between hexanal and ω-hydroxyundecyl triphenylphosphonium bromide. The hydroxy phosphonium salt was obtained from triphenylphosphine and undecamethylene bromohydrine, which was derived via half-bromination of undecamethylene glycol.

The resulting products contained homo-coupled by-products, which were removed by preparative GLC to give (*Z*,*Z*)-5,27-$C_{33:2}$. Purity was checked by capillary GLC (>99%).

RESULTS

Estimation of Cuticular Hydrocarbons. Qualitative analysis was carried out be means of cooled on-column capillary GLC. Compositions of cuticular hydrocarbons from both sexes are shown in Table 1. The average amount of total hydrocarbons extracted in the present experiments were 1.73 μg/female (SEM ±0.086) and 2.01 μg/male (SEM ±0.129), respectively. No qualitative differences were found between males and females.

Purification of Pheromone. Although the crude extract (5.8 mg) from 3660

TABLE 1. CUTICULAR HYDROCARBON COMPOSITION IN *D. pallidosa*

Hydrocarbons	female (N = 18) ($\mu g \pm$ SEM)	Male (N = 12) ($\mu g \pm$ SEM)	Peak No.[a]
Branched alkane			
C_{27}	0.02 ± 0.002	0.01 ± 0.002	1
C_{29}	0.34 ± 0.015	0.34 ± 0.015	2
C_{31}	0.03 ± 0.005	0.02 ± 0.004	7
Diene			
C_{27}	0.13 ± 0.006	0.10 ± 0.003	
C_{31}	0.07 ± 0.003	0.09 ± 0.004	4
	0.06 ± 0.003	0.09 ± 0.001	5
C_{33}	0.17 ± 0.008	0.23 ± 0.019	9
	0.03 ± 0.019	0.05 ± 0.004	8
	0.58 ± 0.031[b]	0.65 ± 0.059[b]	10
Triene			
C_{31}	0.04 ± 0.002	0.07 ± 0.006	6
C_{33}	0.15 ± 0.007	0.25 ± 0.022	11
	0.07 ± 0.004	0.08 ± 0.008	12
Others	0.01 ± 0.001	0.04 ± 0.003	
Total	1.73 ± 0.086	2.01 ± 0.129	

[a] Numbers correspond to those of Figure 1.
[b] (*Z*, *Z*)-5-27-Tritriacontadiene.

flies of both sexes elicited no behavioral response of any kind among males at any dose (0.1, 1, 10 FE/decoy, respectively), the pheromonal activity was first demonstrated in the hexane eluate from the SiO_2 column. The active fraction was concentrated and then applied to the first $AgNO_3/SiO_2$ column. The bioassay revealed high pheromonal activity in the 97:3 hexane–ether fraction. The chromatographic behavior and GLC profiles of eluates suggested that these active fractions consisted of alkenes and alkadienes. Combined active fractions (1.7 mg) were further chromatographed on the second $AgNO_3/SiO_2$ column. The activity was recovered in the first fraction eluted with benzene (active at 10 FE/decoy), and its GLC profile (Figure 1A) consisted of one abundant alkadiene and lesser quantities of several other alkadienes, which was confirmed by MS. This fraction was compared to the cuticular hydrocarbons of a female (Figure 1B) and used for structure elucidation.

Structure Elucidation. The major peak of the active fraction appeared near the standard tritriacontane (C_{33}) region by GLC. The DMDS analysis clearly showed that the double-bond locations of the main compounds were at C-5 and C-27. The diagnostic ions were as follows: m/z 648 ($m^{+}\cdot$, DMDS diadduct), 600 ($m^{+}\cdot -48$), 553 ($m^{+}\cdot -94$), 507 ($m^{+}\cdot -141$), 483(B-48), 469 (C-48), 437 (B-94), 423 (C-94), 131, and 117, respectively, according to Carlson et al.

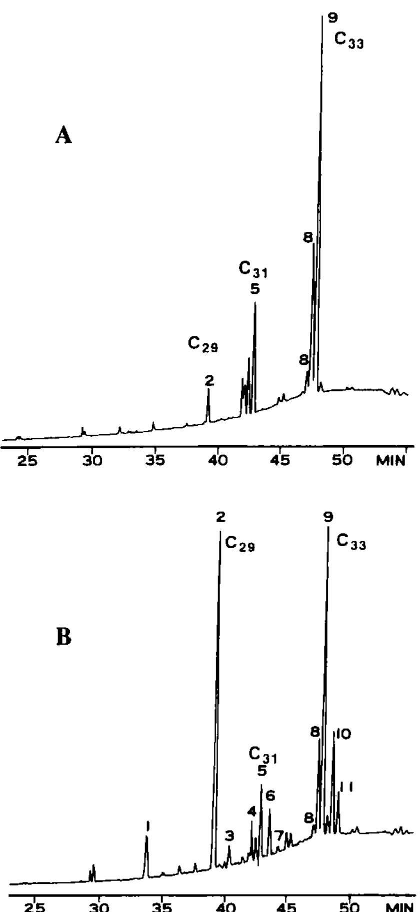

FIG. 1. (A) Gas-liquid chromatogram of a sex pheromone active fraction (fraction 13 from the and $AgNO_3/SiO_2$ column). (B) Gas-liquid chromatogram of a microextract from a *D. pallidosa* female with a FFAP fused silica capillary column (0.25 mm ID × 25 m, df = 0.25 μm). Temperature programmed from 160 to 250°C at 2.5°C/min. Indicated peak numbers correspond to those of Table 1.

(1989). The *Z*-configuration was assigned by the lack of absorption around 970 cm^{-1} in the FT-IR spectrum of hydrocarbon fraction. The synthetic (*Z*,*Z*)-5,27-$C_{33:2}$ was cochromatographed with the purified fraction by GLC and was identical to the predominant peak in GLC (R_t = 46.49 min). As a result, the chemical structure of the major GLC peak in the active fraction was determined to be (*Z*,*Z*)-5,27-tritriacontadiene.

Bioassay. The dose–response experiments for courtship behavior in males, including the synthetic alkadiene (Table 2), indicated that *D. pallidosa* females, dead or alive, gave strong responses and high CI values, while hexane-washed females and males gave drastically decreased activity. The significant difference in activity between a dead female and a washed female imply the importance of sex pheromone. Although dead female and male *D. melanogaster* gave no response at all, washed *D. melanogaster* males used as decoys elicited unexpectedly weak activity (Table 2). Two FE of (Z,Z)-5,27-$C_{33:2}$ clearly showed pheromonal activity with a 48% response and 35.3 CI; even 1 FE gave a good response (35%) and a CI of (26.9) was observed. Five FE gave a 75% response with 41.2 CI, and these values were comparable to those of one dead female (90% and 67 CI). Hence, (Z,Z)-5,27-tritriacontadiene is the main sex pheromone component in *D. pallidosa*.

DISCUSSION

(Z,Z)-5,27-$C_{33:2}$ elicited all elements of courtship behavior; hence it was identified as the major female sex pheromone component of *D. pallidosa*. Since there are nearly equal quantities of (Z,Z)-5,27-$C_{33:2}$ in both males and females (Table 1), there must be another sex recognition mechanism that discourages male–male courtship.

TABLE 2. COURTSHIP RESPONSES (%) AND COURTSHIP INDICES (CI) IN MALE *D. pallidosa*

Source	*N*	Dose	Response	CI
Control				
D. pallidosa				
Live female	20	1[a]	100	75.6 ± 5.47
Dead female	20	1[a]	90	67.1 ± 7.28
Washed female	20	1[a]	30	9.1 ± 3.94
D. melanogaster				
Dead female	20	1[a]	0	0
Dead male	20	1[a]	0	0
Washed male	43	1[a]	7	5.3 ± 2.34
Washed female	20	1[a]	15	1.9 ± 1.53
Synthetic, (Z, Z)-5,27-C_{33}	20	1[b]	35	26.9 ± 10.06
	60	2[b]	48	35.3 ± 5.76
	20	5[b]	75	41.2 ± 9.14

[a] One fly.
[b] Female equivalent (FE).

In the cockroach, *Nauphoeta cinerea*, one of the typical courtship elements is wing-raising behavior by a male. The wing-raising stimulant in the cuticular wax has been identified as a common hydrocarbon present in both sexes (Takahashi and Fukui, 1980). Hydrocarbon fractions from both sexes showed similar wing-raising activities and were of identical chemical composition. The antiaphrodisiac (depressant) was isolated and identified as octadecyl (*Z*)-9-tetracosanoate (nauphoetin) from male cuticular wax (Fukui and Takahashi, 1983). This phenomenon is more common in fly species. Wasteful male–male courting behavior (homosexual behavior) is often observed not only in the laboratory but also under natural conditions. The presence of contact chemicals functioning as its inhibitor were demonstrated biologically in the tsetse fly, *Glossina moritans moritans*, and the house fly, *Musca domestica* (Schlein et al., 1981). In *D. melanogaster* (Canton-S), the male-predominant cuticular hydrocarbon component, 7-tricosene was identified as the inhibitor of male courtship behavior, i.e., functioning as an antiaphrodisiac. Courtship activities of males towards females treated with 7-tricosene were clearly suppressed (Scott, 1986). In the Tai-Y strain of the same species, 7-pentacosene was identified in males as an antiaphrodisiac instead of 7-tricosene (Scott and Jackson, 1988). Thus, presence of the antiaphrodisiac or the courtship inhibitor produced by males might be a common phenomenon among *Drosophila* species. Male-produced antiaphrodisiacs or the courtship inhibiting signals of the present species remain to be studied. Structures of other minor alkadienes in the active fraction and their function relative to the major sex pheromone component also remain obscure.

No male *D. pallidosa* courted a dead female *D. melanogaster*, but some males showed a low CI to both washed females and male *D. melanogaster* (Table 2), suggesting that some chemical substance(s) may inhibit elicitation of interspecific courting in *D. pallidosa*. This fact implies that chemicals present in the cuticular lipids may play an important role in species recognition. If true, we can then identify an interspecific courtship inhibitor in *Drosophila* and elucidate an interspecific recognition mechanism.

The reason for using the washed male *D. melanogaster* as the decoy instead of *D. pallidosa* was that hexane-washed *D. pallidosa* of both sexes gave moderate CI values (Table 2) and further hexane washing did not provide complete elimination of the response. This suggests that visual cues or surface structures of the body may also play an important role in courting.

Our aim in analyzing the sex pheromone in *D. pallidosa* is to clarify the genetic basis of the isolation mechanism in this complex. We are analyzing the sex pheromone in *D. ananassae* a plan to test the sex pheromone on male *D. pallidosa*. The present study is a first step to attain this aim.

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HERBIVORE-INDUCED VOLATILE EMISSIONS FROM COTTON (*Gossypium hirsutum L.*) SEEDLINGS

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Abstract—The effect of herbivory on the composition of the volatile blends released by cotton seedlings was investigated by collecting volatiles from undamaged, freshly damaged (0–2 hr after initiation of feeding), and old damaged (16–19 hr after initiation of feeding) plants on which corn earworm caterpillars (*Helicoverpa zea* Boddie) were actively feeding. A blend of 22 compounds was consistently observed to be emitted by the old damaged plants with nine occurring either only in, or in significantly greater amounts in old damaged, as compared with freshly damaged plants. These were (*Z*)-3-hexenyl acetate, hexyl acetate, (*E*)-β-ocimene, (3*E*)-4,8-dimethyl-1,3,7-nonatriene, (*Z*)-3-hexenyl butyrate, (*E*)-2-hexenyl butyrate, (*Z*)-3-hexenyl 2-methylbutyrate, (*E*)-2-hexenyl 2-methylbutyrate, and indole. The nature of this response is compared with other studies where herbivore-induced volatile responses are also known. The presence of large amounts of terpenes and aldehydes seen at the onset of feeding and the appearance of other compounds hours later suggest that cotton defense mechanisms may consist of a constitutive repertoire that is augmented by an induced mechanism mobilized in response to attack. A number of the induced compounds are common to many plants where, in addition to an immediate defensive function, they are known to be involved in the attraction of natural enemies.

Key Words—Cotton, *H. zea*, feeding deterrence, phytoalexin, semiochemicals, terpenes, tritrophic interactions.

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INTRODUCTION

In the past, studies on plant volatile production have generally dealt with the relationship between the healthy plant and its attractancy or repellency to potential herbivores, or with the effect on herbivore feeding of plant-produced chemicals (Carroll and Hoffman, 1980; Dickens, 1984; Gunasena et al., 1988; Mitchell et al., 1991). Intact healthy plants often produce specific odors that are recognized by certain herbivores searching for potential oviposition or feeding sites, but damage to these plants may result in the release of volatile antifeedants that serve to reduce the damage inflicted by the herbivore (Mihaliak et al., 1987). Plant odors can also be attractive to parasitoids or predators, which then attack the herbivores. Moreover, it has recently been shown that some plants produce a specific blend of volatile chemicals, only in response to herbivore-induced damage, that attract either predators (Dicke and Sabelis, 1988) or parasitoids (Turlings et al., 1990), which then attack the injurious herbivores and relieve the plant. The predators and parasitoids appear to orient to these chemicals, recognizing them as marker cues for their prey or hosts.

This plant response to herbivore damage is likely to be part of a general chemical defense against the herbivores, which is exploited secondarily by natural enemies of the herbivores, thereby adding to the benefits that the plant gains from the emission of volatiles (Turlings and Tumlinson, 1991). In those cases so far described, the volatile blends released by plants in response to herbivore damage are unique to the particular plant species described but contain a number of commonly encountered plant volatiles (Turlings et al., 1993a). Furthermore, prior results with corn seedlings (Turlings et al., 1993b) and preliminary results with cotton (Turlings and McCall, unpublished) indicate that the composition of the induced blend of volatiles does not vary significantly when the plants are attacked by different species of herbivorous insects. This suggests that the plant is responding nonspecifically to insect damage and that this response is characteristic for that particular plant species or variety. Therefore, as part of a series of studies on the interactions among plants, herbivores, and parasitic wasps, the nature of this induced response in cotton plants was investigated. In the present study, the effect of damage by one caterpillar species, corn earworm, *Helicoverpa zea* Boddie (Lepidoptera: Noctuidae), was examined. This was chosen as a complement to studies on the Braconid wasp *Microplitis croceipes* Cresson (Hymenoptera: Braconidae), a parasitoid specialist on corn earworm and related species, and its host location behavior when foraging in cotton and other plant species (McCall et al., 1993). To date, studies on cotton volatiles have dealt with the odors of intact plants (Thompson et al., 1971; Hedin et al., 1975b; Hedin, 1976), or with specific biosynthetic classes of compounds (Minyard et al., 1965, 1966; Pomonis et al., 1980; Chang et al., 1988). Here the complete

odor blend emitted by cotton under attack by caterpillars is described, and the delayed nature of the release is shown.

METHODS AND MATERIALS

Insects. All of the insects used (*Helicoverpa zea* Boddie, CEW) were reared on a pinto bean-based artificial diet using the procedure described by King and Leppla (1984). In all studies, late-second to third-instar larvae were used.

Plant Treatments. Cotton (*Gossypium hirsutum* L., var. McNair 235) was grown in a greenhouse, in styrofoam cups (500 ml), with one seedling per cup. The seedlings used were approximately 20 cm tall at 2–3 weeks old (4 to 6-leaf stage). The plants were brought indoors between 1700 and 1900 hr. In cases where the plant treatment was classed as being old damage, five caterpillars were placed on each plant, and the whole plant and pot were covered with an inverted styrofoam cup in which the base was replaced by fine nylon mesh, and then fitted tightly to the plant pot to retain the caterpillars. On the following morning, the plant was cut close to the base of its stem with a razor blade, the cut end wrapped in wet cotton to prevent dehydration, and immediately placed with the caterpillars in a volatile collection chamber. All volatile collections were made between 0900 and 1200 hr, at 16–19 hr after feeding began. In cases classed as fresh damage, plants were stored overnight as described, but no larvae were introduced until the cut plants were placed in the volatile collection chamber. In both old and fresh damage, extra caterpillars that had been starved overnight were placed with the plants, so that active feeding was occurring during the volatile collections from both treatments.

Collection of Plant Volatiles. The volatile collection system has been described in detail by Turlings et al. (1991a). Briefly, humidified air, purified by an in-line activated charcoal filter, entered four parallel chambers consisting of Pyrex glass tubes (approx. 50 cm long and 115 mm in diameter), each with a glass frit to ensure laminar airflow through the tube. The tubes were assembled from three sections to facilitate cleaning and introduction of the seedlings. Airflow at 300 ml/min was balanced with house air and vacuum, and maintained at slightly higher than atmospheric pressure inside each chamber. Air exited each chamber through a reusable 3.7-mm-ID × 4-cm-long glass collection trap packed with 25 mg Super Q adsorbent (80–100 mesh) (Alltech, Deerfield, Illinois), which was prerinsed prior to each volatile collection with 5–10 ml dichloromethane to remove impurities. Volatiles were collected for 2 hr, after which the collection traps were extracted immediately with 150 μl dichloromethane, and internal standards added (1 μg each of octane and nonyl acetate in 30 μl dichloromethane). A total of four collections comprising a system blank (containing only wet cotton wool), undamaged seedlings (seedlings simply cut

at the base and wrapped in wet cotton wool), fresh damaged seedlings, and old damaged seedlings were run in parallel on each occasion and the airflows through each were equalized with Aalborg flowmeters at the downwind end of the collection traps. Each treatment involving plant damage consisted of three seedlings under attack by a total of 20–25 caterpillars. The experiment was replicated five times.

Chemical Analyses. Collected volatiles were analyzed with a Hewlett-Packard model 5890 GC, or a Varian model 3700 GC, equipped with split-splitless capillary injector systems and flame ionization detectors. All analyses were performed on two fused silica capillary columns, with helium as carrier gas (19 cm/sec). The columns were 50 m × 0.25 mm ID with a 0.25-μm film of bonded methyl silicone (007) and 50 m × 0.25 mm ID with a 0.25-μm film of bonded cyanopropyl methyl silicone CPS-1 (Quadrex Corporation, New Haven, Connecticut) and were operated at an initial temperature of 40°C for 3 min, then programmed at 5°C/min to 180°C. All injections of 2 μl were made in the splitless mode, and split after 30 sec. Data were collected, analyzed, and stored with a Perkin-Elmer chromatographic data system.

Samples were also analyzed by GC–mass spectroscopy (GC–MS) with a Nermag model R1010 mass spectrometer in both electron impact and chemical ionization modes. Both of the columns and the conditions described above were used in GC–MS. Methane and isobutane were used as reagent gases for chemical ionization. The retention times on both columns and the spectra of the natural compounds were compared with those of candidate synthetic compounds. (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 by the Wittig reaction of geraniol and farnesal, respectively, with methylenetriphenyl phosphorane (analogous to Maurer et al., 1986). In the case of the (*Z*)-3-hexenyl and (*E*)-2-hexenyl esters of butanoic and 2-methyl butanoic acids, identifications were further confirmed by cochromatography of the natural compounds with authentic standards. All of the other synthetic standards were obtained from commercial sources.

RESULTS

Identity of Released Volatiles. In volatiles emitted by plants with old damage, 22 compounds were consistently present. Figure 1 shows typical chromatograms for volatile collections made from three seedlings over 2 hr, and Table 1 shows the calculated amounts released from one seedling per hour, based on an integration of the data from five replicates per treatment. None of these compounds were found in blank collections made from wetted cotton wool, and only minute amounts were found in collections from undamaged cotton, some of which may have arisen as a result of slight mechanical damage to the plants during handling.

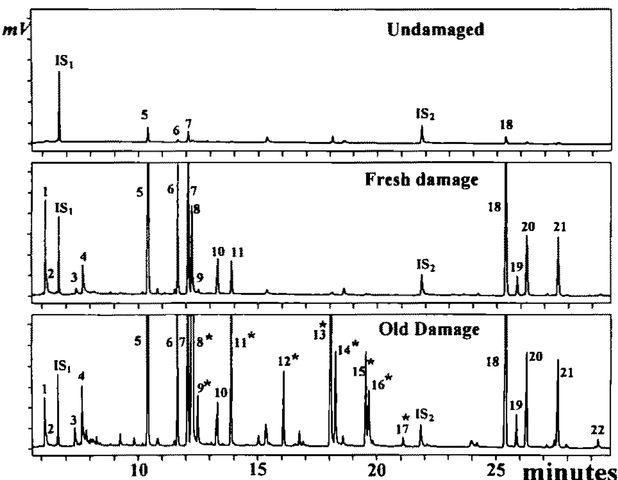

FIG. 1. Gas chromatographic analyses on a methyl silicone capillary column of volatiles collected from groups of three cotton seedlings for a period of 2 hr. Fresh damage: volatiles collected during the 2 hr immediately following initiation of feeding by 20–25 *H. zea* larvae. Old damage: volatile collection commenced at least 15 hr after initiation of feeding. Peak numbers correspond to the compounds named in Table 1 and those peaks marked with an asterisk are present in significantly greater amounts in old damage than in fresh damage. IS_1 and IS_2 represent *n*-octane and *n*-nonyl acetate, respectively.

It was possibly to identify most of these compounds by comparison of the mass spectra and retention times on polar and nonpolar columns with the known synthetic compounds. In the case of α-guaiene and δ-guaiene however, authentic standards of sufficient purity to make positive identification were not available. The seven most intense ions in the electron impact mass spectra of these two tentatively identified compounds were 105 (100), 204 (M^+, 85.4), 93 (84.4), 70 (83.3), 81 (78.1), 91 (77.1), and 107 (66.7) for α-guaiene; and 93 (100), 108 (81.6), 79 (75.5), 107 (68.4), 81 (63.3), 91 (57.1), and 95 (55.1) for δ-guaiene. Additionally, a number of other compounds were occasionally found in the volatile collections. Of these, certain compounds were matched with library mass spectra and provisionally identified as phellandrene, copaene, terpinene, bisabolol, benzaldehyde, and nonanol, but as they were not consistently found in most of the volatile collections, their identities were not confirmed.

Variations in Volatile Blends. Nine of the compounds were found to be released in significantly greater amounts from plants with old damage than from freshly damaged plants (Figure 1, Table 1). Of these, (*Z*)-3-hexenyl acetate, hexyl acetate, (*E*)-β-ocimene, (3*E*)-4,8-dimethyl-1,3,7-nonatriene, and (*Z*)-3-hexenyl butyrate were also found in volatiles of freshly damaged plants (peaks

TABLE 1. AMOUNTS, STANDARD DEVIATIONS, AND RELATIVE PERCENTAGES OF COMPONENTS OF COMPLETE BLENDS FOR UNDAMAGED PLANTS, FRESH AND OLD DAMAGED PLANTS RELEASED BY 1 COTTON SEEDLING PER HOUR, DETERMINED FROM DATA COLLECTED FROM 5 REPLICATES PER TREATMENT[a]

		Undamaged		Fresh damage		Old damage	
Peak	Compound	Amount, ng/hr (SD)	Relative %	Amount, ng/hr (SD)	Relative %	Amount, ng/hr (SD)	Relative %
1	(*Z*)-3-hexenal	2.2 (1.9)	1.2	109.7 (68.3)	2.8	122.5 (605.)	1.6
2	hexanal	0.4 (0.9)	0.2	12.2 (8.6)	0.4	20.0 (13.0)	0.3
3	(*E*)-2-hexenal	0	0	11.2 (9.6)	0.3	41.6 (31.6)	0.5
4	(*Z*)-3-hexen-1-ol	0	0	63.5 (72.3)	1.6	117.2 (35.4)	1.5
5	α-pinene	65.6 (84.5)	35.9	1341.8 (1168)	34.4	1311.5 (371.3)	17.0
6	β-pinene	11.5 (15.1)	6.2	239.3 (203.7)	6.1	229.0 (66.0)	3.0
7	myrcene	27.9 (30.5)	15.3	522.5 (348)	13.4	673.9 (269.0)	8.7
8	(*Z*)-3-hexenyl acetate	11.5 (23.0)	6.3	104.5 (100.2)	2.7	1617.7 (707.3)	21.0[b]
9	hexyl acetate	0	0	3.7 (4.7)	0.1	69.5 (56.4)	1.0[b]
10	limonene	9.3 (10.5)	5.1	100.0 (80.5)	2.6	98.2 (52.0)	1.2
11	(*E*)-β-ocimene	8.2 (16.5)	4.5	46.6 (16.2)	1.2	394.4 (266.0)	5.1[b]
12	(3*E*)-4,8-dimethyl-1,3,7-nonatriene	0	0	20.0 (37.6)	0.5	206.0 (92.2)	2.6[b]
13	(*Z*)-3-hexenyl butyrate	0	0	1.7 (3.3)	0.1	494.6 (415.2)	6.4[b]
14	(*E*)-2-hexenyl butyrate	0	0	0 0	0	130.9 (127.7)	1.6[b]
15	(*Z*)-3-hexenyl 2-methylbutyrate	0	0	0 0	0	110.0 (131.2)	1.4[b]
16	(*E*)-2-hexenyl 2-methylbutyrate	0	0	0 0	0	77.1 (98.5)	1.0[b]
17	indole	0	0	0 0	0	26.8 (12.3)	0.4[b]
18	(*E*)-β-caryophyllene	30.0 (35.6)	16.4	835.7 (420.6)	21.5	1231.5 (370.4)	16.0
19	α-guaiene	0.9 (1.8)	0.5	63.8 (51.6)	1.6	90.8 (34.2)	1.2
20	α-humulene	9.1 (11.3)	5.0	220.4 (102.9)	5.6	325.7 (98.5)	4.2
21	δ-guaiene	5.7 (5.6)	3.1	197.5 (180.3)	5.1	302.0 (101.7)	4.0
22	(3*E*, 7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene	0.6 (1.2)	0.3	6.7 (11.9)	0.2	19.5 (15.0)	0.3

[a] Details of each treatment are given in the text.
[b] Significantly different from fresh-damage values (Mann-Whitney test: $P < 0.05$).

8, 9, 11, 12, and 13), but in significantly lower quantities ($P < 0.05$, Mann–Whitney test). (*E*)-2-Hexenyl butyrate, (*Z*)-3-hexenyl 2-methylbutyrate, (*E*)-2-hexenyl 2-methylbutyrate, and indole (peaks 14, 15, 16, and 17) were never found in volatiles from fresh damage. Although peak 22, (3*E*,7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene, also occurred in increased amounts in the old damage, this difference was not significant ($P = 0.09$).

DISCUSSION

This study represents the first attempt to describe the total blend of volatiles released by cotton plants following herbivore damage. Earlier studies described either the constituents of cotton essential oil (Minyard et al., 1965, 1966; Hedin et al., 1975a; Kumamoto et al., 1979), or volatile compounds from intact cotton plants (Thompson et al., 1971; Hedin et al., 1975b; Hedin, 1976; Pomonis et al., 1980; Chang et al., 1986, 1988). What is immediately apparent from this study is the enormous increase in volatile release following caterpillar feeding and the subsequent change in relative proportions of the compounds within the blend as feeding continues. The increase in the particular compounds in old damaged seedlings is very striking in some cases, with (*E*)-2-hexenyl butyrate, (*Z*)-3-hexenyl 2-methylbutyrate, (*E*)-2-hexenyl 2-methylbutyrate, and indole appearing only in older damaged plants, and others occurring in significantly greater amounts. The latter is best illustrated by the dramatic increase in (*Z*)-3-hexenyl acetate from 2.1% of the total blend in fresh damage to 21% in old damage (Table 1). Increases of this nature following herbivore damage have previously been recorded from corn seedlings following caterpillar damage (Turlings et al., 1990) and in lima beans following mite damage (Dicke et al., 1990a) (see below).

A number of the compounds recorded here are not mentioned in the previous literature on cotton volatiles (compounds 1, 8, 9, 12, 13, 14, 15, 16, 17, and 22 in Figure 1). The green leaf compounds hexyl acetate and (*Z*)-3-hexenyl acetate have been found in volatiles of cowpea (Lwande *et al.*, 1989) and corn (Buttery and Ling, 1984; Turlings *et al.* 1991a), respectively, and both are found in strawberry (Hamilton-Kemp et al., 1989). Peak 15, (*Z*)-3-hexenyl 2-methylbutyrate, is also known from strawberry leaves (Hamilton-Kemp et al., 1989). The related methylene monoterpene, (3*E*)-4,8-dimethyl-1,3,7-nonatriene and methylene sesquiterpene, (3*E*,7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene have been reported from cardamom oil (*Elettaria cardamomum*) (Maurer et al., 1986), from corn seedlings (Turlings et al., 1991a) under attack by caterpillars, and from lima beans leaves (Dicke et al., 1990a) and cucumber leaves (Dicke et al., 1990b) under attack by acarine herbivores. Many of the compounds identified in volatiles from cotton leaves in the present study are

also known to occur in the volatiles from flowers and bolls, although in different relative proportions (Turlings et al., 1993a), and the differences between fresh and old damage shown here are also seen in those plant parts (McCall and Turlings, unpublished).

(*Z*)-3-Hexenal is highly volatile and is seldom reported as a green leaf volatile elsewhere, although Turlings et al. (1991a) found high proportions in corn volatiles, probably as a result of using this volatile collection technique. This relatively simple, nondestructive, and highly efficient [as evidenced by the retrieval of (*Z*)-3-hexenal] procedure allows a more accurate determination of the identity and proportions of compounds emitted by the plant. This is preferable to identification of compounds obtained by extraction or more destructive methods, which lead to the identification of compounds present in the plant in various forms, or in the worst case, artifacts produced by degradation of plant-contained compounds. The composition of such extracts is unlikely to be relevant to the blend actually emitted by the plant and ultimately detected by insects.

Many of the compounds detected in cotton at the onset of, or induced by, herbivore infestation are known feeding deterrents (Zummo et al., 1984; Mihaliak et al., 1987; Gunasena et al., 1988; Hedin et al., 1991), or are known to have a defensive function against other unrelated pathogens (Zeringue and McCormick, 1989, 1990). The subsequent appearance of, or increase in, specific compounds only after 16 hr, suggests a more specific role for these induced compounds. Corn seedlings under attack by caterpillars show a dramatic increase in certain terpenoids 16 hr after the onset of damage caused by herbivory (Turlings et al., 1990), in response to the oral secretions of the herbivore (Turlings et al., 1993b). A similar response was also shown to occur in lima bean (Dicke and Sakelis, 1988). However, the presence within the cotton plant of high quantities of terpenoids at the initiation of herbivore feeding suggests that, unlike the faster growing annuals corn and lima bean, which may rely primarily on an induced response that is activated when attacked and becomes effective only after a number of hours (Turlings and Tumlinson, 1991), the relatively slow growing perennial cotton already has effective constitutive defense resources in place. Coley et al. (1985) suggested that slower growth rates favor larger investments in antiherbivore defenses, whereas plants with faster growth rates may utilize more mobile or flexible defenses. Results from the present study suggest cotton may utilize both means of defense. In fact, certain of the compounds induced in cotton, (*Z*)-3-hexenyl acetate and (3*E*)-4,8-dimethyl-1,3,7-nonatriene, are also found following herbivore damage in lima bean (Dicke and Sabelis, 1988), in corn (Turlings et al., 1991a), and in cowpea (Turlings et al., 1993a), and (*E*)-β-ocimene and indole are induced in lima bean and corn, respectively, suggesting a common role for these compounds. Moreover, there is evidence that production of terpenes and other antifeedants varies seasonally,

with peak production coinciding with fruiting and consequential *Heliothis zea* attack (Hedin, 1976; Zummo et al., 1984).

Turlings and Tumlinson (1992) showed that the induced response to herbivores in corn was not limited to the sites of damage but occurred throughout the plant, with undamaged leaves also releasing induced compounds. In this study a systemic response by cotton was not investigated. However, Karban and Carey (1984) showed that new growth on cotton seedlings that had been infested previously with mites was more resistant to subsequent infestations by the same and novel mite species than were seedlings that had never been infested. Thus, cotton may be capable of a systemic chemical response to herbivore damage. Further research will be required to determine whether release of volatiles by herbivore damaged cotton is systemic.

We also have shown that damaged cotton plants were the most important source of volatile cues that foraging parasitic wasps, *M. croceipes*, used to locate potential host sites (McCall et al., 1993). Other studies on lima bean (Dicke and Sabelis, 1988) and corn (Turlings et al., 1990, 1991b) found that it is the herbivore-induced volatiles in particular that are exploited by natural enemies to locate their hosts or prey. Although other workers have shown that parasitic wasps respond to volatiles of cotton (Elzen et al., 1984, 1986; Baehrecke et al., 1989; Li et al., 1992), they did not take into account the delay in the appearance of the induced response in the plant. Thus, whereas *M. croceipes* will respond in simple flight tests to individual green leaf odors (Whitman and Eller, 1990), characteristic of fresh herbivore or simple mechanical damage, they prefer old damage to fresh damage in choice tests, regardless of previous experience (McCall et al., 1993). The results of both that and the present studies suggest that this parasitic wasp has an innate predisposition to orient to herbivore-induced volatiles, which overrides positive learning experiences. Considering, then, the role of delayed herbivore-induced volatile responses in host-location by natural enemies on cotton and other plants, precise timing of plant damage must be considered crucial in such behavioral studies. The compounds making up the green leafy odor are common to many different plant species (Visser et al., 1979). Although they may be of secondary importance in the close range location of the freshest damage, where the hosts are currently likely to be, it is the induced compounds that are the more reliable and consistent indicators of the presence of herbivores. Natural enemies are faced with a problem in obtaining cues for hosts or prey, which need to be both reliable and detectable for efficient foraging (Vet and Dicke, 1992). Within the complex of odors originating from the plant, the herbivore, and the herbivore's waste products, the plant-derived odors are by far the most abundant and are the most attractive to foraging parasitic wasps (Turlings et al., 1991a). In nature the herbivore-specific volatiles within the plant odor blend would allow the endan-

gered plant to advertise itself more accurately and prominently to searching beneficial insects.

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FATHEAD MINNOWS USE CHEMICAL CUES TO DISCRIMINATE NATURAL SHOALMATES FROM UNFAMILIAR CONSPECIFICS

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Abstract—Naturally occurring shoals of fathead minnows (*Pimephales promelas*) were captured and individuals given the choice between shoalmates and unfamiliar conspecifics in a two-choice discrimination test. When presented with chemosensory cues alone or with both chemosensory and visual cues, minnows exhibited a significant preference for shoalmates versus unfamiliar conspecifics. With visual cues alone, there was no significant discrimination of shoalmates. A second set of trials was conducted to ensure that minnows were choosing natural shoalmates and not just individuals with which they were held in the laboratory. When given the choice between unfamiliar conspecifics and shoalmates from which they were separated for a minimum of two months, minnows exhibited a significant preference for shoalmates. Taken together, these data suggest that fathead minnows are able to discriminate among conspecifics on the basis of familiarity using chemosensory cues, even after a relatively long separation. The ability to discriminate among conspecifics may facilitate: (1) the maintenance of kin groups or groups that share similar foraging or predator avoidance patterns or (2) the recognition of former shoalmates after some period of separation.

Key Words—Fathead minnow, *Pimephales promelas*, familiar recognition, alarm signaling, kin selection.

INTRODUCTION

Discrimination of conspecifics can occur at a variety of levels (Fletcher, 1987); from the recognition of members of a specific stock or population (i.e., Olsén,

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1986; Stabell, 1987) to the recognition of specific individuals (i.e., Waas and Colgan, 1994). The majority of recent work dealing with fishes has focused on either individual recognition (i.e., Miklosi et al., 1992; Waas and Colgan, 1994) or kin recognition (i.e., Quinn and Busack, 1985; Brown and Brown, 1992). In addition to demonstrating the ability to discriminate among classes of individuals, several authors have determined which sensory modality is involved in such discriminations (i.e., VanHavre and FitzGerald, 1988).

Paradise fish (*Macropodus opercularis*) have been shown to bias their social behavior towards individuals with which they have had prior interactions, suggesting that they are able to discriminate familiar from unfamiliar conspecifics (Miklosi et al., 1992). Juvenile bluegill sunfish (*Lepomis macrochirus*) are also able to discriminate familiar from unfamiliar conspecifics (Brown and Colgan, 1986). Since Brown and Colgan presented visual and chemosensory cues together, either cue may be sufficient to allow for this discrimination or both may be required.

VanHavre and FitzGerald (1988) have shown that threespine sticklebacks (*Gasterosteus aculeatus*) can discriminate familiar from unfamiliar individuals on the basis of chemosensory and visual cues together, but not by visual cues alone. They did not test chemosensory cues alone. Furthermore, threespine sticklebacks have also been shown to discriminate among specific individuals with a group of familiar conspecifics (individual recognition) on the basis of visual cues alone (Waas and Colgan, 1994). This suggests that, in the case of threespine sticklebacks, different levels of recognition may be mediated by different sensory modalities.

Fathead minnows (*Pimephales promelas*) are small, schooling cyprinid fish, common to lakes and streams in central North America (Scott and Crossman, 1973). If they are able to discriminate between classes of conspecifics, this may provide a mechanism for kin discrimination (Brown and Colgan, 1986) or for increased shoaling efficiency and/or cohesiveness (VanHavre and FitzGerald, 1988). We designed this study to determine: (1) if fathead minnows can discriminate among conspecifics on the basis of familiarity, and (2) if chemosensory or visual cues alone are sufficient to allow for this recognition. The results are discussed in the context of kin discrimination and the potential for kin selection to act as a selective force favoring alarm signalling in fathead minnows.

METHODS AND MATERIALS

Fish Collection. Naturally occurring shoals of fathead minnows were collected from Pike Lake, an oxbow lake of the South Saskatchewan River in central Saskatchewan. Fish were collected using a beach seine along a 3-m section of shoreline. One or two seine hauls were taken until a minimum of 50

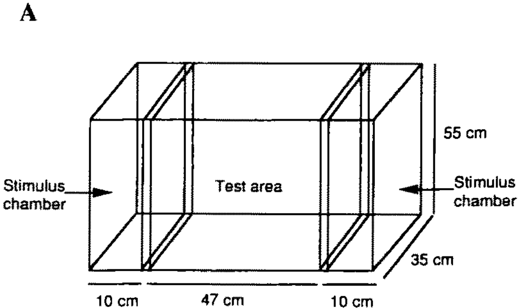
minnows per site were collected. Six discrete shoals were collected and no two were less than 200 m apart.

Each natural shoal was divided into two equal subgroups and placed into separate 37-liter aquaria. Fish were maintained under a 12L:12D cycle for a period of at least four weeks prior to testing. The sides of the tanks were covered with black plastic to prevent visual communication between tanks. Fish were fed daily with Nutrafin flakes.

Experiment 1 Treatment Conditions. Four identical glass aquaria (67 × 35 × 55 cm high) were used as testing tanks. The water depth was maintained at 40 cm. Barriers were placed 10 cm in from each end (Figure 1A) to create chambers for groups of stimulus fish (stimulus chambers). We divided the remainder of the tank into three equal sections by drawing grid marks on the exterior of the tank. An airstone was placed along the center of the back wall.

Minnows were tested in one of three treatment conditions: (1) visual cues only (visual trials), (2) chemosensory cues only (chemical trials), and (3) both

A



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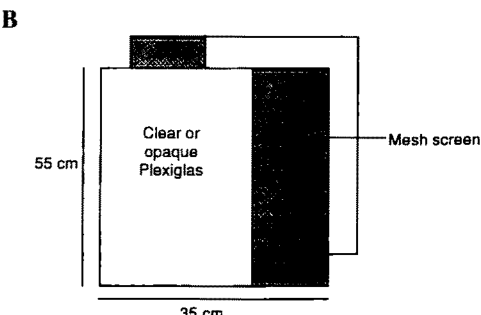

FIG. 1. Schematic diagram of test tank (A) and barriers used in the chemosensory only and combined cues trials (B). Drawings not to scale.

visual and chemosensory cues (combined trials). The test tanks were the same for all conditions with the exception of the construction of the barriers. In the visual trials, pairs of Plexiglas sheets (approximately 3 mm thick) were mounted 1.5 cm apart, 10 cm from each end of the tank. These barriers were sealed with silicone so there was no exchange of water between the stimulus and testing chambers. Fish could see through the barriers.

In the chemical trials, the barriers consisted of opaque Plexiglas frames, in which opaque Plexiglas sheets extended across two thirds of the total width, with the remaining third being covered with mesh screen. The barriers were mounted in pairs, so that the mesh portions were on opposite sides of the tank (Figure 1B). This allowed diffusion of water between the stimulus chamber and the test chamber, but did not allow visual communication between chambers. The barriers used for the combined trials were similar to those used in the chemical traits, but were constructed of clear Plexiglas, allowing for both chemosensory and visual communication between the chambers.

Experiment 1 Test Protocol. Pairs of shoals were chosen at random, from which the stimulus groups and the test fish were selected. Groups of five stimulus fish from both the shoalmate and unfamiliar groups were placed into each stimulus chamber and given a 24-hr acclimation period prior to testing. We randomly chose one shoal of the pair to be the shoalmate group. Stimulus fish were matched for size (i.e., mean length shoalmate stimulus group = mean length unfamiliar stimulus group).

A single test fish was placed into a mesh cylinder (15 cm diameter, 45 cm high) located in the center of the tank. Although fathead minnows are generally considered a shoaling species, we tested a single fish in order to ensure that the test fish was motivated to join one of the two possible stimulus groups (i.e., join an existing shoal). After a 1-hr acclimation period, we removed the cylinder and observed the fish for a 10-min observation period. We recorded two behavioral measures: (1) initial and final choice, and (2) time spent in each of the three sections of the tank. For initial and final choices, a fish was considered to have made a choice once half of its body had crossed the boundary between two sections. The section nearest the stimulus chamber containing fish from the same shoal as the test fish was designated as the shoalmate section and the section nearest the chamber containing fish from another shoal was designated as the unfamiliar section. The middle section was designated as the no-choice section.

After each trial, test fish were marked by clipping the caudal fin and returned to their original holding tank after a trial. Each fish was tested only once. Twenty minnows were tested in each treatment condition. We drained and cleaned test tanks between trials to remove any residual chemical cues. First and final choices were compared using a binomial probability test (Siegel, 1956). Overall differences in the time spent in each section of the test tank were compared using a

Friedman's analysis of variance (Siegel, 1956) and individual differences were compared using a Wilcoxon's signed ranks test (Siegel, 1956). All statistics were conducted using StatView SE software on a Macintosh computer.

Experiment 2 Testing Protocol. The tests described above did not allow us to determine if fathead minnows discriminated between natural shoalmates or simply individuals with which they have been held in the laboratory. In order to control for this, we tested individual fathead minnows to determine if they are able to discriminate between unfamiliar conspecifics and natural shoalmates that had been held in another tank. At the time of testing, subgroups of each shoal had been isolated for a minimum of two months (range two to five months). Using the test tanks that allowed for chemical cues only, we tested 20 individual minnows as previously described. The stimulus groups were picked at random, and the test fish was arbitrarily chosen from the second subgroup of one of the stimulus groups. We recorded the same behavioral measures and subjected the data to the same statistical treatments as described for experiment 1.

RESULTS

Experiment 1. When the trials began, the test fish typically swam around the tank for a short period (approximately 30 sec) and then adopted a stationary position near one of the stimulus chambers. There was no significant difference in any condition in terms of initial choice for shoalmate versus unfamiliar stimulus groups (Table 1). In the combined and chemical trials, test fish were observed in the shoalmate area of the test tank significantly more often than in the unfamiliar area at the end of the trial (final choice; Table 1). No significant difference in final choice was observed in the visual trials (Table 1).

Similar results were observed when we compared the amount of time spent in each section of the test tank. Significant overall differences were observed in the combined and chemical trials (Table 2, Figure 2), but not in the visual trials

TABLE 1. NUMBER OF INITIAL AND FINAL CHOICES FOR SHOALMATES VERSUS UNFAMILIAR CONSPECIFICS[a]

	Initial choice			Final choice			
Treatment	Shoal	Unfam	P	Shoal	Unfam	NC	P
Combined trials	8	12	0.25	16	3	1	0.001
Chemical trials	12	8	0.25	13	5	2	0.02
Visual trials	12	8	0.25	10	10	0	0.59

[a]Binomial test; Shoal = shoalmate, Unfam = unfamiliar, NC = no choice.

TABLE 2. OVERALL AND INDIVIDUAL COMPARISONS OF AMOUNT OF TIME SPENT IN EACH SECTION OF TEST TANK FOR EACH TRIAL CONDITION[a]

Treatment	Overall (χ^2)	Shoal vs. Unfam (Z)	Shoal vs. NC (Z)	Unfam vs. NC (Z)
Combined trials	14.8*	−3.17*	−3.58*	−0.22[ns]
Chemical trials	9.3*	−3.02*	−2.80*	−0.97[ns]
Visual trials	2.4[ns]			

[a]Abbreviations as in Table 1. χ^2 = Friedman's analysis of variance; Z = Wilcoxon's signed ranks test; * = $P \leq 0.05$; ns = $P > 0.05$.

(Table 2, Figure 2). In the combined and chemical trials, individual minnows spent significantly more time in the compartment nearest shoalmates (Table 2, Figure 2) versus either the unfamiliar or the no choice sections. No difference was found between the amount of time spent in the no-choice and the unfamiliar sections (Table 2, Figure 2). Since no overall difference was found in the visual trials, no individual comparisons could be calculated.

Experiment 2. Individual test fish responded in a similar manner as described for the chemical trials in experiment 1. No significant difference was observed in the initial choice data (9 shoalmate, 11 unfamiliar; $P = 0.412$), but there was a significant difference in the number of shoalmate versus unfamiliar final choices (14 shoalmate, 5 unfamiliar; $P = 0.03$).

An overall difference was found in the amount of time spent in each section of the test tank ($\chi^2_{(df=2)} = 6.30$, $P \leq 0.05$; Figure 3). The test fish spent significantly more time in the shoalmate section than the unfamiliar section ($Z = -2.15$, $P \leq 0.02$; Figure 3) or the no-choice section ($Z = -2.91$, $P \leq 0.01$; Figure 3). No significant difference was seen in the amount of time spent in the no choice versus the unfamiliar sections ($Z = -0.60$, $P = 0.48$; Figure 3).

DISCUSSION

These data demonstrate that fathead minnows are able to discriminate individuals from naturally occurring shoals (i.e., familiar) from unfamiliar conspecifics, but only when chemosensory cues are present. When visual cues alone were presented, the test fish did not exhibit a significant preference for shoalmates versus unfamiliar conspecifics. The ability to recognize familiar from unfamiliar conspecifics has been argued potentially to increase the benefits obtained from shoaling. VanHavre and FitzGerald (1988) suggest that by recognizing familiar or related conspecifics, individuals might form schools faster and

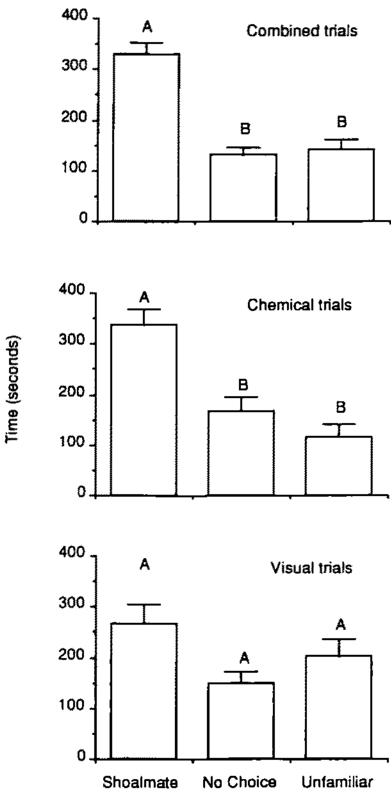

FIG. 2. Mean (+1SE) time in seconds spent in each of the three compartments for the three conditions when given the choice between shoalmates and unfamiliar conspecifics. Different letters denote significant differences at $P \leq 0.05$, Wilcoxon's signed ranks test (Z).

more cohesively when under a predation threat. Waldman (1982) has argued that this may serve as a selection pressure for similar discrimination abilities in American toad (*Bufo americanus*) tadpoles. Chivers et al. (1994) suggest that there may be significant benefits to schooling with familiar versus unfamiliar conspecifics in fathead minnows. They demonstrated that groups of familiar minnows exhibit increased antipredator behaviors (i.e., increased shoal cohesion and increased frequency of predator inspection visits) following exposure to a chemical stimuli from a known predator or to a visual threat of a model predator, compared to groups of unfamiliar minnows.

The results of experiment 2 suggest that individuals can discriminate shoalmates from naturally occurring shoals even after two months of isolation. This

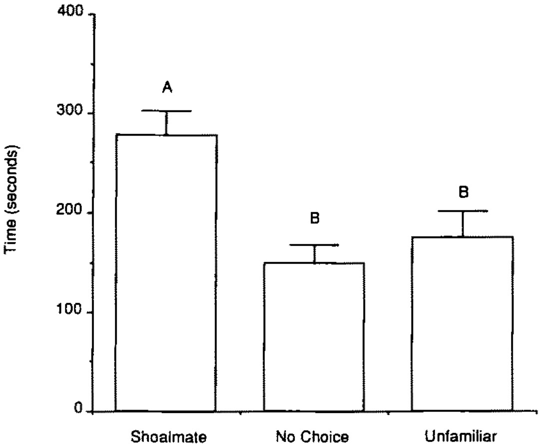

FIG. 3. Mean (+1SE) time in seconds spent in each of the three compartments of the test tank when given the choice between shoalmates housed separately and unfamiliar conspecifics. Different letters denote significant differences at $P \leq 0.05$, Wilcoxon's signed ranks test (Z).

would provide a mechanism by which shoalmates could be separated and later rejoin shoals. Given the potential benefits of shoaling with familiar conspecifics (Chivers et al., 1994), this discrimination ability might allow individuals to maximize these benefits. In addition, the observation that shoals may break up and reform into mixed groups would not preclude long-term shoalmate associations, given that they can recognize shoalmates after relatively long periods of time.

The ability to discriminate shoalmates based on chemosensory cues alone would allow individuals to obtain these benefits while not having to remain in constant visual contact with shoalmates. Fathead minnows have been shown to rely heavily upon chemosensory cues for various aspects of predator recognition and avoidance. The best known use of chemical signals is likely the alarm response, as a result of the detection of an alarm pheromone. The release of alarm pheromone by an injured minnow elicits a stereotypic fright response in nearby conspecifics (Smith, 1986, 1992). Minnows have been shown to use chemosensory cues to learn the location of dangerous or risky habitats (Chivers and Smith, 1994a) and the identity of potential predators such as northern pike (*Esox lucius*) (Chivers and Smith, 1994b). Mate attraction by both males and females appears to be based in part on chemosensory cues (Cole and Smith, 1987, 1992). In addition, pike that have been consuming minnows, but not swordtails or breeding male fathead minnows that lack alarm substance cells (Smith, 1973), are chemically labeled as a dangerous predator to pike-naive

minnows (Mathis and Smith, 1993a,b; Brown et al., 1994). Given this previously documented reliance on chemosensory cues, it is not surprising that shoalmate recognition appears also to be strongly dependent on chemosensory cues. We cannot, however, rule out the secondary use of visual cues.

Two social contexts in which familiarity-based recognition systems may be favored are dominance hierarchies and kinship-based recognition (Brown and Colgan, 1986). If kin groups do not disperse upon hatching, then familiarity among individuals may be correlated with kinship. We can refer to this phenomenon as familiarity-based kin recognition. The possibility of familiarity-based kin recognition is important in these fish, because it has been suggested that cyprinid alarm signaling may be kin selected (Smith, 1977, 1992). If an individual is injured and releases the alarm pheromone during a predation event, it might warn others within the immediate area of the potential predation risk. If conspecifics within the immediate area are related, then the individual can maintain its inclusive fitness by increasing the probability of survival of kin (Wilson, 1987; Smith, 1977, 1992).

Naish et al. (1993) examined this possibility in the European minnow (*Phoxinus phoxinus*) using mitochondrial DNA and multilocus DNA fingerprinting techniques. They failed to find evidence of higher relatedness within rather than between aggregations. This cannot, however, be taken as evidence against the existence of kin-selected benefits associated with alarm signaling. It is possible that an aggregation may consist of many sibling groups, hence still allowing for the possibility of increased inclusive fitness potential by warning relatives within a mixed kin group shoal. In addition, the results of experiment 2 suggest that individual minnows retain the ability to discriminate among conspecifics even after long periods of separation, possibly compensating for mixing of shoals under natural conditions.

Kin discrimination can occur as the result of a variety of mechanisms, including direct familiarity (Fletcher, 1987). A variety of species have been shown to bias their social behavior towards kin based on previous interactions or direct familiarity, including ground squirrels (Schwagmeyer, 1988; Porter and Blaustein, 1989), deermice (*Peromyscus maniculatus*) (Dewsbury, 1982), snow geese (*Anser caerulescens*) (Cooke, 1978), bank swallows (*Riparia riparia*) (Beecher et al., 1981), and spadefoot toad tadpoles (*Scaphiopus multiplicatus*) (Pfennig, 1990).

The ability to recognize and aggregate preferentially with related conspecifics has also been demonstrated in a variety of species (e.g., rainbow trout and Atlantic salmon) (*Oncorhynchus mykiss* and *Salmo salar*) (Brown and Brown, 1993a,b), white-footed deermice (*Peromyscus leucopus*) (Grau, 1982), and anuran tadpoles (Blaustein and Waldman, 1992). If minnows are more likely to remain with the same group for an extended period of time, then familiarity-based kin-biased shoaling may occur, as argued for bluegills (Brown and Col-

gan, 1986). Ferguson and Noakes (1981) provide evidence that common shiners (*Notropis cornutus*) have more homogeneous gene frequencies within, rather than between, groups. This can be taken as evidence for kin-biased aggregation tendencies.

In summary, fathead minnows appear to be able to discriminate among conspecifics based on familiarity and can do so using chemosensory cues. Given their life histories, this ability may result in kin-biased shoaling, which, in turn, may serve as mechanism for the kin selection of alarm signaling in this cyprinid fish.

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BEAVER (*Castor canadensis*) RESPONSES TO MAJOR PHENOLIC AND NEUTRAL COMPOUNDS IN CASTOREUM

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Abstract—North American beaver (*Castor canadensis*) mark their territories with castoreum, a chemically complex secretion from their castor sacs. The phenolic and neutral fractions of castoreum have been shown to elicit specific behavioral responses from beavers in a field setting. Our objective was to identify compounds/mixtures that evoked responses similar to those stimulated by castoreum. We assayed recently identified phenolic compounds, some phenolics that had been determined to be biologically active in previous studies, the neutral compound borneol, and combinations of phenolic compounds, neutral compounds, and the two combined. Biological activity was measured by the elicitation and extent of specific responses and their strength (duration, frequency, and proportion of beavers responding). Generally, single compounds stimulated fewer responses than mixtures. A 26-compound mixture of phenolic and neutral compounds elicited responses in a similar proportion of trials as castoreum. However, responses to castoreum were stronger than to any synthetic sample. Further investigation of different measures of response, namely, elicitation, completeness, and strength, are deemed necessary to fully decipher the design of social odors.

Key Words—*Castor canadensis*, beaver, castoreum, communication, social odors, phenolic compounds, neutral compounds, territorial behavior, response measures.

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INTRODUCTION

Species-specific mammalian olfactory signals, termed social odors or pheromones, contain diverse types of chemical compounds serving a variety of functions (Gorman, 1976; Jemiolo et al., 1985; Singer et al., 1976, 1986; Smith et al., 1985; Klemm et al., 1987; Belcher et al., 1988). Communicative scents often include numerous compounds that appear to play no role in the olfactory message. The remaining compounds may work together through addition, synergism, redundancy, or suppression to construct the signal's meaning, which is elucidated typically by observing the responses of the receiving animals. Deciphering the role or necessity of individual components in a biologically active fraction of a social odor requires extensive assays of compounds, singly and in mixtures.

The North American beaver, *Castor canadensis*, is an excellent model for field studies of mammalian social odors because beavers rely heavily on scent, but less so on visual or auditory communication; perform a highly detectable and stereotypical scent marking behavior; can be live-trapped and tagged for identification; are easily located and observed at close range; and are found in large enough populations for meaningful data acquisition. Beavers live in family units and are considered to be monogamous (Svendsen, 1989 and references therein). Their year-round territories are demarcated by mud mounds on the banks of their ponds. Beaver mounds are marked with urine and castoreum from the castor sacs, and possibly with anal gland secretion. While marking may serve multiple purposes, recent studies have supported a territorial function as primary (Houlihan, 1989; Welsh and Müller-Schwarze, 1989).

Yet, we still have an incomplete understanding of the functional composition of beavers' territorial signals. For instance, are these signals individual identifiers, species-specific social odors, or pheromones that couple explicit messages (e.g., warning, ownership) with specific responses (e.g., avoidance, scent marking)? Individual markers may be key compounds or more likely arrays of compounds in different ratios. Responses to synthetic odors that may represent an individual are difficult to predict without an understanding of the individual being mimicked. If a social odor, composed of redundant components, signals "beaver," then many compounds or mixtures may evoke similar activity. The social odor hypothesis predicts mixtures should generally be more active than single compounds. However, if a territorial pheromone, consisting of one or a few specific compounds, exists, then most single compounds or mixtures from biologically active fractions should evoke little activity. The relative activity of compounds versus mixtures cannot be explicitly predicted without an understanding of the pheromone's chemical composition. Furthermore, pheromones and species-level social odors should elicit similar responses from year to year,

whereas individual cues (and subsequent responses) may change on much shorter time scales.

Although beaver castoreum is a chemically complex secretion consisting of well over 100 compounds (Lederer 1946, 1950; Maurer and Ohloff, 1976), only specific chemical fractions evoke responses by beavers. Placement of whole or partial fractions of these secretions on human-made mud piles releases typical scent marking behavior (Müller-Schwarze et al., 1983, 1986), which includes oriented sniffing from the water, visitation to the experimental scent mound (ESM), and overmarking of the ESM. By definition, land visits to the ESM are indicative of greater biological activity than oriented sniffing from the water. Beavers were attracted to ESMs containing the phenolic and neutral components of castoreum but not the carboxylic acids or amines (Svendsen and Huntsman, 1988). Several single castoreum compounds, especially phenolics, were active alone and in mixtures (Müller-Schwarze and Houlihan, 1991; Müller-Schwarze, 1992).

A primary goal of this research was the continued search for castoreum components that evoked similar responses (activity) as whole castoreum. Activity was measured in the ability to elicit specific responses, the completeness of land visit responses to the ESM, and the strength of these responses. Therefore, recently identified and/or abundant phenolic compounds, the neutral compound borneol, and progressively larger mixtures of phenolic compounds, a mixture of neutral compounds, and combined mixtures of the two were assayed. The compound 4-ethylphenol exhibited strong activity in a previous study (Müller-Schwarze and Houlihan, 1991) and was retested at four concentrations to further investigate its candidacy as a territorial pheromone. Our focus was on assaying phenolic and neutral compounds with the intention of better understanding the overall design of the castoreum odor signal in a territorial context.

METHODS AND MATERIALS

Study Area. All field experiments were conducted during May–September, 1989–1992, in Allegany State Park, Cattaraugus County, New York State, on an unharvested beaver population that had been live-trapped, ear-tagged, and observed since 1985. Sixteen to 20 beaver sites, consisting of one to several small ponds located on streams, were used for behavioral observations each year. The average family size was 5.5 animals (SD = 2.9, range 1–14).

Assumptions of Bioassay Design. Measuring the responses of the territorial resident to the odors or components of those odors from potential conspecific intruders is the basis of this bioassay design. The observed behaviors may reveal functionally relevant information, and any samples eliciting biological activity may truly play an important role in the social odor. Before attributing responses

by beavers to the experimental stimuli, we examined our four basic assumptions about the continuity of behavior: (1) the responses of beavers did not vary significantly over time within a year, or (2) across years; (3) beaver responses to castoreum components were not the result of associative learning to the castoreum control; and (4) beavers from different family units (sites) responded similarly to the same samples. Previous studies have shown assumptions 1 and 2 to be acceptable (Müller-Schwarze and Houlihan, 1991; Müller-Schwarze, 1992). In 1989, assumption 3 was examined by observing the responses to 4-ethylphenol at sites that had not yet been exposed to castoreum. This compound was selected because it was biologically active in a 1987 study at Allegany State Park (Müller-Schwarze and Houlihan, 1991). In the 1987 study, castoreum was assayed at sites prior to other samples. Comparing responses to 4-ethylphenol between the two years would allow us to test whether responses were the result of associative learning to castoreum. In regards to the final assumption, Houlihan (1989) demonstrated that neither family size nor the number of beaver sites along a stream were important variables in the responses of beavers to ESMs. However, other factors may contribute to response differences among sites. To test the null hypothesis that beavers at different sites responded to the same samples in a similar manner, beaver sites that had been used in assays of the same samples over the four years of this study were assembled into seven groups of three to nine sites each. Comparisons of the observed ("sniff from water" and land visit) and overnight land visit responses were made among sites within these groups.

General Methods. Beaver sites were surveyed for activity each spring prior to bioassays. Beavers were trapped with Hancock Live-Traps, weighed, and immobilized with a 1:2 mixture by volume of Rompun (xylazine) and Ketaset (ketamine) (0.67 and 6.7 mg/kg body weight, respectively). Sedated beavers were sexed (Young, 1936; Osborn, 1955; Schulte et al., in review), assigned to an age class (adult, 2-year-old, 1-year-old, kit) based on weight and size, and individually tagged with anodized aluminum ear tags in unique color combinations for both ears. From 1985 to 1992, 245 beavers were tagged. Of the beavers observed during the experiments, 70% were recognizable by tags, including 86% of the adults. Untagged beavers were identified by distinguishing physical marks and assigned to an age class based on their relative size.

Stimulus Preparation. We assayed individual compounds and selected mixtures of compounds that have been identified as components of castoreum (Tang et al., 1993, in preparation). A few commercially available castoreum constituents used in a previous study (Müller-Schwarze and Houlihan, 1991)—4-ethylphenol, 4-propylphenol, salicylaldehyde—and the neutral compound borneol were retested. The amount of each sample assayed and the composition of mixtures of phenolic and/or neutral compounds are given in Table 1. The ratio of compounds in mixtures was based on their ratio in a sample of 16 pooled,

TABLE 1. NAMES, ABBREVIATIONS (ABBR.), YEAR ASSAYED, AND SUMMARIZED BIOACTIVITY OF SAMPLES USED IN FIELD BIOASSAYS, 1989–1992

			Activity[a]		
Name	Abbr.	Year	Str	Eli	Com
A. Single Compounds (mg/trial)					
Acetovanillone	AC	—			
Borneol (0.56; 0.48)	BO	90; 92	I; I	I; I	M; P
2-catechol (0.014)	CT	89	I	I	I
2,6-dimethoxy-4-methylphenol (0.11)	DC	90; 92	I; I	I; I	I; I
3,5-dimethoxyphenol	DP	—			
4-ethylguaiacol (0.83)	EG	90	I	I	I
4-ethylphenol (0.69)	EP	89	P	I	P
4-ethylphenol (0.032)	E1	90	I	I	I
4-ethylphenol (0.32)	E2	90	I	I	I
4-ethylphenol (3.2)	E3	90	I	I	I
4-ethylphenol (32)	E4	90	I	I	I
4-(4′-hydroxyphenyl)-2-2-butanone	HB	—			
Indanol	IN	—			
m-cresol (0.023)	MC	89	P	I	I
4-methylguaiacol (0.033)	MG	90	M	P	I
o-cresol (0.11)	OC	89	P	I	I
4-propylguaiacol (0.025)	PG	90	P	I	I
Phenol	PH	—			
4-propylphenol (0.37)	PP	89	I	I	I
Salicylaldehyde (0.025)	SA	89	I	I	I
B. Mixtures					
BO + EG + MG + PG	BG	90	I	I	I
OC + MC + CT + SA (4 phenols)	FP	89	P	P	I
FP + EP + PP (6 phenols)	SP	89	I	I	P
SP + PH + HB + IN + AC (10 phenols)	TP	90	I	I	I
TP + G (13 phenols)	TG	92	I	I	P
TP + BG (13 phenols + borneol)	TBG	90; 92	I; P	I; P	I; P
TP + EG + MG + PG + DC (14 phenols)	P	91; 92	I; I	I; I	A; P
P + B (14 phenols + borneol)	PB	92	I	I	P
BO + 11 other neutrals[b]	N	91	M	I	P
P + N (1:1 ratio)	P + N	91	A	A	P
P at 5-fold concentration	5P	91	M	P	P
N at 5-fold concentration	5N	91	A	M	P
5P + 5N (1:1 ratio)	5P + 5N	91	A	M	A

TABLE 1. CONTINUED

Name	Abbr.	Year	Activity[a] Str	Eli	Com
C. Odor controls					
Castoreum (maximum response)	CA	89–92	A	A	A
Ethanol (minimum response)	OH	89–92	I	I	I
D. Blank (no applied odor)	BL	91; 92	I; I	I; I	I; I

[a]Activity — not tested; Str = strength of observed land visit is a composite bioactivity based on average duration of first land visit, number of land visits, and proportion of beaver visiting sample versus controls; Eli = elicitation of observed land visit compared proportion of trials in which land visits were observed to sample versus controls; Com = completeness of overnight land visit is a categorial comparison of extent (none, paw, scratch, flat/remark) of modification to ESMs marked with a sample versus controls. See Table 2 for explanation of activity codes where I = inactive; P = potentially active; M = mildly active; A = active. Assayed amounts of mixtures were 1/30 gland equivalent, except for three 5-fold concentrates, with the ratio of compounds based on those in 16 pooled, male castor sacs (Tang et al., 1993).

[b]The 12 neutral compounds were borneol, benzaldehyde, benzyl alcohol, 6-methyl-1-heptanol, *trans*- and *cis*-linalool oxide, 4,6-dimethylheptane-1-ol, *trans*-pinocarveol, isopinocamphone, verbenone, (1R)-myrtenol, and 3,4-dimethoxy-acetophenone.

male beaver castor sacs (Tang et al., 1993). Four concentrations of 4-ethylphenol (1000-fold range) and two concentrations (five fold range) of three mixtures were also assayed.

Each year, ethanol and castoreum served as minimum and maximum response elicitors, respectively. Ethanol was the solvent (usually 0.25 ml/trial) for all samples, except the castoreum control in 1990–1992, which was purchased as quill (dried beaver glands ground into small granules) from a trapping supplier and presented in dry form. Responses to castoreum dissolved in ethanol (1989) and to quill did not differ significantly. Blank (mud only) mounds were used in 1991 and 1992. Earlier studies showed no significant responses to either ethanol or blank ESMs (Müller-Schwarze et al., 1986; Müller-Schwarze and Houlihan, 1991).

Stimulus Presentation. For the bioassays, an ESM was constructed by scooping mud from the pond with a small container, held by gloved hand. The ESM was approximately 30 cm diameter × 10 cm high and located about 50 cm from the water's edge at a bank where beaver were able to exit from the water. A size 18 cork (3.6 cm top diameter) was placed in the center of the mound, even with the surface. The sample was applied to the cork with a disposable pipet. In order to detect subtle disturbances to the mound overnight, one-half of a tongue depressor was placed vertically into the mound (as by

Svendsen, 1980) adjacent to the cork. The placement of the tongue depressor does not interfere with beaver inspecting or remarking the mound.

Experimental Design. A minimum of eight trials and a maximum of 16 trials at different sites were performed for each sample during a single season. A trial was equivalent to a single-day, single-site assay. Samples were randomly assigned to beaver sites in 1989 (except 4-ethylphenol) and 1992 using a random number table. In 1990 and 1991, a Latin square design structure was implemented to balance the testing of the chemically similar samples over time at different sites. Beaver sites were grouped by proximity in the same watershed into clusters of four (1990) or three (1991) sites. Samples were also grouped by fours or threes based upon their similarity in chemical composition or type (i.e., yielding a 4 × 4 or 3 × 3 Latin square). The matrix size was determined by the number of available observers each season. Control trials were run at the beginning and end of assaying the samples in each square.

Behavior Observations. Behavioral bioassays were performed from June to September in 1989 and May to August in 1990–1992, following the procedures described by Müller-Schwarze and Houlihan (1991). The ESM was built and the scent applied at about 1700 hr, generally 0.5–1 hr before the first beaver emerged. Each beaver was observed from the time it emerged until nightfall (2000-2130 hr, except in September 1989: 1900–2000 hr). A computer program created by P. Houlihan and modified by C. Sack for the Tandy 102 portable computer automatically records the time of each behavior entered. A field notebook and a stopwatch were available to record observations in case of computer failure. When one or more beavers approached the sample, observations were focused on these individuals (i.e., focal location). An exhaustive ethogram was created, but the behaviors of greatest interest included: swim (fast, slow), float, tail-slap, dive, sniff from water (general or directed at ESM), land visit, and activity at the ESM (sniff, scratch, mark). Weather conditions, such as precipitation, air temperature, and wind direction, and unusual observations were noted.

The ESM was checked the next morning for signs of visitation during the night, separated into four categories: (1) intact; (2) paw prints on an otherwise undisturbed ESM; (3) scratch marks, movement of the applicator, and/or removal of the cork; and (4) flattening or marking (fresh castoreum odor) of the mound. Activity at the ESM that could be attributed to other animals (e.g., whitetail deer, raccoon) was discarded. Marking was discernible by sniffing the mound and an area of 50 cm radius around the ESM.

Format of Data. For each year of the study, biological activity was determined by comparing responses to each sample with the responses to castoreum (maximum response control) and to ethanol (minimum response control). The responses to samples was also compared to the results from the controls pooled over the four years.

The data were organized into two general types: observed responses and overnight land visits. The observed responses were sniffing from the water (directed towards and within 5 m of ESM) and land visits. In a sniff response, beavers orient toward the ESM and noticeably sniff by raising their lower jaw slightly out of the water. Land visits occurred when a beaver exited the water, moved directly to the ESM, and sniffed and/or contacted the ESM. Three numerically continuous parameters, and one categorical parameter, of the observed responses were used: (1) average duration in seconds per beaver responding per trial; (2) average number of responses, calculated as the number of sniffs or land visits per beaver responding per trial; (3) the proportion of the number of beaver responding to the total number of beaver seen per trial; and (4) classifying the trials as no response, sniff from water only, or land visit. Because beavers live in family units, duration of land visits was determined both for the first individual to respond and for all responding members of the family unit combined. The three continuous parameters were calculated by first using all trials, then a second time by including only those trials in which responses were observed. During observations of land visits, beavers would often sniff the ESM without flattening or overmarking it. Therefore, an observed land visit does not necessarily include a scent-marking response. Responses by kits were not included in the observed analysis.

The data from overnight land visits were analyzed by the categories of response (intact, paw, scratch, flat/re-mark). Each trial was placed in one category only, representing the most complete response recorded. If a beaver left no trace of its presence on or near the ESM, then the ESM was labeled intact, even if a beaver had visited the ESM during the observations. This maintained the independence of the observed and overnight measures of land visit response. Trials in which no beaver were seen were included only in the overnight data set, and only if beaver were determined to still reside at the site.

Statistics. For each response measure, castoreum controls were compared across all four years using analysis of variance (ANOVA) for the continuous data and the log likelihood goodness of fit test (G) for the categorical data. Because the assumptions of normality and equality of variance for ANOVA were often not met with the raw data sets, Kruskal-Wallis tests were performed by year. Each sample was compared by Wilcoxon's rank sum test to its maximum and minimum controls from the same year. Contingency table analysis was used for comparisons of the categorical data. The Yates correction in 2×2 contingency tables and the Williams correction for larger tables were utilized to provide a conservative test of the null hypothesis (Sokal and Rohlf, 1981). Exact P values (Mehta and Patel, 1992), calculated for these likelihood ratio tests, agreed quite well with the P values approximated from the G statistic (i.e., the conclusions drawn from the data analyses were the same).

The Statistical Analysis System (SAS, Cary, North Carolina) and the BIOM package of statistical programs (Rohlf, 1987) were used for all data analyses.

Bioactivity Criteria. As minimal requirement for activity, we stipulated that the responses to a sample were statistically different ($\alpha = 0.05$) from the responses to the ethanol control. Bioactivity was determined by considering the proportion of trials in which a particular response was observed (elicitation) or for overnight land visits the extent to which the ESM was disturbed (completeness) and the duration, frequency, and proportion of beaver sniffing from the water or visiting the ESM (strength) for the observed responses. Each sample was categorized into one of four activity levels for each response measure (Table 2). Bioactivity measures of completeness and strength included a component of response elicitation because trials with no responses were considered as values of zero to ensure appropriate sample sizes for the statistical analyses (Table 1).

RESULTS

Examination of Assumptions of Bioassay Design. Responses by beaver to the experimental samples did not appear to change across time, either within a season or between years. All regressions of sniff from water and land visit responses to the castoreum control against time were nonsignificant (all $P > 0.13$). The responses to castoreum across the four years also did not differ statistically, although responsiveness appeared to diminish in 1992 (Table 3A). In addition, the preparation of the castoreum control (ethanol solvent in 1989 versus dry quill in 1990–1992) had no effect on the response. Responses to the ethanol control did not differ within or between years (Table 3B). Responses to ethanol also did not differ from those to the blank ESMs. Since the beaver did not respond differently to the controls in the four years, their disposition to respond to all other stimuli was assumed not to differ either.

Animals may respond to a placed sample because of its association in time or space with a biologically meaningful chemical signal. We tested this by

TABLE 2. ACTIVITY LEVELS ASSIGNED TO SAMPLES (TABLE 1) BASED ON PAIRWISE WILCOXON RANK SUM TESTS (COMPARISON ERROR RATE = 0.05) WHERE IDENTICAL LETTERS INDICATE NO STATISTICALLY SIGNIFICANT DIFFERENCE

Ethanol	Sample	Castoreum	Activity level
a	a	b	Inactive (I)
a	ab	b	Potentially active (PA)
a	b	c	Mildly active (MA)
a	b	b	Active (A)

TABLE 3. RESPONSE BY TRIAL TO CASTOREUM AND ETHANOL CONTROLS[a]

	Responses by trial						
	Observed[b]			Overnight[b]			
	None	Sniff	LV[c]	Intact	Paw	Scratch	Flat
A. Castoreum							
1989	3	1	4	1	1	2	4
1990	3	0	8	1	0	1	11
1991	2	3	10	3	4	3	5
1992	3	2	6	4	4	0	3
1989-1992	11	6	28	9	9	6	23
B. Ethanol							
1989	7	1	0	7	0	0	1
1990	11	0	0	11	0	0	0
1991	11	1	0	10	3	0	1
1992	6	2	1	10	0	0	0
1989-1992	35	4	1	38	3	0	2

[a] Values in boxes are number of trials for each response category. For castoreum (CA) the years were not statistically different using the G (Williams) value for contingency table analysis and exact or nearly exact (Monte Carlo) P values for observed ($G = 4.96$, $df = 6$, $P = 0.60$) or for the overnight visitation ($G = 16.8$, $df = 9$, $P = 0.06$); the same was true for ethanol, observed ($G = 3.07$, $df = 6$, $P = 0.37$), Overnight ($G = 4.13$, $df = 6$, $P = 0.10$); for ethanol the paw + scratch categories were combined because of zeros. For each year, CA was significantly different from OH, as were the combined totals from the four years (combined: observed $G = 43.2$, $df = 2$, $P < 0.001$; overnight $G = 48.8$, $df = 3$, $P < 0.001$).

[b] See text for complete explanation of categories.

[c] LV = land visit to ESM. Land visits may have been preceded by sniffs, but trials in the sniff column had no land visits.

assaying the single compound 4-ethylphenol (EP) both before any castoreum samples were assayed (1989, this study) and after assays with castoreum conducted at Allegany State Park in 1987 by Müller-Schwarze and Houlihan (1991). The similar levels of response to EP between the two studies (if anything slightly stronger in 1989) suggested that association with the castoreum control was not the cause of the responses. In the 1987 study, beavers were observed to visit the ESM in 10% of the trials compared to the 12.5% recorded in 1989. Overnight visitations were made in 25% of the trials in 1987 compared to 62.5% in this study. Thus, beavers did not respond to 4-ethylphenol as a result of association with castoreum in the sequence order of assays. If these results were generalizable for all samples, then the order of the sample relative to castoreum in the experimental design should not have affected the response of the beaver.

Furthermore, beavers from different sites responded similarly to the chemical samples. While beavers from separate sites did vary in their amount and

degree of responsiveness, responses were not statistically different among sites for most site groups. For instance, of the 16 sites used most frequently throughout this study, beavers at one site exhibited a reduced level of response, while beavers at a second site displayed greater responsiveness. Beavers at the remaining 14 sites behaved similarly to the same samples both within and among years. Therefore, the bioactivity of samples cannot be attributed to the response characteristics of the beavers at the sites where the assays were performed, but rather should be generalizable across beavers from most sites, at least in our study area.

Observed Sniff from Water Responses. Responses to the samples differed mainly in their probability of evoking sniffing and not the strength of the response (i.e., duration or frequency of sniffs or the proportion of beavers sniffing). Beavers sniffed ethanol in only four of 40 trials (10%) compared to 31 of 45 (69%) for castoreum ($G = 30.5$, 1 *df*, $P < 0.001$). Considering only trials in which sniff responses were observed, the average duration of sniffing ESMs containing ethanol was nearly as long (9.1 sec ± 5.4) as castoreum (11.2 sec ± 1.1). The number of responses to ethanol were two few to statistically compare with responses to castoreum (Figure 1). Sniffing was elicited in at least 50% of the trials by only six samples—one single compound (EP) and five mixtures: EP 50%; six-phenol mixture (SP) 50%, 12-neutral mixture (N) 56%, 12-neutral mixture at fivefold higher concentration (5N) 78%, 26-compound mixture of phenolic and neutrals (P + N) 56%, and 26-compound mixture at fivefold higher concentration (5P + 5N) 67%. The mean sniff duration ranged

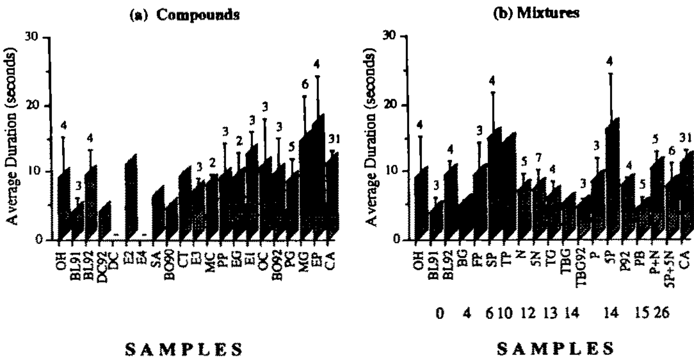

FIG. 1. Duration of sniffing from water by beaver to compounds, mixtures, and controls for trials with responses only. Compounds are ordered based on activity. Mixtures are ordered by increasing number of compounds in sample (shown along abscissa in b). Bars are one standard error. Sample sizes shown above error bars. Samples lacking error bars had only one value; samples with (-) had no response. See Table 1 for key to abbreviations of samples.

from 4 to 17 sec with greater means having correspondingly high variations (Figure 1). While very few samples evoked sniffing in a majority of the trials, once initiated, the duration of sniff responses by beavers were not highly discriminatory among the samples. The same was true for frequency of sniffs and the proportion of beaver sniffing (results not shown).

Observed Land Visits. During land visits beavers directly sniffed the ESM and surrounding area, often followed by beavers standing upright and rotating their heads while sniffing the air. On some instances beavers scratched at the ESM, occasionally flattening and/or overmarking it.

Most of the samples triggered only few or no observed land visits. Hence, determination of bioactivity based upon strength of the observed land visits (Table 1) was made by including all no response trials (e.g., duration of land visit equal to zero) in the analyses. Five mixtures [four phenolics (FP), P + N, 5P, 5N, 5P + 5N] were visited in 30% or more of the trials. Only one single compound, 4-methylguaiacol, evoked a land visit in more than one trial (three trials, 23%). Land visits to 4-ethylphenol (EP; E1–E4; concentrations and years pooled) were observed in three of 52 trials (6%).

Typically, land bouts lasted 20–45 sec. Only the mean duration of first land visits are shown (Figure 2) because land visits occurred just once per trial to each of the compounds and to most of the mixtures (Figure 3). Thus, there were no important differences in the duration of the first land visit versus all land visits (i.e., individual versus family response). On average, less than 50% of the beavers observed per trial responded to the sample (Figure 4). The two exceptions were to castoreum and a single trial with a land visit to 4-propylguaiacol (PG).

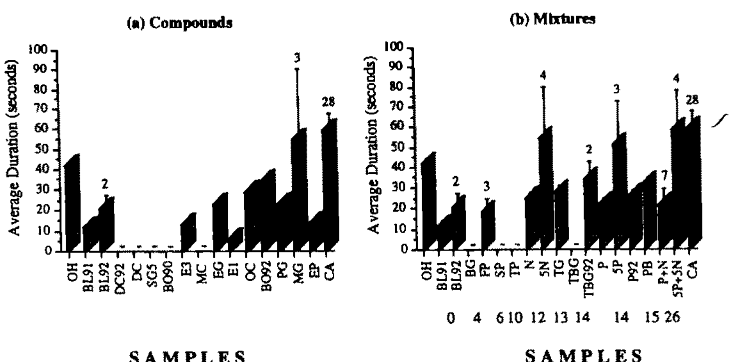

FIG. 2. Duration of first land visit by beaver to compounds, mixtures, and controls for trials with responses only. Samples ordered and format same as in Figure 1. Five compounds are grouped as SG5 = CT, E2, E4, PP, and SA. Sample sizes (above error bars) differ from Figure 1.

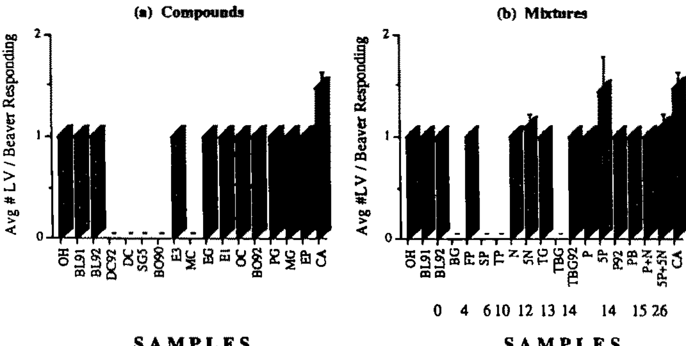

FIG. 3. Average number of land visits (LV) by beaver to compounds, mixtures, and controls for trials with responses only. Samples ordered and format same as in Figure 1. Sample sizes and abbreviations as in Figure 2.

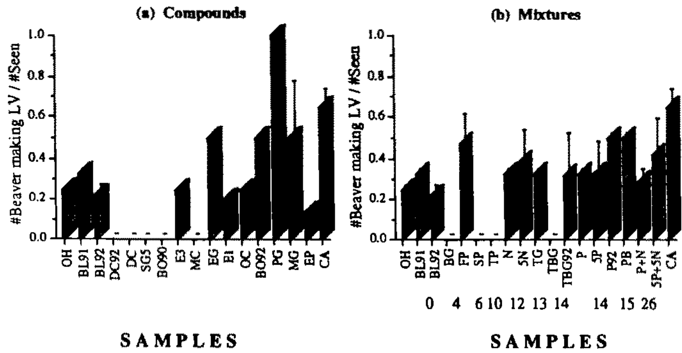

FIG. 4. Proportion of beavers making land visits (LV) to compounds, mixtures and controls for trials with responses only. Samples ordered as in Figure 1. Sample sizes and abbreviations as in Figure 2.

For the three mixtures tested at two concentrations (N, 5N; P, 5P; P + N, 5P + 5N), no significant differences were found in the observed responses by beavers between any of the concentration pairs. However, both the number of land visits and their durations in response to the high concentrations of the phenolic compounds (5P), neutral compounds (5N), and the two combined (5P + 5N) were more similar to castoreum than the responses to any other samples (Figures 2 and 3).

Relationship of Sniffing to Observed Land Visit. In general, the observed response variables were not linearly correlated (Table 4). However, the response

TABLE 4. CORRELATION MATRIX OF OBSERVED RESPONSE MEASURES, 1989–1992[a]

Response variables	Sniff duration	Sniff number	Sniff Pro	First LV duration	LV number	LV Pro
SF Dur	1.00	0.16	−0.09	0.16	−0.04	−0.10
SF Num	0.22**	1.00	0.08	−0.16	0.50**	0.28
SF Pro	−0.04	0.16	1.00	0.04	0.25	0.77**
LV Dur	0.24	−0.04	0.09	1.00	0.02	0.29*
LV Num	−0.03	0.49**	0.41	0.15	1.00	0.22
LV Pro	−0.04	0.31*	0.81**	0.32*	0.32*	1.00

[a] Sniff (SF) = sniffing from water; LV = land visit; For each response type, duration (Dur) and number (Num) of responses, plus the proportion (Pro) of beaver responding/seen was determined. Only trials in which a sniff from water or land visit were observed are included in this analysis. Values in the top half of the matrix are Pearson product-moment correlations. Spearman rank correlation coefficients are shown in the bottom half of the matrix. The only high correlations are between the duration of first and all land visits, and between the proportion of beavers sniffing and making land visits. Sample size: N = 137 for sniffing from water and N = 70 for land visit variables. For tests rejecting the null hypothesis that the correlation coefficient = 0: $^{*}P < 0.05$; $^{**}P < 0.001$.

variables that were highly correlated were between sniff from water and land visit variables and not within either type. Specifically, the proportion of beaver from a family that made a land visit was positively correlated with the proportion of beaver sniffing (r = 0.77, P = 0.0001). Sniffing preceded land visits in 71% of the trials. Thus, most beavers that made a land visit sniffed from the water first in an observable fashion. In addition, the average number of land visits was correlated with the average number of sniffs from the water (r = 0.50, P = 0.0003). The average number of sniffs and land visits per beaver was often just one in any trial, which may explain this correlation.

Overnight Land Visits. Strength (as defined by duration, frequency, and proportion of beaver visiting) of the overnight land visits could not be determined. Based on only the proportion of trials in which the condition of the ESM overnight was categorized as intact, pawed, scratched, or flattened/overmarked (completeness), the activity of the samples was determined (G statistic, 3 df): 10 compounds and three different mixtures were inactive; two compounds (EP; BO92) and nine mixtures were partially active; one compound (BO90) and no mixtures were mildly active; and no compounds and two mixtures (P, 5P + 5N) were active (Table 1). Three different mixtures were visited in a majority of their trials: P (88.9%) and 5P (55.6%); 5N (66.7%); P + N (66.7%) and 5P + 5N (55.6%).

Castoreum evoked overnight land visits in 38 of 47 trials (81%), while ethanol elicited land visits in only five of 43 trials (11.6%). In 29 trials, there were no overnight land visits to blank ESMs. Of the single compounds, only

4-ethylphenol (EP) was visited in over half its trials (5/8, 62.5%). However, responses to the four concentrations of EP tested the next year (1990) elicited fewer overnight land visits (18–37% of the trials). There was no pattern of response related to concentration.

Responses to borneol (BO90, BO92) showed the greatest contrast in the observed versus the overnight measures. Beavers were observed to visit borneol only once in 22 trials (4.5%) and only sniffed from the water in four trials. Yet, borneol was visited overnight in 10 of 27 overnight trials (37%), flattened in four cases, and overmarked three of those times.

In 1992, the responses by beavers to all of the samples, including castoreum, diminished, possibly because it was an exceptionally wet season. The responses to each sample were compared to those elicited by the controls over all four years of the study (castoreum, 1989–1992; ethanol, 1989–1992). Based on the degree of manipulation to the ESM overnight, the 14-phenolic mixture (P92) and borneol (BO92) were potentially active and other samples were inactive. Comparing the experimental samples from 1989 through 1991 to the pooled controls did not alter any of the results.

Bioactivity of 14-Phenol and 12-Neutral Mixture. The responses to the 26-compound mixture (pooling responses to the two concentrations) and to castoreum (1989–1992) were compared. The proportion of trials in which both observed and overnight responses were elicited by the 26-compound mixture was statistically indistinguishable from the same proportions in responses to castoreum (G statistic, 1 df, both $P > 0.10$). This was true for only this mixture. In addition, for trials with an overnight land visit, the extent of ESM modification (paw, scratch, flat) overnight in response to this mixture or castoreum did not differ significantly ($P > 0.90$, $G = 0.09$, 2 df). However, observed land visits to the 26-compound mixture were shorter ($P = 0.02$, Wilcoxon), fewer in number ($P = 0.01$), and made by a smaller proportion of beavers ($P = 0.005$) than land visits to castoreum.

DISCUSSION

A primary objective of this study was to identify castoreum compounds and/or mixtures of compounds that elicited scent-mark inspection behavior similar to that evoked by castoreum. A synthetic mixture of 26 castoreum constituents elicited land visit responses to the ESM in a similar proportion of trials as the castoreum control. However, observed land visits to this mixture (or any other sample) relative to castoreum were shorter in duration, fewer in number, and performed by a smaller proportion of beavers. Yet, the degree of modification of the ESM was not significantly different between ESMs marked with the 26-compound mixture and those with castoreum. The 26-compound mixture

may have provided a clearer or simpler signal that required less inspection to elicit particular responses than the more chemically elaborate castoreum. This may be analogous to the responses observed to castor fluid from familiar resident versus less familiar nonresident beaver (Schulte, 1993). In Schulte's 1993 study, beaver made shorter land visits to ESMs marked with the castoreum from the resident adult male than ESMs marked with castoreum from nonresident adult males. Thus, perhaps the 26-compound mixture is part of a recognizable, core beaver signal, yet lacking individualistic qualities that may evoke longer investigation.

The assumptions of behavioral continuity in time and space for our field studies conducted over several years at Allegany State Park appear reasonable. Our assays performed at numerous sites yielded results that were not affected by the order in which the samples were tested. Biological activity of the controls within a season and among years varied more in the range of behaviors elicited than in the relative amount of responses. At no time did beavers respond more to ethanol or blank ESMs than to castoreum. However, some intriguing differences in responses over time were apparent. For instance, the compound 4-ethylphenol (EP), shown to be active in a previous study (Müller-Schwarze and Houlihan, 1991), evoked a strong sniff from water response and some scent-marking behavior by beavers in the 1989 season of this study. Yet, the four concentrations tested in 1990 were generally inactive. Active samples (i.e., potential pheromones) should stimulate responses across both time (e.g., within a season or between years) and sites. Failure to do so may indicate a novel compound response, or more interestingly, some temporal-spatial significance to the meaning of the signal. The likelihood that many of the compounds in castoreum are diet-related, and therefore transitory, may be very important in this regard.

We offer no support for a single compound serving as a territorial signal, yet many more compounds need to be assayed. In agreement with the social odor hypothesis, mixtures were more active than single compounds, and larger mixtures were more active than smaller ones, with some exceptions. Methods for evaluating overall bioactivity will be discussed in a separate paper (Schulte et al., in review).

Mixtures with more compounds may have stimulated activity in more trials either because the odor image (Albone, 1984) was enhanced, the compounds carried a redundant message, or the different compounds played distinct roles in the signal (or some combination). The odor image concept suggests a gestalt process of information transfer in that compounds do not play particular roles by themselves, but together they produce a meaningful signal (Beauchamp et al., 1976). Redundant compounds may form a clearer signal and thereby increase detectability (Krebs and Dawkins, 1984) and subsequent responsiveness (i.e., elicitation) but not affect the completeness or strength of the response. Addi-

tionally, specific tasks may be carried out by certain compounds, or types of compounds, in the transmission of the message (Albone, 1984). For instance, some more volatile compounds may attract attention, while other components, possibly with longer retention times on the substrate, provide more detailed information (Alberts, 1992).

There was some hint of this latter pattern in the results. The phenolic, 4-ethylphenol, elicited more sniffing from the water than the neutral compound borneol; however, overnight land visits to borneol were more frequent and more complete than those to 4-ethylphenol. ESMs marked with borneol may have been chanced upon during foraging bouts, rather than directly attracting beavers from the water. If certain types of compounds (e.g., neutral versus phenolic compounds) play specific roles in signal transmission and/or meaning, then differences in the type of responses (i.e., sniff versus land visit) might be expected. Yet, responses to the mixtures of 12 neutral compounds (N, 5N) were not very different from those to the 14-phenolic compounds (P, 5P). Two phenolic compounds (EP, MG) and phenolic mixtures (SP, 5P) did evoke some long bouts of sniffing, but sniff responses to these samples were highly variable. Furthermore, some of the "inactive" phenolic compounds and mixtures, plus the mixture of three guaiacols and borneol (BG), stimulated sniffing but very few to no land visits (observed or overnight). More information on responses to neutral compounds is needed to examine their role in the castoreum signal and to determine if responses to neutral and phenolic compounds contrast in biologically relevant ways.

A complex secretion such as castoreum may serve several functions either simultaneously or over time. This olfactory fingerprint may identify individuality without coding for species, age, sex, status, or other information separately (e.g., Voznessenskaya et al., 1992). Understanding the signal structure and message in beaver scent mounds may help to reveal the specific function(s) of marking behavior. Separating the response components of elicitation, completeness, and strength may be helpful in deciphering the meaning and chemical composition of this signal. Elucidating the potential role of scent marking in beaver dispersal, territory maintenance, and mating behavior could be greatly beneficial in improving our ability to manage beaver populations and wetlands and in furthering our knowledge of the chemical ecology of mammalian social odors.

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CHIRAL ESTERS: SEX PHEROMONE OF THE BAGWORM, *Oiketicus kirbyi* (LEPIDOPTERA: PSYCHIDAE)

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Abstract—Gas chromatographic-electroantennographic detection (GC-EAD) analyses of pheromone extract of female bagworms, *Oiketicus kirbyi* (Guilding), revealed five EAD-active compounds. Retention index calculations, GC-mass spectrometry in both full-scan and selected-ion monitoring modes and GC-EAD analyses of authentic standards identified the compounds as 1-methylbutyl octanoate (MBO), 1-methylbutyl nonanoate (MBN), 1-methylbutyl decanoate (MBD), 1-methylpentyl decanoate (MPD), and 1-methylbutyl dodecanoate (MBDD). Of these five chiral esters, MBD was most abundant in extracts and elicited the strongest antennal response. In field experiments in Costa Rica, (*R*)-MBD attracted *O. kirbyi* males, whereas (*S*)-MBD in combination with (*R*)-MBD inhibited response. *R* but not *S* enantiomers of MBO, MBN, and MBDD strongly synergized attraction to (*R*)-MBD. (*S*)-MBO and (*S*)-MBDD were inactive, whereas (*S*)-MBN was inhibitory. (*R*)-, (*S*)- and racemic MPD were inactive. Blends of (*R*)-MBD in ternary combination with either (*R*)-MBO and (*R*)-MBN or (*R*)-MBN and (*R*)-MBDD were as attractive as the five-ester blend. Five- and four-ester blends were equally attractive, suggesting redundancy of pheromone components for attraction of males. The multiple sex pheromone component blend of chiral esters in *O. kirbyi* may have evolved to maintain species-specific communication in bagworm communities of tropical Americas.

Key Words—Lepidoptera, Psychidae, *Oiketicus kirbyi*, bagworm, sex pher-

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omone, pheromone chirality, 1-methylbutyl octanoate, 1-methylbutyl nonanoate, 1-methylbutyl decanoate, 1-methylbutyl dodecanoate.

INTRODUCTION

The bagworm, *Oiketicus kirbyi* (Guilding) (Lepidoptera: Psychidae), is a major defoliator of oil palm plantations in the tropical Americas (Genty et al., 1978). As with other bagworms, *O. kirbyi* has an unusual life history (Stephens, 1962; Ponce et al., 1979; Villanueva and Granda Paz, 1986; Campos Arce et al., 1987; Rhainds et al., 1995). Upon encountering a suitable host, wind-dispersed larvae envelop themselves in a self-constructed bag, which they enlarge throughout their development. Following eclosion, vermiform, apterous females stay within their pupal case and protecting bag, awaiting the arrival of winged males. To copulate, the male inserts his extensible abdomen through the bag into the female pupal case. Mated females lay a single egg mass within their pupal case.

As in other bagworms (Leonhardt et al., 1983; Bosman and Brand, 1971; Boguang, 1981), female *O. kirbyi* expel pheromone-impregnated scales (hairs) out of the pupal case into the lower part of the bag for attraction of males (Acosta, 1986). (1*R*)-1-Methylbutyl decanoate (MBD) has been identified as a sex pheromone component of *Thyridopteryx ephemeraeformis* (Haworth) (Leonhardt et al., 1983), but sex pheromones of other bagworms are as yet unknown. We report the identification and field testing of the sex pheromone of *O. kirbyi*.

METHODS AND MATERIALS

Laboratory Analysis. Bags containing *O. kirbyi* pupae were collected in commercial oil palm plantations in Coto, Costa Rica, and sent to Simon Fraser University. Pupae were removed from their bags and kept separately in Petri dishes (9 cm diameter) at 25°C under a photoperiod of 12L:12D. Pheromone-impregnated scales expelled by a female out of the pupal case were extracted in 150 μl of hexane for 10 min. Extracts of 100 females were combined and subjected to gas chromatographic–electroantennographic detection analyses (GC-EAD) (Arn et al., 1975) on three fused silica columns (30 × 0.25 or 0.32 mm ID) coated with DB-23, DB-210 (J&W Scientific, Folsom, California 95630), or SP-1000 (Supelco, Bellafonte, Pennsylvania 16823). Coupled GC-mass spectrometry (MS) (Hewlett-Packard 5985B) in selected ion monitoring (SIM) mode, using a DB-210 column with isobutane for chemical ionization (CI), was conducted to confirm the identification of EAD-active components in scale extracts. Full-scan CI mass spectra of synthetic candidate compounds were obtained to select diagnostic ions. In sequence, 200 pg of synthetic compounds, a hexane blank, and a concentrated pheromone extract were then analyzed in SIM mode,

each time scanning for the diagnostic ions. Synthetic candidate pheromone components were further subjected to GC-EAD analyses to compare their EAD activity with those of female-produced compounds.

Synthesis of Pheromone Components. Chiral esters were synthesized with >90% yield from chiral alcohols and alkanoyl chlorides in pyridine. Final products were purified by silica gel chromatography using hexane–ether (10%) as eluents. Enantiomeric excess of (1*R*)- and (1*S*)-1-methylbutanol (each ≥96%), as well as (1*R*)- and (1*S*)-1-methylpentanol (each ≥96%) (Aldrich Chemical Co., Milwaukee, Wisconsin), was determined by derivatization (Slessor et al., 1985) and GC analyses [fused silica, DB-5 coated column (30 × 0.25 mm); temperature: 90°C and 100°C isothermal for 2-pentanol and 2-hexanol derivatives, respectively]. Mass spectra of synthetic compounds were obtained on a Hewlett Packard 5895B mass spectrometer equipped with a fused silica column (30 m × 0.25 mm ID) coated with DB-210. NMR spectra (Bruker WU-400 spectrometer) were taken in $CDCl_3$ at 400 MHz (*J* values in hertz).

(1R)-1-Methylbutyl Octanoate ((R)-MBO). EI-MS [*m*/*z*] (%)]: 214 (M^+, 1), 171 (10), 145 (87), 144 (40), 128 (14), 127 (100), 101 (16), 87 (19), 85 (10), 84 (20), 73 (12), 71 (25), 70 (58), 60 (12), 57 (45), 55 (30), 43 (42), 42 (13), 41 (27). ^1H NMR ($CDCl_3$): δ: 4.91 (1H, hex, *J* = 7 Hz), 2.26 (2H, t, *J* = 7 Hz), 1.60 (3H, m), 1.45 (1H, m), 1.30 (10H, m), 1.17 (3H, d, *J* = 7 Hz), 0.90 (3H, t, *J* = 7 Hz), 0.80 (3H, t, *J* = 7 Hz). (*S*)-MBO gave almost identical spectra.

(1R)-1-Methylbutyl Nonanoate ((R)-MBN). EI-MS [*m*/*z* (%)]: 228 (M^+, 1), 159 (79), 158 (50), 142 (17), 141 (100), 129 (18), 115 (15), 98 (15), 87 (17), 73 (13), 71 (44), 70 (53), 69 (13), 60 (11), 57 (25), 55 (30), 43 (37), 42 (12), 41 (23). ^1H NMR ($CDCl_3$): δ: 4.90 (1H, hex, *J* = 7 Hz), 2.26 (2H, t, *J* = 7 Hz), 1.60 (3H, m), 1.42 (1H, m), 1.28 (12H, m), 1.17 (3H, d, *J* = 7 Hz), 0.89 (3H, t, *J* = 7 Hz), 0.86 (3H, t, *J* = 7 Hz). (*S*)-MBN gave almost identical spectra.

(1R)-1-Methylbutyl Decanoate ((R)-MBD). EI-MS [*m*/*z* (%)]: 242 (M^+, 1), 173 (83), 172 (44), 156 (11), 155 (100), 143 (10), 129 (25), 87 (11), 85 (11), 71 (31), 70 (39), 69 (10), 57 (12), 55 (16), 43 (30), 41 (18). ^1H NMR ($CDCl_3$): δ: 4.89 (1H, hex, *J* = 7 Hz), 2.24 (2H, t, *J* = 7 Hz), 1.59 (3H, m), 1.40 (1H, m), 1.26 (14H, m), 1.16 (3H, d, *J* = 7 Hz), 0.88 (3H, t, *J* = 7 Hz), 0.84 (3H, t, *J* = 7 Hz). (*S*)-MBD gave almost identical spectra. Spectroscopic data were consistent with those previously reported (Leonhardt et al., 1983).

(1R)-1-Methylpentyl Decanoate ((1R)-MPD). EI-MS [*m*/*z* (%)]: 256 (M^+, 1), 173 (73), 172 (49), 156 (15), 155 (100), 129 (26), 101 (12), 85 (33), 84 (55), 83 (11), 73 (11), 71 (22), 69 (22), 57 (20), 56 (14), 55 (23), 43 (32), 41 (20). ^1H NMR ($CDCl_3$): δ: 4.86 (1H, hex, *J* = 7 Hz), 2.26 (2H, t, *J* = 7 Hz), 1.60 (3H, m), 1.46 (1H, m), 1.26 (16H, m), 1.20 (3H, d, *J* = 7 Hz), 0.95

(3H, t, J = 7 Hz), 0.86 (3H, t, J = 7 Hz). (S)-MPD gave almost identical spectra.

(1R)-1-Methylbutyl Dodecanoate ((1R)-MBDD). EI-MS [m/z (%)]: 270 (M^+, 1), 202 (13), 201 (100), 200 (66), 184 (11), 183 (86), 157 (16), 129 (14), 85 (11), 83 (12), 71 (21), 70 (31), 57 (14), 55 (15), 43 (22), 41 (13). ^{1}H NMR ($CDCl_3$): δ: 4.90 (1H, hex, J = 7 Hz), 2.25 (2H, t, J = 7 Hz), 1.60 (3H, m), 1.44 (1H, m), 1.24 (18H, m), 1.20 (3H, d, J = 7 Hz), 0.90 (3H, t, J = 7 Hz), 0.87 (3H, t, J = 7 Hz). (S)-MBDD gave almost identical spectra.

Field Bioassay. Experiments were conducted in commercial oil palm plantations in Coto, Costa Rica. Green Unitraps (Phero Tech Inc., Delta, British Columbia) were suspended from oil palms 3 m above ground in randomized complete blocks, with traps and blocks at 18 to 27-m intervals. Traps were baited with cotton balls impregnated with hexane solutions of candidate pheromone components. A small Diclorvos cube (Green Cross, Division of Ciba Geigy Canada Ltd., Mississauga, Ontario) placed on the bottom of traps assured retention of captured moths. After 1 to 2 days, baits were changed and captured male *O. kirbyi* counted. Blocks were rerandomized when a predetermined number of males had been captured.

The first experiment compared attraction of (R)- and (S)-MBD (1000 μg), alone and in combination. The second experiment tested (R)-MBD (10,000 μg) alone and combined with (S)-MBD at respective ratios of 1:1, 1:0.1, 1:0.01, 1:0.001, and 1:0.0001. Experiment 3 tested (R)-MBD (1000 μg) alone and combined with either one or both enantiomers of MBO at 1:0.1 and 1:0.01 ratios. Experiments 4–6 tested (R)-MBD (1000 μg) alone and in 1:0.1 ratio with either one or both enantiomers of MBN (experiment 4), MPD (experiment 5), and MBDD (experiment 6). Experiments 7–9 each tested (R)-MBD in pentanary combination with (R)-MBO, (R)-MBN, (R)-MPD, and (R)-MBDD, and in all binary (experiment 7), ternary (experiment 8), and quaternary combinations (experiment 9) with these chiral esters. Experiment 10 tested (R)-MBD (1000 μg) alone and in ternary combinations with (R)-MBN and (R)-MBO at respective ratios of 1:1, 1:0.1, 1:0.01 and 1:0.001. A final dose–response experiment tested (R)-MBD alone and in ternary combinations with both (R)-MBO and (R)-MBN at a 1:1:1 ratio, employing doses of 10, 100, 1000, and 10,000 μg.

Statistical Analysis. Statistical analyses were conducted with the SAS statistical package (SAS Institute Inc., Cary, North Carolina). Numbers of male *O. kirbyi* captured in Unitraps were compared using a nonparametric analysis of variance (Friedman's test) followed by the Student-Newman-Keul's (SNK) test. In all analyses, $P < 0.05$ levels of significance were used.

RESULTS

Laboratory Analysis. GC-EAD analyses of female *O. kirbyi* pheromone extract on a DB-210 column consistently revealed five EAD-active compounds (Figure 1). Coupled GC-MS of the most abundant and major EAD-active com-

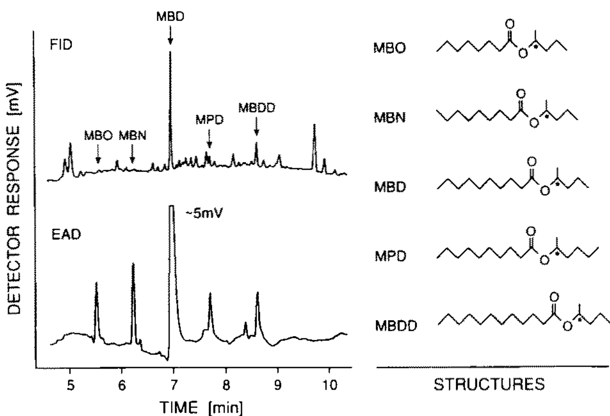

FIG. 1. Flame ionization detector (FID) and electroantennographic detector (EAD: male *O. kirbyi* antenna) responses to female *O. kirbyi* pheromone extract, chromatographed on a DB-210 column (1 min at 100°C, 15°C/min to 180°C, 2°C/min to 220°C). MBO = 1-methylbutyl octanoate; MBN = 1-methylbutyl nonanoate; MBD = 1-methylbutyl decanoate; MPD = 1-methylpentyl decanoate; MBDD = 1-methylbutyl dodecanoate. Retention indices of MBO, MBN, MBD, MPD, and MBDD were respectively: 1643, 1745, 1846, 1942, 2050 (DB-210); 1651, 1756, 1858, 1949, 2064 (DB-23); 1588, 1688, 1788, 1876, 1990 (SP-1000). *Indicates chiral center of molecule.

ponent gave the same mass spectrum as MBD, a previously identified pheromone component of *T. ephemeraeformis* (Leonhardt et al., 1983). Identical retention and mass spectrometric characteristics of and comparable antennal responses to synthetic and female-produced MBD confirmed the presence of this compound in female *O. kirbyi* pheromone extract.

Retention indices of the four minor EAD-active compounds (Figure 1) suggested they were homologous MBO, MBN, MPD, and MBDD. Equivalent amounts of synthetic and female-produced esters elicited similar antennal responses. GC-MS-CI-SIM analyses of pheromone extract and synthetic MBO, MBN, MPD, and MBDD resulted in retention time and ion ratio matches of synthetic and female-produced compounds, except for MBN; synthetic MBO [*m/z* (%)]: 145 (100), 215 (M+1, 63), extract: 145 (100), 215 (64); synthetic MPD: 173 (100), 257 (M+1, 45), extract: 173 (100), 257 (40); synthetic MBDD: 201 (100), 271 (M+1, 53), extract: 201 (100), 271 (49). Diagnostic ions *m/z* 159 and 229 for MBN were detected at the correct retention time, but the ion ratio could not be accurately determined due to a coeluting compound also containing *m/z* 229 (but not *m/z* 159).

Field Bioassay. In a 12-replicate experiment, (*R*)-MBD attracted on average 11.7 bagworm males per trap, while the *S* enantiomer was unattractive and in combination with (*R*)-MBD inhibited response. Unbaited traps did not attract

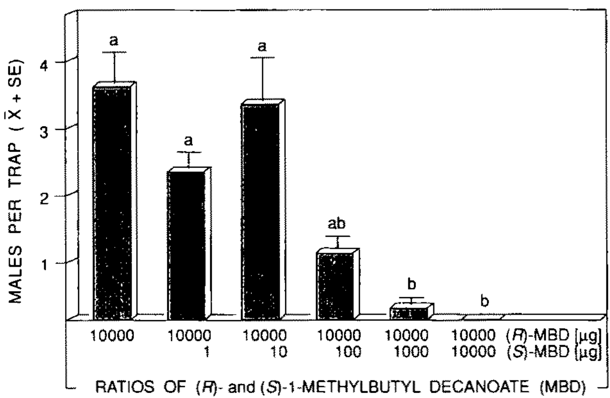

FIG. 2. Mean number and standard error (SE) of male *O. kirbyi* captured in Unitraps baited with different ratios of (*R*)- and (*S*)-MBD. April 5–9, 1993, Coto, Costa Rica, $N = 8$. Bars superscripted by the same letter are not significantly different, SNK test, $P < 0.05$.

any males. Attraction of males to (*R*)-MBD proportionally decreased as the amount of (*S*)-MBD in the lure increased (Figure 2). The *R* but not *S* enantiomers of either MBO, MBN, or MBDD strongly enhanced attraction to (*R*)-MBD (Figure 3). (*S*)-MBO and (*S*)-MBDD were inactive, whereas (*S*)-MBN added to its antipode reduced attraction (Figure 3). (*R*)-, (*S*)-, and racemic MPD were behaviorally benign. A five-ester blend was more attractive than any of four binary blends containing (*R*)-MBD (Figure 4). Blends of (*R*)-MBD in ternary combination with either (*R*)-MBO and (*R*)-MBN or (*R*)-MBN and (*R*)-MBDD were as attractive as the five-ester blend (Figure 5). Five- and four-ester blends were equally attractive (Figure 6). Increasing the amount of synergistic (*R*)-MBO and (*R*)-MBN relative to (*R*)-MBD increased attraction (Figure 7); attraction increased as the amount of pheromone increased from 10 to 10,000 μg (Figure 8).

DISCUSSION

Chiral lepidopteran sex pheromone components comprise mono- and diene epoxides in geometrids, noctuids, and arctiids (Mayer and McLaughlin, 1991; Arn et al., 1992; Gries et al., 1993a; Szöcs et al., 1993), methyl-branched hydrocarbons in lyonetiids (Francke et al., 1987, 1988) and geometrids (Gries et al., 1991, 1993b,c, 1994), a methyl-branched epoxide in lymantriids (Bierl et al., 1970, 1975), and a methylbutyl ester in psychids (Leonhardt et al., 1983). While chirality of female-produced pheromone components has rarely been

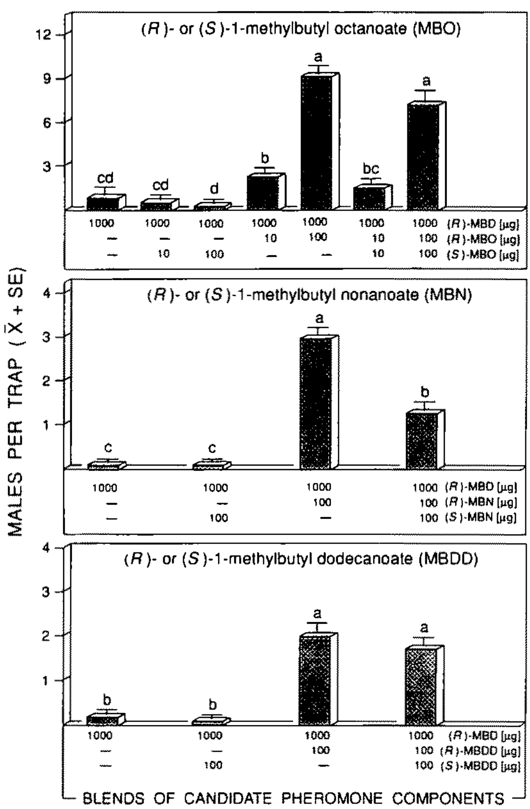

FIG. 3. Mean number of male *O. kirbyi* captured in Unitraps baited with (*R*)-MBD alone and in combination with optical isomers of MBO (November 1–15, 1992, $N = 15$), MBN (October 15–23, 1993, $N = 10$), and MBDD (October 14–28, 1993, $N = 10$), Coto, Costa Rica. Bars superscripted by the same letter are not significantly different, SNK test, $P < 0.05$. Compound abbreviations as in Figure 1.

determined (Szöcs et al., 1993), electroantennogram, wind-tunnel, and field bioassays of synthetic optical isomers alone and in combination indicate that usually one isomer is attractive, whereas the antipode is inactive (Li et al., 1993a,b; Klimetzek et al., 1976), synergistic (Millar et al., 1991) or inhibitory (Cardé et al., 1977; Plimmer et al., 1977; Millar et al., 1991; Szöcs et al., 1993). Blends of two or three chiral components of sex pheromone have been documented in geometrids. (3*Z*,9*Z*,6*R*,7*S*)-Epoxy-nonadecadiene and (6*Z*,9*Z*,3*S*,4*R*)-epoxy-nonadecadiene synergistically attract *Probole amicaria* (Herrich-Schäffer) (Millar et al., 1990). (5*R*,11*S*)-5,11-Dimethylheptadecane,

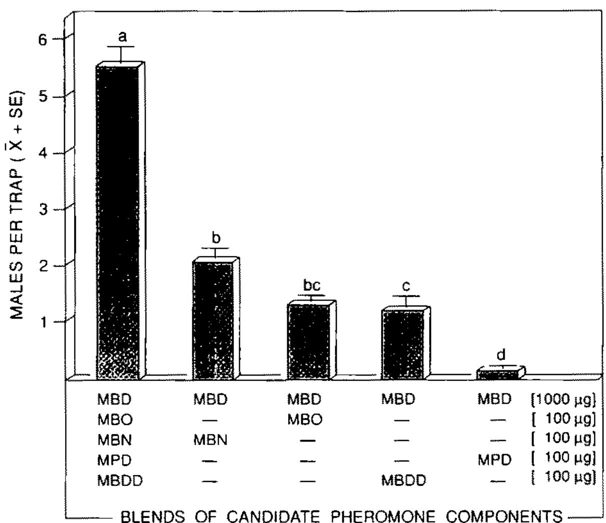

FIG. 4. Mean number of male *O. kirbyi* captured in Unitraps baited with (*R*)-MBD in binary and pentanary combinations with (*R*)-MBO, (*R*)-MBN, (*R*)-MPD, and (*R*)-MBDD. November 1–8, 1993, Coto, Costa Rica, $N = 10$. Bars superscripted by the same letter are not significantly different, SNK test, $P < 0.05$. Compound abbreviations as in Figure 1.

(2,5*R*)-2,5-dimethylheptadecane, and (7*R*)-7-methylheptadecane comprise the ternary sex pheromone blend of the western hemlock looper, *Lambdina fiscellaria lugubrosa* (Hulst) (Li et al., 1993a,b). Of the four chiral esters in *O. kirbyi*, *R* enantiomers are attractive, whereas *S* enantiomers are behaviorally benign (MBO, MBDD) or inhibitory (MBD, MBN).

Even though both *T. ephemeraeformis* and *O. kirbyi* bagworms utilize (*R*)-MBD as a major sex pheromone component, their pheromones are distinct. While (*S*)-MBD is behaviorally benign in *T. ephemeraeformis* (Leonhardt et al., 1983), it inhibits response of *O. kirbyi* (Figure 2). Contrasting with the single ester sex pheromone of *T. ephemeraeformis*, female *O. kirbyi* produce a blend of four chiral esters. Distinct communication channels of *T. ephemeraeformis* in the eastern United States (Sheppard and Stairs, 1976) and *O. kirbyi* in Central and South America (Genty et al., 1978) may be attributed to reproductive isolation and different environmental conditions (Cardé and Baker, 1984). The likely more diverse bagworm fauna in the tropical Americas may have provided selective forces for *O. kirbyi* to evolve a multiple component blend of chiral esters for species-specific communication.

Enhanced attraction to (*R*)-MBD when combined in either two-, three-,

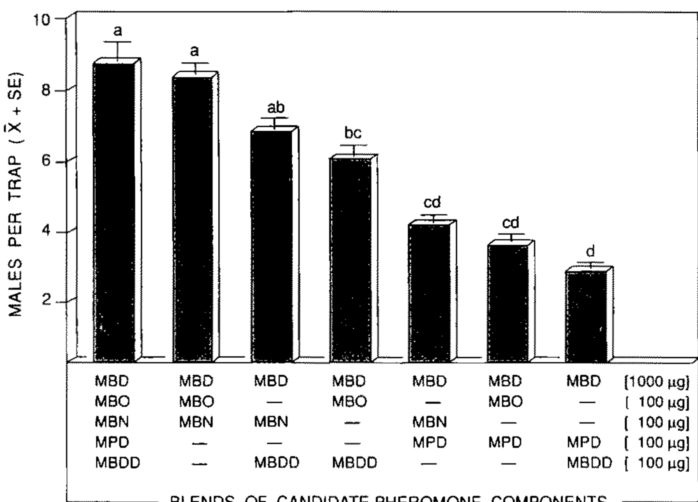

FIG. 5. Mean number of male *O. kirbyi* captured in Unitraps baited with (*R*)-MBD in ternary and pentanary combinations with (*R*)-MBO, (*R*)-MBN, (*R*)-MPD, and (*R*)-MBDD. November 2–22, 1993, Coto, Costa Rica, $N = 20$. Bars superscripted by the same letter are not significantly different, SNK test, $P < 0.05$. Compound abbreviations as in Figure 1.

four-, and five-component blends with (*R*)-MBO, (*R*)-MBN, (*R*)-MPD, and (*R*)-MBDD (Figures 3–6) indicated that all compounds except MPD are sex pheromone components. Equal attraction of two ternary and all four- and five-component blends suggests redundancy of pheromone components for male attraction. Redundancy of sex pheromone components was first documented in the cabbage looper moth, *Trichoplusia ni* (Hubner), with various pheromone blends compensating for the lack of one or more components (Linn et al., 1984). While individual components may be redundant for the attraction of males, they may serve to inhibit response by sympatric congeners.

Most female moths actively release sex pheromone from abdominal glands for attraction of males (Percy-Cunningham and MacDonald, 1987). Cessation of calling behavior and decline of pheromone production after mating (Richerson and Cameron, 1974; Raina, 1984; Webster and Cardé, 1984; Giebultowicz et al., 1991) has been associated with transfer of sperm [*Lymantria dispar* (L.)] (Giebultowicz et al., 1991) or male accessory gland secretion [*Heliothis zea* (Boddie)] (Raina, 1984) into the spermetheca. Calling female *O. kirbyi*, in contrast, expel their pheromone-impregnated scales at once out of the pupal case into the lower part of the bag (Rhainds, unpublished). As pheromone dissipation from these scales is independent of the female's mating status, it is unclear

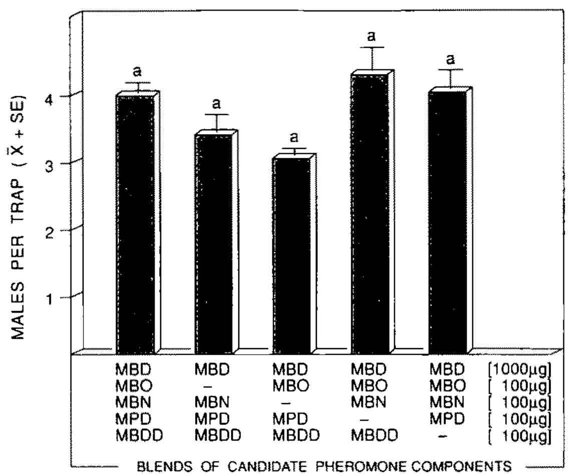

FIG. 6. Mean number of male *O. kirbyi* captured in Unitraps baited with (*R*)-MBD in quaternary and pentanary combinations with (*R*)-MBO, (*R*)-MBN, (*R*)-MPD, and (*R*)-MBDD. November 4–15, 1993, Coto, Costa Rica, $N = 10$. Bars superscripted by the same letter are not significantly different, SNK test, $P < 0.05$. Compound abbreviations as in Figure 1.

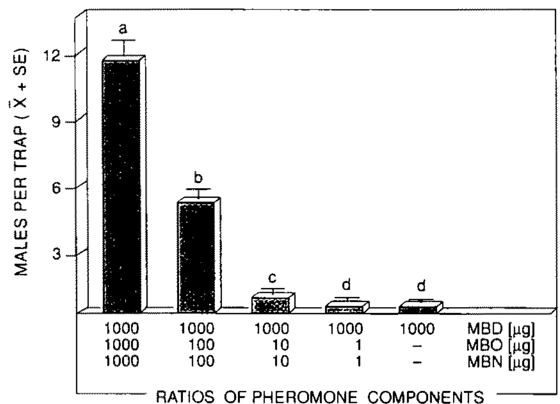

FIG. 7. Mean number of male *O. kirbyi* captured in Unitraps baited with (*R*)-MBD, (*R*)-MBO, and (*R*)-MBN in different ratios. November 12–21, 1993, Coto, Costa Rica, $N = 10$. Bars superscripted by the same letter are not significantly different, SNK test, $P< 0.05$. Compound abbreviations as in Figure 1.

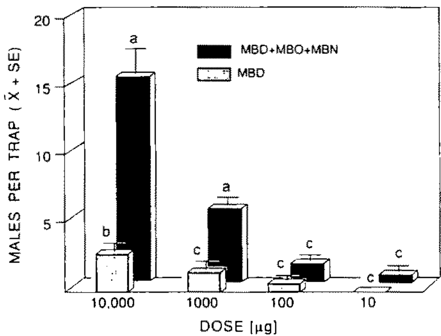

FIG. 8. Mean number of male *O. kirbyi* captured in Unitraps baited with (*R*)-MBD alone and in combination with (*R*)-MBO and (*R*)-MBN (1 : 1 : 1) at increasing doses. November 19–27, 1993, Coto, Costa Rica, $N = 10$. Bars superscripted by the same letter are not significantly different, SNK test, $P < 0.05$. Compound abbreviations as in Figure 1.

whether or how mate-seeking males discriminate between bags containing virgin or mated females. We hypothesize that females produce an anti-sex pheromone after mating, that males mark mated females with an inhibitory compound, or that the pheromone rapidly dissipates from scales, rendering the female attractive for a short duration.

Assessment of bagworm populations in oil palm plantations currently involves counting larvae on one leaf per tree per hectare (Chinchilla, 1992). This approach is not reliable or cost-effective (Mackenzie, 1976; Wan and Hoh, 1992; Chinchilla, 1992). If proven to be efficacious, future monitoring of *O. kirbyi* populations could employ pheromone-based trapping with a multiple-ester lure. Successful use of MBD for biorational control of *T. ephemeraeformis* (Klun et al., 1986) indicates excellent potential for pheromone-based mating disruption of *O. kirbyi* populations.

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FEEDING BEHAVIOR OF GRAMINIVOROUS GRASSHOPPERS IN RESPONSE TO HOST-PLANT EXTRACTS, ALKALOIDS, AND TANNINS

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Abstract—Secondary metabolites exhibit the potential to direct food selection by grass-feeding (graminivorous) grasshoppers. We examined the effects of plant extracts and representative secondary metabolites on the feeding behavior of two such grasshoppers, *Ageneotettix deorum* (Scudder) and *Phoetaliotes nebrascensis* (Scudder). Three alkaloids and two tannins were bioassayed for their activity as feeding deterrent allelochemicals, as were extracts from the foliage of the graminoids commonly eaten by these grasshoppers: *Agropyron smithii* Rydb., *Andropogon hallii* Hack., *Andropogon scoparius* Michx., *Bouteloua gracilis* (H. B. K) Lag. ex Griffiths, *Carex heliophila* Mack. and *Stipa comata* Trin. & Rupr. Alkaloids strongly deterred feeding but tannins only exhibited a weak effect, even when present at four times the concentration of total phenolics typical for these graminoids. Host-plant extracts also exhibited weak effects, such that we found no evidence for either strong deterrence or phagostimulation. Our results for alkaloids and host-plant extracts are consistent with the view that grass-feeding grasshoppers may be restricted to graminoids because of: (1) the presence of deterrents in nonhosts and (2) the absence of deterrents in hosts. However, our data for tannins show that these are unlikely to be effective barriers to herbivory by these grasshoppers.

Key Words—Phenolics, graminoids, feeding preference, Nebraska Sandhills grassland, *Ageneotettix deorum*, *Phoetaliotes nebrascensis*, Orthoptera, Acrididae.

INTRODUCTION

Some grasshoppers consume a diet containing only graminoids (grasses and other monocots of similar growth form, such as sedges and rushes). Among

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grasshoppers, most species feed solely on graminoids or on forbs but seldom on both (Joern, 1983). While a variety of secondary metabolites have been thought to explain insect feeding on forbs (Harborne, 1988), we do not have an equivalent understanding of the grass-feeding habit. At present, explanations of graminivory in grasshoppers rest on two observations. First, effective allelochemical deterrents have been demonstrated in many nonhost plants (Bernays and Chapman, 1975, 1977, 1978). Second, graminoids contain only low levels of secondary metabolites (Gibbs, 1974; Jung et al., 1979), which are unlikely to have deterrent or toxic effects (Bernays and Chapman, 1977, 1978; Owen and Wiegert, 1981; McNaughton, 1983) or strong phagostimulant effects. On this basis, it is argued that graminoids become host plants by default (Bernays and Chapman, 1977, 1978): Deterrents rather than phagostimulants are considered to have primary importance in host selection, and graminoids provide a refuge from chemically deterrent nonhosts. Here, we question whether this explanation for host range can be sustained for two species of graminivorous grasshoppers native to a Nebraska Sandhills grassland.

Claims that grasses lack effective chemical defenses continue to be controversial (Redak, 1987; Vicari and Bazely, 1993). Certainly, grasses contain some secondary metabolites. For example, we have documented that low but readily detectable levels of phenolic secondary metabolites can be found in several of the dominant graminoid species of Sandhills Prairie vegetation (Mole and Joern, 1993). Indeed, the chemical diversity of phenolics in these species may be sufficient to enable their use as cues for host-plant recognition. It is also possible that phenolics exert a more general activity as feeding deterrents. For example, *Bouteloua gracilis*, the most preferred grass by grasshoppers at our field site, contains the lowest level of phenolics (Joern, 1985; Mole and Joern, 1993), while two of the least preferred grasses contain relatively high levels of phenolics.

In our earlier analysis (Mole and Joern, 1993), we identified in these graminoids the presence of simple phenolics (not tannins) and suggested that these were unlikely to be especially toxic or deterrent to herbivores. Even so, dose–response ratios are herbivore-specific and cannot be confidently inferred from chemical data alone. For this reason, we examine possible feeding-deterrent responses by the graminivorous grasshoppers *Ageneotettix deorum* (Scudder) and *Phoetaliotes nebrascensis* (Scudder) collected from the same site as the plant material analyzed in our previous study (Mole and Joern, 1993).

We employed a behaviorally based bioassay (Chapman et al., 1988) because of the possibility that secondary metabolites may act as proximate cues to guide food selection without producing direct physiological effects (Bernays and Chapman, 1987; Bernays, 1991). We emphasize phenolics in these tests because we failed to detect other classes of plant secondary metabolites in our previous study of graminoids (Mole and Joern, 1993).

We began the present study with bioassays for grasshopper responses to commercially available alkaloids and tannins. Plants from the Sandhills grassland that are not in the diet of these grasshoppers often contain secondary metabolites (including tannins and alkaloids; Mole and Joern, unpublished results). On the basis of previous work (Bernays and Chapman, 1977, 1978; Bernays, 1987), we predicted that these substances should have feeding deterrent effects on graminivorous grasshoppers. We obtained clearly deterrent effects with some of these substances, and we compare these responses to the effects elicited by extracts of hostplants.

METHODS AND MATERIALS

Grasshoppers and Plant Material. These were collected from Arapaho Prairie, a 1280-acre reserve representative of the larger Sandhills grasslands of western Nebraska (Arthur County, sections 31 and 32, T18N, R38W, 18,000 sq. mi) (Weaver, 1965; Kaul, 1975). This grassland may be considered as either the most western extension of the tallgrass prairie (French, 1979) or as a unique mixture of tall- mid-, and shortgrass prairie types. A detailed description of the site is provided by Barnes and Harrison (1982).

Two grasshopper species, *Ageneotettix deorum* and *Phoetaliotes nebrascensis*, were collected as adults during August 1993 and maintained in the lab for several days prior to their use in these experiments. They were fed a diet of live rye foliage and wheat bran and were kept in cages of approximately 70 individuals, in a room illuminated with daylight and kept at 25°C. To allow grasshoppers to thermoregulate and attain preferred body temperatures, 40-W light bulbs were illuminated next to each cage for a 6-h thermoperiod each day.

Extracts of foliage from five grasses and one sedge were evaluated in this study: *Agropyron smithii* Rydb., *Andropogon hallii* Hack., *Andropogon scoparius* Mich., *Bouteloua gracilis* (H.B.K.) Lg. ex Griffiths, and *Stipa comata* Trin. & Rupr., and the sedge was *Carex heliophila* Mack. Samples were collected during July and August in 1993 from the valley site, using our standard collection and preservation techniques (Mole and Joern, 1993). Samples were returned to the lab, lyophilized and then ground in a Wiley mill to pass through a 40-mesh screen before use.

Bioassay Protocol. Feeding responses to plant extracts and other potential allelochemicals were determined in choice test experiments similar to those of Chapman et al. (1988). We paired (1) glassfiber disks impregnated with sucrose and the test material with (2) disks impregnated with either sucrose alone or sucrose plus a plant extract known to be low in phenolics (see below for details). Disks were weighed before and after feeding so that we were able to determine the weights of each disk consumed by a grasshopper.

Adult female grasshoppers were presented with pairs of disks in individual cylindrical wire screen cages (12 cm high × 8 cm diameter). We presented the disks (Whatman GF/A, 2.1 cm diameter) on inverted push pins glued to the floor of the cages and positioned across the diameter of the cage, approximately equidistant from each other and the side of the cage. An inverted vial of water with a damp cotton wool plug was attached to the side of the cage. Water was equidistant from the two disks and at the opposite side of the cage relative to the light source. Grasshoppers in these cages were kept under the same conditions of light and temperature as the main grasshopper colony from which they had been taken. We used individual grasshoppers only once in these experiments. Grasshoppers were placed in the cages early in the 6-h thermoperiod and were removed at the end of the period on the following day, or earlier if 25% or more of either disk had been eaten. Cages were checked hourly during the thermoperiod, less frequently during the remaining daylight hours and not at all overnight. Most individuals of *P. nebrascensis* completed the trial within a few hours of being placed in the cages, while individuals of *A. deorum* usually required an overnight stay. Little if any feeding occurred overnight. Very few entire disks were consumed, so that an alternative choice for feeding existed at all times during the trials.

Disk Preparation and Chemical Analyses. We extracted plant material in 50% methanol, a frequently used solvent for simple phenolics (Waterman and Mole, 1994). This provided plant extracts for application to the glassfiber disks as well as enabling the analysis of total phenolics. We used the method of Price and Butler (1977) for this assay, with tannic acid as the standard (Mole and Joern, 1993; Waterman and Mole, 1994).

For preparing disks, extracts were made using a ratio of 100 μl of extractant to 18 mg dry ground plant material. After overnight extraction at room temperature, we filtered the extract and applied it to disks for immediate use. This procedure extracted substances from a weight of plant material equivalent to one glassfiber disk into 100 μl of solvent.

Besides plant extracts, we also tested the effects of the alkaloids gramine (Sigma), nicotine (Sigma), and eserine (physostigmene, Aldrich) and the tannins tannic acid (Sigma) and quebracho tannin. All disks contained sucrose as a phagostimulant, applied at levels of either 2.5% or 5% of the weight of the disk. Disks paired in the bioassays always contained equal levels of sucrose. Other substances were added at levels given in Tables 1–4 below, and disks were dried between applications. No influence of the level of sugar on the disks was seen in the experiments, so we have dropped this as a factor in the analysis of our results.

Experimental Design. For each level of each substance or extract tested, we used the same level of replication. Twelve grasshoppers were used: six in trials where paired disks each contained 2.5% sucrose and six where they con-

tained 5% sucrose. We tested extracts and test substances at several concentrations on the disks. Alkaloids were tested at a 1% level, a somewhat high level, but one within the natural range for alkaloids in foliage (Bernays and Chapman, 1977; Bernays, 1991). Tannins were tested at levels of 1%, 2%, and 4%, which ranges from the natural level of total phenolics in prairie graminoids to four times this level (Mole and Joern, 1993 and present results). Plant extracts were also tested at one, two, and four times their normal level in the plants.

In experiments with plant extracts containing phenolics, preferences were evaluated in two sets of experiments: (1) one where the control disk contained sucrose only, and (2) one where the control contained sucrose plus a plant extract low in phenolics. Our goal in using this second type of control was to provide control disks with nonphenolic plant constituents to match those in disks impregnated with phenolic containing plant extracts. The object was to minimize differences between the disks with respect to potentially phagostimulant nonphenolics while maintaining a difference in the phenolics applied to each disk. The plant species used for these extracts low in phenolics were *A. smithii*, *B. gracilis*, and *S. comata*. We used a factorial design in these experiments such that extracts of each of *A. hallii*, *A. scoparius*, and *C. heliophila* were paired with each of *A. smithii*, *B. gracilis*, and *S. comata* at equal levels of replication.

Data Analysis. We calculated two variables in our analysis of consumption from the disks: Difference, the weight removed from the control disk subtracted from the weight removed from the disk containing the test substance or extract high in phenolics (see Diff. in Tables 1–4), and Diff% where Diff. is expressed as a percentage of the total weight removed from both disks in the experiment (see Diff% in Tables 1–4). For Difference, positive values indicate a preference for the test material, i.e., relatively less of the control disk was consumed. Negative values indicate deterrence; i.e., more of the control disk was consumed. We eliminated trials when no material from either disk was consumed (see Tables 1–4 for cases where $N < 12$). Diff. was approximately normally distributed, and we used parametric statistics to analyze individual experiments; t tests were performed using DOS SYSTAT 5.0 (Wilkinson, 1990).

RESULTS

Alkaloids. Both grasshopper species avoid the alkaloid-containing disks, irrespective of the particular alkaloid present. Furthermore, this deterrence is virtually complete (see Table 1). This follows because the difference in disk mass removed from the alkaloid-containing disk, versus the control disk, is negative and significantly different from zero in all cases (see Diff. in Table 1). For eserine and gramine, the deterrence is complete because there is effectively

TABLE 1. FEEDING RESPONSES TO ALKALOIDS AND TANNINS[a]

	A. deorum					*P. nebrascensis*				
	Diff.	SEM	*N*	*P*	Diff%	Diff.	SEM	*N*	*P*	Diff%
Alkaloids										
Eserine	−9.0	2.9	5	*	100	−10.7	1.9	10	***	98
Gramine	−11.3	2.1	10	***	100	−10.2	1.5	11	***	100
Nicotine	−6.9	1.8	8	**	90	−6.5	2.7	10	*	48
Tannic Acid										
1%	−4.3	1.9	11	*	58	+2.5	1.4	11	NS	39
2%	−1.6	2.0	7	NS	24	+1.7	1.5	12	NS	31
4%	−1.2	1.6	9	NS	29	−3.3	1.7	11	NS	60
Quebracho extract										
1%	−0.9	1.5	11	NS	12	−1.3	1.8	11	NS	19
2%	+1.8	1.1	12	NS	28	−2.4	1.0	11	*	40
4%	−3.8	1.4	10	*	61	−0.6	1.0	12	NS	29

[a] Diff. = difference between weights of disks consumed (mg), test substance − control. SEM = standard error of Diff. N = number of trials. P = probabilities that Diff. = 0 from t test; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; NS, not significantly different. Diff% = Diff./weight removed from both disks (mg) × 100%.

zero consumption of the alkaloid disks, such that Diff. is close or equal to 100% of the total consumption from both disks (see Diff% in Table 1). Although significant deterrence was obtained in response to nicotine, deterrence is not as complete because some nicotine was consumed by both species (Diff% is less than 100% in Table 1). In addition, only 75% (54 of 72) of grasshoppers starting these trials with alkaloids fed at all. This is fewer than in all the remaining experiments and perhaps results from undetected sampling of the alkaloids that reduced feeding.

Tannins. The two tannins did not effectively deter feeding (Table 1). Taken individually, significantly less of the tannin-containing disk was consumed in only three of 12 tests (Table 1). This contrasts with the uniformly and highly significant aversion seen in trials involving all three alkaloids. When the trials with tannins are considered together, the typically negative value of Diff. (nine of 12 trials) provides only weak support for deterrence (binomial test of the hypothesis that Diff = 0; $P = 0.053$). Overall, this indicates relatively slight deterrence by these polyphenolics. Unlike tests with alkaloids, there is no evidence for complete deterrence, nor is there a pattern of dose-dependent deterrence as the quantity of tannin is increased.

Total Phenolics in Plant Extracts. Mean levels of total phenolics were relatively high in three graminoids: *A. hallii* 1.43% (SE: 0.06%), *A. scoparius* 1.61% (SE: 0.05%), and *C. heliophila* 1.40% (SE: 0.06%). These levels were

about twice those of the other three species: *Agropyron smithii* 0.81% (SE: 0.07), *Bouteloua gracilis* 0.50 (SE: 0.02), and *Stipa comata* 0.55% (SE: 0.04).

Plant Extracts, Sucrose-Only Controls. When we tested extracts from graminoids rich in phenolics against controls without any plant extract (Table 2), we found evidence for both weak phagostimulation and weak deterrence. *A. scoparius* attracted positive responses compared with the neutral responses to its congener *A. hallii*. The sedge, *C. heliophila*, provides the only clear evidence for a deterrent effect. Here, all but one of the values for Diff. are negative, and three of these are statistically significant. While the deterrent effect of this extract is clear, deterrence is far from complete. Over 30% of the disk material consumed in these trials was from the *C. heliophila* extract-containing disks.

Plant Extracts, Low Phenolic Controls. When tested against extracts from plants low in phenolics, extracts higher in phenolics are uniformly and slightly deterrent to feeding. None of the 18 trials exhibited statistical significance, although. Diff. is negative in all but one case. We interpret this as evidence for deterrence because the binomial probability of 17/18 trials producing Diff. < 0 is 0.0001. This deterrence can only be regarded as slight because the grasshoppers are clearly feeding from both disks in these trials (Diff% is not close to 100% in Table 3).

The striking aspect of these results (Table 3) is their uniformity: The differences between *A. scoparius*, *A. hallii*, and *C. heliophila* that were evident

TABLE 2. RESPONSES TO PHENOLICS: SUCROSE CONTROLS[a]

	A. deorum					*P. nebrascensis*				
	Diff.	SEM	*N*	*P*	Diff%	Diff.	SEM	*N*	*P*	Diff%
Andropogon halli										
1×	−0.5	1.9	12	NS	8	−1.1	1.6	11	NS	17
2×	−2.3	3.1	9	NS	30	+1.2	1.1	12	NS	22
4×	−1.7	2.2	9	NS	35	+0.5	1.0	11	NS	6
Andropogon scoparius										
1×	+3.4	0.8	10	**	65	+1.6	1.2	11	NS	25
2×	+1.4	2.4	7	NS	27	−0.2	1.1	11	NS	4
4×	−2.2	1.6	10	NS	44	+3.8	1.1	10	**	76
Carex heliophila										
1×	−4.3	1.9	11	*	58	+0.9	1.1	12	NS	18
2×	−1.6	2.0	7	NS	24	−2.3	0.7	10	**	42
4×	−1.2	1.6	9	NS	29	−2.6	1.0	10	*	42

[a]Diff. = difference between weights of disks consumed (mg), test substance − control. SEM = standard error of Diff. *N* = number of trials. 2P = probabilities that Diff. = 0 from *t* test; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; NS, not significantly different. Diff% = Diff./weight removed from both discs (mg) × 100%.

TABLE 3. RESPONSES TO PHENOLICS: PLANT EXTRACT CONTROLS[a]

	A. deorum					*P. nebrascensis*				
	Diff.	SEM	*N*	*P*	Diff%	Diff.	SEM	*N*	*P*	Diff%
Andropogon halli										
1×	−2.1	1.5	9	NS	44	−0.9	5.0	9	NS	16
2×	−3.0	1.6	11	NS	58	−4.2	7.3	11	NS	58
4×	−1.2	2.0	10	NS	24	−0.3	4.6	11	NS	6
Andropogon scoparius										
1×	−2.7	1.9	12	NS	28	−6.3	3.6	11	NS	74
2×	−0.6	1.6	10	NS	10	−4.8	2.9	11	NS	66
4×	−2.4	1.5	10	NS	46	−1.1	5.1	11	NS	20
Carex heliophila										
1×	−1.3	1.5	11	NS	30	−2.9	3.9	10	NS	65
2×	−1.4	2.5	9	NS	22	−1.2	4.0	11	NS	18
4×	+2.1	2.5	7	NS	38	−3.0	4.5	11	NS	49

[a]Diff. = difference between weights of disks consumed (mg), test substance − control. SEM = standard error of Diff. N = number of trials. P = probabilities that Diff. = 0 from t test; $^{*}P < 0.05$; $^{**}P < 0.001$; NS, not significantly different. Diff% = Diff./weight removed from both discs (mg) × 100%.

with sucrose-only controls are not seen here. In Table 4, we present an analysis of the same raw data used for Table 3 but grouped by the plant extract used on the control disk. This is largely the mirror image of Table 3, as all but one of the values of Diff. are positive (indicating preference). The important point is that these results indicate uniformity in the grasshoppers' responses to *A. smithii*, *B. gracilis*, and *S. comata*.

DISCUSSION

Our results indicate that six dominant Nebraska Sandhills graminoids each lack effective chemical deterrents to feeding by *A. deorum* and *P. nebrascensis* (Tables 2–4). *P. nebrascensis* naturally consumes all of the plant species for which extracts were tested, and it is unclear whether individuals of this species have an innate tolerance of their allelochemicals or whether they acclimated to them by experience in the field. *A. deorum* encounters these same species in the field but, in contrast, *A. deorum* does not routinely include the two *Andropogon* species in its diet and only consumes trace quantities of *C. heliophila* (Joern, 1985). Despite this, it also feeds on disks laden with extracts of all three of these species. These results are consistent with the view that grasses generally lack the necessary levels of secondary metabolites for effective chemical defense

TABLE 4. RESPONSES TO (CONTROL) EXTRACTS LOW IN PHENOLICS[a]

	A. deorum					*P. nebrascensis*				
	Diff.	SEM	*N*	*P*	Diff%	Diff.	SEM	*N*	*P*	Diff%
Agropyron smithii										
1×	+1.8	1.6	10	NS	38	+5.0	2.4	11	NS	82
2×	+0.8	1.6	10	NS	15	+1.1	4.4	12	NS	20
4×	+1.3	2.0	8	NS	27	0.7	4.4	12	NS	11
Bouteloua gracilis										
1×	+3.0	0.9	12	**	45	+1.7	5.6	10	NS	23
2×	+3.4	2.3	9	NS	47	+5.0	6.3	11	NS	56
4×	+1.1	2.0	11	NS	21	+0.6	6.0	10	NS	11
Stipa comata										
1×	+1.1	2.5	10	NS	14	+4.0	4.9	10	NS	56
2×	+1.2	1.7	11	NS	25	+1.0	4.0	10	NS	15
4×	−0.1	2.2	8	NS	2	+3.2	2.3	11	NS	62

[a] Diff. = difference between weights of disks consumed (mg), test substance − control. SEM = standard error of Diff. N = number of trials. P = probabilities that Diff. = 0 from t test; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; NS, not significantly different. Diff% = Diff./weight removed from both discs (mg) × 100%.

(Gibbs, 1974; Jung et al., 1979; Bernays and Chapman, 1977; Owen and Wiegert, 1981; McNaughton, 1983; Mole and Joern, 1993), and they contradict recent reevaluations of this position (Redak, 1987; Vicari and Bazely, 1993). We stress that this position does not deny the existence of phytochemical deterrents in grasses. Our present results and those of Bernays and Chapman (1975) indicate that graminoids can possess mildly deterrent allelochemicals.

Importantly, the two grasshoppers that we have studied and the *Chorthippus parallelus* studied by Bernays and Chapman (1975) are in different subfamilies (Oedipodinae, Gomphocerinae and Melanoplinae), so that the similar results now available for each are independent and not confounded by any close systematic relationships.

The present behavioral results support and strengthen conclusions based on the phytochemical survey data used in previous studies (Gibbs, 1974; Jung et al., 1979; Mole and Joern, 1993) because the use of crude plant extracts in bioassays gave us the potential to detect allelochemicals that were below the detection limits of the chemical tests used previously, but that may have been sensed by grasshoppers. They also allowed for the detection of deterrent chemicals in biosynthetic groups not considered in these previous studies.

Graminivorous grasshoppers differ in their dietary choices when feeding on graminoids in natural grassland vegetation (Joern, 1985). These host preferences are species-specific and do not merely reflect plant abundance. In many

plant–insect-herbivore systems, feeding attractants or phagostimulants may be just as important as deterrents in host-plant choice, and in many well characterized systems such substances are phenolic secondary metabolites (e.g., silkworm–mulberry interaction, several pine–bark beetle systems; Harborne, 1988). However, our present evidence indicates that phenolics do not act as strong phagostimulants, nor are any previously undetected allelochemicals active in this way. Using plant extracts versus sucrose controls, we only saw suggestive evidence for phagostimulation with one plant species (*A. scoparius*; Table 2). This result is unlikely to be ecologically relevant. *A. scoparius* is not a major component of either grasshopper's natural diet, and the phagostimulatory response was not seen when extracts low in phenolics were used as controls (Table 3). If there is one caveat to these results, it is that extremely nonpolar substances may have been insoluble in our extract; however, we doubt that these results would differ if a more lipophilic solvent were used. In overview, we suggest that it is unlikely that phagostimulation by phenolics occurs in the field. In this, we support previous statements to the effect that host-specific phagostimulants do not play a dominant role in host-plant selection by graminivorous grasshoppers (Bernays, 1987).

Weak phagostimulation from host-plant extracts low in phenolics (Table 4) allows for the possibility that these do contain phagostimulants, but it seems unlikely that these will be phenolics. Irrespective of their chemical nature, any possible phagostimulants exhibit only weak activity and seemingly do not appear to provide clear cues to plant species identity. One group of substances able to play this role comprises the primary metabolites such as free amino acids and mono- or disaccharides. These are sufficiently soluble in 50% methanol to have been active in our extracts, and they are nonphenolic metabolites common to all species. Such a role is all the more plausible because both *A. deorum* and *P. nebrascensis* are known to increase feeding in response to physiologically appropriate levels of sugars and amino acids (Behmer and Joern, 1994a,b).

Graminivorous grasshoppers may be excluded from nongraminoid nonhosts by allelochemical deterrents, and our results with alkaloids (Table 1) are consistent with this view. Indeed, they complement previous work with *Locusta migratoria* (Bernays and Chapman, 1977). In contrast, the responses to tannins in our experiments indicate that these may be ineffective as chemical barriers to the consumption of nonhosts by graminivorous grasshoppers. This result does not imply that the defenses of nonhosts are ineffective, as secondary metabolites other than tannins may prevent herbivory by these grasshoppers, even in plants that contain tannins.

Our result for tannins is important because tannins are often considered a major class of allelochemical defense in dicotyledonous plants (Bernays et al., 1989). They have also been considered as deterrents to graminivorous grasshoppers in previous work (Bernays and Chapman, 1977). However, as these

authors reported that 1% tannic acid and 4% condensed tannin were only ca. 50% deterrent to feeding, we would not interpret their data to indicate complete or substantial deterrence.

A considerable amount of data on the concentration of tannins in foliage has become available in recent years, especially for condensed tannins. An analysis of data from five such studies (Gartlan et al., 1980; Waterman et al., 1983; Janzen and Waterman, 1984; Marks et al., 1985; Rogers et al., 1990) indicates that the distribution of condensed tannin levels is far from bell-shaped. Instead, the natural distribution of condensed tannin levels is markedly skewed towards low values, with 25% of tannin-containing dicots having foliage where levels are between 0.5 and 2.5% condensed tannin. Given this result, our data, and that of Bernays and Chapman (1977), we suggest that many dicots that do contain condensed tannins contain incompletely deterrent or acceptable levels (i.e., less than 4%).

Very little quantitative data exist on tannin levels in graminoids that only contain condensed tannins. For grasses (Poaceae), presence–absence data are consistent with the view that tannins are rarely found (present in the leaves of only 7% of 136 species tested; Mole 1993). However, in other graminoids they are more frequent (Mole, 1993). Tannins are found in 42% of sedges (177 tested) and 17% of rushes (53 tested). This result, combined with the skewed distribution of condensed tannin contents in the dicots, suggests that graminivorous grasshoppers will frequently encounter species with acceptable levels of tannin, some of which will be graminoids. Overall, these data suggest an allelochemical continuity between graminoids and nonhosts such that condensed tannins are an unlikely barrier to the exploitation of nonhost plants.

Hydrolyzable tannins do not seem to pose a major barrier to the exploitation of nonhosts, either. These tannins exhibit a relatively restricted taxonomic distribution, and they generally play a minor role in the protein precipitation capacity of plant extracts (Mole, 1993). Where they do occur in quantity, our present results indicate that they have no strong deterrent effects.

In summary, our data support the idea that graminivorous grasshoppers do not encounter strong deterrents or plant species-specific phagostimulants in their host plants, and to this extent we support the views of Bernays and Chapman (1977, 1978). With respect to deterrence in nonhosts, our data for alkaloids also support these views. However, our data for tannins indicate that this class of secondary metabolite is unlikely to be among effective deterrents to these grasshoppers. The present study also indicates that the mechanism of host-plant choice among graminoid hosts by grasshoppers is a largely open question.

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INHIBITION OF SALVINIA (*Salvinia molesta* MITCHELL) BY PARTHENIUM (*Parthenium hysterophorus* L.). I. EFFECT OF LEAF RESIDUE AND ALLELOCHEMICALS[1]

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Abstract—Parthenium (*Parthenium hysterophorus* L.) leaf residue (LP, leaf powder) inhibited salvinia (*Salvinia molesta* Mitchell) biomass and the number of healthy fronds at 0.25% (w/v) and killed the treated plants at and above 0.75% (w/v) in about 5–15 days, depending on the quantity of the residue. At the lethal dose, the LP caused an abrupt desiccation of above-water plant parts, probably due mainly to root dysfunction. This was concurrent with the loss of dehydrogenase activity in, and an increase in solute leakage from, the roots and loss of chlorophyll *a*, *b*, and total chlorophyll contents in the fronds, resulting in death of the treated plants. The LP appears inhibitory to salvinia through affecting macromolecules—proteins, lipids, and nucleic acids. The inhibitory activity of LP at the lethal dose suspended in water was completely lost when allowed to stand for 30 days under outdoor conditions and promoted growth of the salvinia plants placed in it. The standard allelochemicals, including those present in parthenium LP, except parthenin and *p*-hydroxybenzoic acid, did not inhibit growth up to 100 ppm. However, parthenin and *p*-hydroxybenzoic acid killed salvinia plants at 100 and 50 ppm, respectively. Since *p*-hydroxybenzoic acid is unlikely to be present at such a high concentration, parthenin appears to be one of the main allelochemicals responsible for the inhibitory effect of parthenium leaf residue on salvinia.

Key Words—*Salvinia molesta*, growth inhibition, *Parthenium hysterophorus*, leaf residue, membrane integrity, dehydrogenase activity, chlorophyll, water absorption, allelochemicals, phenolics, parthenin.

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INTRODUCTION

Parthenium (*Parthenium hysterophorus* L.) is an aggressive tropical weed of the Asteraceae, endemic to the Americas, and has spread to Africa, Australia, and Asia (Towers et al., 1977). The weed commonly infests pastures, wastelands, and agricultural fields. The weed has spread throughout India, posing serious threats to the environment and may affect natural diversity and cause extinction to natural flora. The plants and their residues show a range of biological activities including affecting various surrounding plants, some economically important, through allelopathy (Kanchan, 1975; Kanchan and Jayachandra, 1979a,b; Sharma et al., 1976, Mersie and Singh, 1987, 1988; Pandey et al., 1993a,b). The allelopathy is caused by the predominant allelochemicals, mainly sesquiterpene lactones and phenolics (Towers et al., 1977; Picman, 1986; Kanchan and Jayachandra, 1979a,b) released from aerial and underground parts of the plants and their residue into the soil environment. Interestingly, the parthenium plant residue showed inhibitory effects on an aquatic weed, water hyacinth, at a much lower level than that required to inhibit growth of wheat seedlings in aquaculture bioassay. Such inhibitory activity indicated the potential for possible biological control of water hyacinth by the strongly allelopathic terrestrial weed (Pandey et al., 1993a,b). Parthenium plant residue either inhibited growth of water hyacinth at lower doses or killed the plants at higher doses, resulting in clearing the water surface (Pandey et al., 1993a,b). However, growth of wheat seedlings continued even at much higher levels of parthenium residue than the lethal dose for water hyacinth.

Salvinia (*Salvinia molesta* Mitchell) is a rapidly growing South American free-floating fern (Forno and Harley, 1979) that has become a serious weed in Africa, Southeast Asia, and Australia (Harley and Mitchell, 1981). This weed is now a major problem in irrigated lowland rice (*Oryza sativa* L.) in some parts of the world (Pablico et al., 1989). In India, it is a major problem in the state of Kerala, where it chokes canals, rivers, ponds, and even paddy fields, affecting the life of more than 5 million people (Joy, 1978). The present investigation was undertaken to study the inhibitory effects of a strongly allelopathic terrestrial weed, parthenium leaf residue, and the possible implications of such inhibition in the management of salvinia through biological control. Attempts have been made to study some selected physiological processes associated with and the allelochemicals involved in inhibition.

METHODS AND MATERIALS

Salvinia Plants. Salvinia (*Salvinia molesta* Mitchell) plants were obtained from the collection of Dr. K.P. Jayanth, Indian Institute of Horticultural Research, Bangalore, India. The plants were grown and maintained in an aqueous

medium that consisted of a 5% (w/v) mixture of farmyard manure powder and field soil (3 : 1, v/v) in water in 20-liter plastic tubs under outdoor conditions. The growing medium was changed at regular intervals, and the plants from this culture were used for the experiments.

Collection and Preparation of Parthenium Leaf Residue. Parthenium leaf residue (LP, leaf powder) was prepared according to the procedure described earlier (Pandey et al., 1993a).

Analysis of Medium. LP was suspended in water at 0.25–1.25% (w/v) and allowed to stand under outdoor conditions for 24 hr. Samples were drawn and analyzed for pH, electrical conductivity (EC), water potential (Ψ_w), and OD at 215 and 340 nm, corresponding to parthenin, a sesquiterpene lactone in the parthenium plant by the procedure described earlier (Pandey et al., 1993a). Total phenolics were measured colorimetrically using the Folin-Denis reagent method (Swain and Hillis, 1959). Inhibitory activity of the samples was tested by using the wheat (*Triticum aestivum* L. var. Sujata) coleoptile growth bioassay in the seeds germinated for 48 hr at 30°C in the dark as described earlier (Pandey et al., 1993a).

Inhibitory Effect of Parthenium Leaf Residue on Salvinia. Leaf residue (LP) was dispersed in 20 liters of tap water in plastic tubs to make suspensions of 0.25, 0.50, 0.75, 1.00, and 1.25% (w/v, the convention used throughout). Plants grown in tap water served as controls. Preweighed salvinia plants with uniform fronds were loaded in each of the tubs and allowed to grow. Tap water was used because such a large quantity was required, its quantity in the tubs being kept constant by regularly replenishing the water lost due to evapotranspiration. Biomass and healthy frond numbers (HFN) were monitored. A frond that did not show desiccation and drying from the margins or appear dull green and flaccid was considered to be a healthy frond.

Effect of pH, Salt, and Water Stress on Salvinia Plants. Preweighed salvinia plants were placed in 1 liter of tap water (EC 0.750 mS/cm, pH about neutrality) with pH adjusted at 1–14 by adding HCl or NaOH solution and were allowed to grow. The HFN and biomass were monitored. For salinity stress studies, salvinia plants were allowed to grow in tap water or in NaCl concentrations ranging from 2.40 to 22.4 mS/cm, and HFN and biomass were monitored. The effect of water stress on salvinia plants was studied by allowing the plants to grow in water or in polyethylene glycol (PEG) -6000 solutions with different water potentials (Ψ_w) (Michael and Kaufmann, 1973) ranging from about −0.013 to −0.250 MPa, and HFN and biomass were monitored.

Absorption of Water and Change in Fresh Weight Over a Brief Period. Preweighed plants were placed in 1 liter of aqueous medium containing a lethal dose of LP and allowed to grow. Plants similarly kept in tap water served as the control. Water was similarly kept for measuring evaporative loss from the free open surface. The plants were removed, weighed, and the volume of aqueous

medium was measured at each observation. Then the volume of the open medium or water was replenished to 1 liter and the plants were replaced. The quantity of water absorbed by the plants was calculated by measuring the evaporative loss.

Solute Leakage from Roots. The roots (about 1 g) from the control and LP-treated plants were sampled, washed with distilled water, blotter-dried, weighed, and steeped in 100 ml distilled water at 30°C for 4 hr. Then the roots were removed, the steep water was filtered through Whatman No. 1 filter paper, and OD at 264 nm (corresponding to UV-absorbing materials, e.g., amino acids, nucleotides, polypeptides, etc.) was measured. An OD of 0.01 was considered as one unit of UV-absorbing substances and was expressed on a per gram root (fresh weight) basis. This was taken as an index of solute leakage and, thus, of cellular membrane integrity.

Dehydrogenase Activity in Roots. For dehydrogenase activity determination, about 0.5 g roots were soaked in 5 ml of 1% (w/v) 2,3,5-triphenyl tetrazolium chloride in darkness at 30 ± 1°C for 4 hr, formazan was extracted, and OD at 520 nm was read following the method described earlier (Pandey et al., 1993a). An enzyme unit was defined as 0.01 OD at 520 nm and was expressed on a per gram root (fresh weight) basis.

Determination of Chlorophyll a, b, *and Total Chlorophyll.* Freshly sampled frond disks (about 5 cm^2) were weighed and ground with a small quantity of acid-washed silica sand in 25 ml of 80% (v/v) acetone, using a mortar and pestle as described earlier (Pandey et al., 1993a). Optical density of the final extract was measured at 645, 652, and 663 nm and chlorophyll *a*, *b*, and total chlorophyll were calculated considering equal area equal weight by the method of Arnon (1949).

Isolation and Purification of Parthenin. Parthenin was isolated and purified by the procedure described by Picman et al. (1980) with modifications (P.V. Subbarao, personal communication, 1992). Dried plant material was refluxed with methanol in a Soxhlet apparatus for 12 hr. The crude methanolic fraction was evaporated to dryness and extracted with petroleum ether. The residue was extracted with chloroform. The chloroform fraction was evaporated to dryness, dissolved in hot ethanol and an equal volume of 4% lead acetate, and allowed to stand for 20 min. This was filtered through diatomaceous earth. The supernatant was concentrated to half the volume and extracted with chloroform. The chloroform layer, comprising the terpene fraction, was dehydrated with anhydrous sodium sulfate, evaporated to dryness, dissolved in benzene–acetone (2 : 1, v/v), and subjected to chromatography over a silica gel column equilibrated with benzene. The column was washed with benzene and eluted successively with portions of a solvent containing increasing amounts of acetone in benzene (10–100%, v/v). The fractions were evaporated to dryness and analyzed by TLC on silica gel-G plates using benzene–acetone (4 : 1, v/v) as a solvent system. The

compounds were located on TLC plates by exposure to iodine vapors or spraying with 5% aqueous $KMnO_4$. The fractions yielded one major and a few minor compounds, which were detected on TLC plates. The fractions containing the major compound parthenin, as identified by cochromatography with parthenin, were pooled and subjected to preparatory TLC using a benzene–acetone (4:1) solvent system. The areas corresponding to the major compound on individual TLC plates were scraped and eluted with chloroform. Pooled chloroform eluate was evaporated to dryness. The residue was dissolved in a small amount of ethyl acetate, the compound was twice crystallized by dropwise addition of cyclohexane in cold, and the purified toxin was obtained and used in the experiments.

Effect of Standard Allelochemicals. Allelochemicals tested included all the major constituents reported in parthenium plant residue, viz., *p*-hydroxybenzoic acid, anisic acid, cinnamic acid, salicylic acid, coumaric acid, fumaric acid, tannic acid, gallic acid, chlorogenic acid, vanillic acid, caffeic acid, ferulic acid, and parthenin, which was isolated and purified in the laboratory. For preparing required concentrations of 10, 25, 50, and 100 ppm, the allelochemicals were, wherever necessary, dissolved in a small quantity of acetone or ethanol or directly prepared in distilled water. The solutions were then made up to one half the total volume with distilled water and the remaining half with the nutrient medium described by Jain et al. (1989). The small quantity of solvent used for dissolving the allelochemicals did not affect growth of the salvinia plants. The plants grown in half-strength nutrient medium served as controls. The HFN and biomass were monitored.

In all experiments the plants were grown under outdoor conditions. All experiments and determinations were repeated at least three times. The data were statistically analyzed for indices of significance (LSD) using a completely randomized block design. Treatments causing death of salvinia plants (i.e., 100% reduction in value) in five days were not included in the statistical analysis.

RESULTS

Analyses of the medium gave the following results. The LP at 0.25–1.25% did not change the pH of the medium much to either side of neutral (range 7.0 $\pm$ 0.6). Electrical conductivity (EC) ranged from 0.7 to 2.7 mS/cm. Water potential (Ψ_w) ranged from -235×10^{-4} to -940×10^{-4} MPa. Total phenolic acids were 18–108 ppm. Optical density at 215 and 340 nm (corresponding to absorption maxima of parthenin), probably indicating the presence of parthenin, and inhibitory activity as shown by reduction in wheat coleoptile length (after 48 hr) bioassay showed a steady increase with the increase of LP in the medium.

Both the number of healthy fronds (HFN) and biomass increased in the control (Figure 1a,b). At 0.25 and 0.50%, the LP inhibited both HFN and biomass. The treatment with LP was relatively more inhibitory to HFN than to biomass. While at lower levels (0.25% and 0.50%) the LP dramatically reduced HFN, a slight increment in biomass continued at 0.25% and was reduced marginally at 0.50%. However, at and above 0.75%, the treatment drastically affected the plants. The results showed very high LSD values due to much fluctuation in the response of plants to LP and, in part, probably due to elimination of treatments causing death (i.e., 100% reduction) from statistical analyses. The LP killed treated plants in about five days and resulted in the subsequent sinking of dead plants. Appearance, disappearance, persistence, and magnitude of the symptoms depended on the light, concentration of the LP, and treatment duration. The inhibitory activity of LP at 0.25% ceased completely in aqueous medium in about 30 days under outdoor conditions as freshly placed plants in the medium showed a rapid increase both in HFN and biomass (data not presented). In the controls, although initial growth was rapid, it subsequently became static, probably due to unavailability of nutrients.

Salvinia plants were able to grow in a wide range of pHs, from 3 to 10. The growth was inhibited at pH 11. At extreme pHs viz., 1, 2, and 12, the plants were killed, showing bleaching and desiccation in about one to five days following death. Salinity levels below 12 mS/cm did not inhibit growth of salvinia. In the range above 12 and below 22.4 mS/cm, depending on the EC, growth of the treated plants was marginally affected. At and above 22.4 mS/

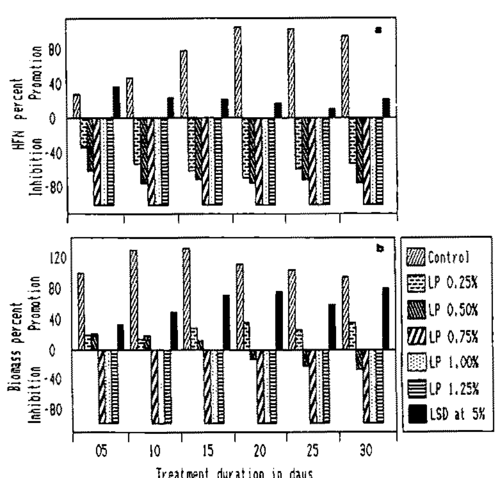

FIG. 1. Effect of parthenium leaf residue (LP, leaf powder) on (a) number of healthy fronds (HFN) and (b) biomass of salvinia.

cm, the plants were inhibited drastically, resulting in death. Results of the effect of Ψ_w on salvinia plants showed that growth of the treated plants was unaffected from −0.15 to −0.25 MPa except that at the latter some leaflets of the treated plants initially showed desiccation along the frond margins; subsequently, the dryness disappeared.

LP treatment at the lethal dose reduced water use by the plants. Over a 24-hr treatment period, the water use per gram fresh weight was 2.24 ± 0.16 ml in control plants and 1.85 ± 0.29 ml in LP-treated plants. Biomass increased considerably in the control plants (by about 10.18 ± 0.62%) and decreased (by about 19.9 ± 8.48%) in the treated plants.

Treatment with LP at the lethal dose, 0.75%, for 12 hr caused massive leakage of solutes from the roots (Figure 2a). The leakage was further enhanced

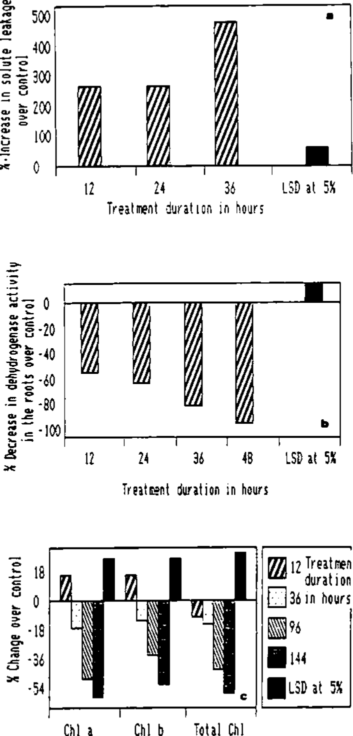

FIG. 2. Effect of parthenium leaf residue (LP) on solute leakage from: (a) dehydrogenase activity in (b) roots, and chlorophyll *a*, *b*, and total chlorophyll (c) in the fronds of salvinia.

by treatment for 36 hr, showing massive loss of cellular membrane integrity due to the treatment.

LP at the lethal dose drastically reduced dehydrogenase activity in the roots (Figure 2b). The treatment for 12 and 48 hr caused about 50% and near total loss of dehydrogenase activity, respectively, in the roots.

Chlorophyll *a*, *b*, and total chlorophyll were considerably reduced in the fronds of the plants treated with LP at the lethal dose (Figure 2c).

At 50 ppm, none of the allelochemicals tested, except *p*-hydroxybenzoic acid, was lethal for salvinia plants (Figure 3). *p*-Hydroxybenzoic acid caused bleaching, browning, and desiccation, followed by death of the treated plants in about 5–15 days. Parthenin proved to be a potential inhibitor at 50 ppm, as it almost contained the HFN and biomass increment over the original value. At 100 ppm (Figure 3), parthenin was also lethal. Although, except for gallic and ferulic acids, all other allelochemicals were slightly inhibitory to biomass, their effect on HFN was relatively less. Total phenolic acids in the medium at lethal dose of LP were about 64 ppm. Except for *p*-hydroxybenzoic acid at 50 ppm and parthenin at 100 ppm, none of the other allelochemicals was lethal at concentrations up to 100 ppm.

DISCUSSION

The Ψ_w and EC values of the medium at even the highest concentration of the LP (i.e., 1.25%) were far lower than those that can cause water and salinity stresses to the salvinia plants. Similarly, the LP in aqueous medium did not change the pH much to either side of neutrality, whereas the salvinia plants were able to grow in a wide range of pH from 3 to 10. Thus, LP at the lethal dose did not result in water, salinity, or pH stress. The inhibitory activity of LP could be attributed solely to the allelochemicals present. An increase in inhibitory activity of the medium with an increase in the level of LP was evident from the results of the bioassay for inhibitory activity and was probably due to a rise in phenolic acids and sesquiterpene lactones, as these are the dominant classes of allelochemicals in the residue of the parthenium leaf (Kanchan and Jayachandra, 1980; Picman, 1986).

The phytotoxic allelochemicals leached out of the LP into the aqueous medium inhibited growth of salvinia plants at lower doses and killed them at higher doses. An immediate effect of the treatment was a dull green appearance of the fronds, drastic loss of turgidity, and resultant flacid texture, as could be easily felt by touching. This was followed and accompanied by desiccation and browning of the frond margins followed by a change of color of the entire frond to pale brown or black. The symptoms started appearing after 6–8 hr of exposure to sunlight. The appearance of these symptoms was much delayed in the dark;

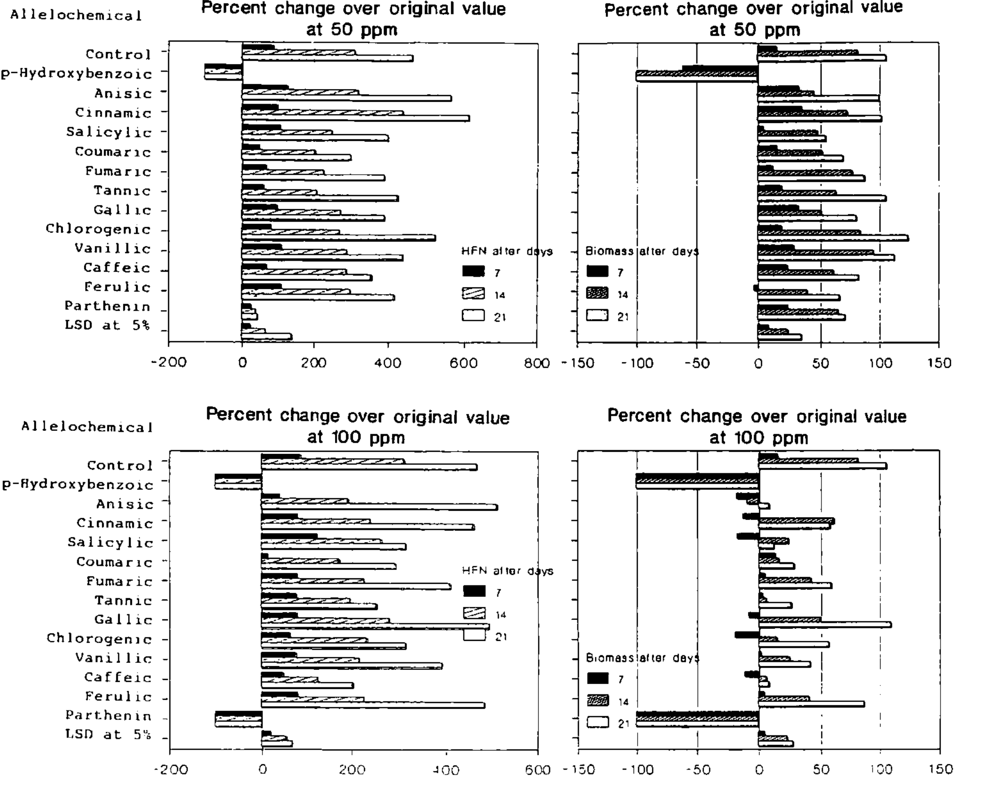

FIG. 3. Effect of different allelochemicals (parthenin and organic and phenolic acids) on number of healthy fronds (HFN) and biomass of salvinia. Except for parthenin, all allelochemicals are acids. A 100% reduction in biomass indicates lethal dose.

nevertheless they appeared, and the plants were affected as under outdoor conditions (unpublished work). It was interesting that untreated plants used more water than treated ones and that the fronds of the plants immersed in the medium containing a lethal dose of LP in 1-liter glass beakers kept under outdoor conditions retained a lush green color for up to 20–30 days. Roots and margins of some of the fronds showing death and rotting (data not presented) indicate that root dysfunction induced a reduction in water supply to the fronds and sustained evapotranspiratory loss of water, probably contributing to the relatively rapid desiccation and death of the treated plants. The visual effects of LP treatment on the fronds were accompanied by a drastic loss of membrane integrity in the roots, as evidenced by excessive loss of solutes even in a relatively short period. There was a concomitant loss of dehydrogenase activity in the roots and loss of chlorophyll *a*, *b*, and total chlorophyll contents in the fronds when compared with untreated controls. Cellular membranes play important roles in living systems and are essential for the maintenance of structure and function. In addition to serving as a permeability barrier, they play vital roles in the compartmentalization of cellular components. Furthermore, cooperative enzymes of a metabolic pathway may be linked together in association with, or integrated into, membrane structures. Massive loss of membrane integrity due to allelochemicals leaching out of the LP and into the medium appears to be one of the main factors resulting in inhibition of salvinia. Likewise, loss of dehydrogenase activity in roots may indicate loss of respiration (Mackay, 1972). Damage to membranes, loss of dehydrogenase activity in roots, and reduced chlorophyll contents in the fronds indicate that the allelochemicals may have acted by affecting the macromolecules—proteins, lipids, and nucleic acids. Allelochemicals other than *p*-hydroxybenzoic acid and parthenin were either slightly inhibitory or did not inhibit the growth of salvinia. *p*-Hydroxybenzoic acid was a more effective allelochemical than parthenin in inhibiting and killing salvinia plants, as the former was lethal at 50 ppm and the latter at 100 ppm. The maximum levels of phenolic acids in the medium at 0.75% LP, the lethal dose, 24 and 72 hr after suspending the plant residue, were about 75 and 107 ppm, respectively (Pandey et al., 1993a,b). The level of phenolic acids did not increase further, rather it decreased subsequently (data not presented). Water-soluble compounds involved in allelopathy in LP include caffeic acid, vanillic acid, ferulic acid, and parthenin (Kanchan and Jayachandra, 1980). Thus, *p*-hydroxybenzoic acid is unlikely to be present at such a high concentration. Hence, other allelochemicals, including parthenin, appear to be mainly responsible for inhibition of salvinia by parthenin leaf residue.

Consistent with earlier findings with water hyacinth (Pandey et al., 1993a,b), the aquatic weed salvinia also showed relatively more sensitivity to the inhibitory effect of allelochemicals when compared with wheat as a reference material. There is a strong possibility that salvinia could be biologically managed

or controlled and replaced in a natural ecosystem by a strongly allelopathic terrestrial weed having allelochemicals with strong inhibitory activity similar to *p*-hydroxybenzoic acid and parthenin.

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INHIBITION OF SALVINIA (*Salvinia molesta* MITCHELL) BY PARTHENIUM (*Parthenium hysterophorus* L.). II. RELATIVE EFFECT OF FLOWER, LEAF, STEM, AND ROOT RESIDUE ON SALVINIA AND PADDY

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Abstract—The relative effect of parthenium (*Parthenium hysterophorus* L.) plant residue on growth of salvinia and paddy seedlings was studied. The inhibitory activity of the residue as shown by its effect on the number of healthy fronds (HFN) and biomass was in the order: flower and leaf > stem and root. The flower (FP) and leaf (LP) residue was lethal at and above 0.75% (w/v, the convention used throughout), and inhibitory at lower doses. The stem (SP) and root (RP) residue supported growth of salvinia at lower doses and were slightly inhibitory at higher (1.25%) dose. All the above residue types supported the growth of paddy seedlings except at 1.25%, the highest concentration tested, which was slightly inhibitory. The amounts of chlorophyll *a*, *b*, total chlorophyll, and carotenoid pigments in the leaves of the paddy seedlings grown in the medium were comparable to the amounts in the leaves of seedlings grown in distilled water. This demonstrates beneficial effects of the treatments. The study shows that salvinia is more sensitive to allelochemicals released by FP and LP into the aqueous medium. Both salvinia and paddy responded similarly to SP and RP by supporting growth at lower doses, probably due to lower levels of inhibitors. The results are discussed with reference to the possible role of allelopathy by parthenium on the population dynamics of aquatic weeds in natural ecosystems.

Key Words—*Parthenium hysterophorus*, *Salvinia molesta*, *Oryza sativa*, biomass, inhibitory activity, chlorophyll, carotenoid.

INTRODUCTION

Parthenium (*Parthenium hysterophorus* L.) is an annual weed endemic to the Americas that has spread to Africa, Australia, and Asia (Towers et al., 1977). The weed has become a serious problem for agriculture and the environment.

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Sesquiterpene lactones and phenolics form the main water-soluble inhibitory allelochemicals in the plant's parts (Kanchan and Jayachandra, 1980). Parthenium leaves and inflorescence contain the highest quantity of parthenin, followed by the stem and roots, successively, while total phenolics were highest in leaves followed by inflorescence, roots, and stem, successively (Kanchan and Jayachandra, 1980). Investigations of the inhibitory effect of parthenium residue on water hyacinth (Pandey et al., 1993a,b) showed that the leaf and flower powder were lethal at relatively lower concentration, and stem residue was lethal at a double dose, killing the treated plants in about a month. Root residue was inhibitory at a relatively much higher dose. On the other hand, the stem and root residue at lower doses supported growth of the treated plants, probably due to lower levels of inhibitors, allowing utilization of nutrients from the residue. It was observed that in aquaculture at different levels of parthenium plant residue, water hyacinth plants were much more sensitive to inhibitory activity, implying that water hyacinth could possibly be controlled by using a strongly allelopathic terrestrial weed. Studies have confirmed that parthenium leaf residue is more toxic to aquatic weeds—water hyacinth and salvinia—than to wheat seedlings (Pandey et al., 1993a; Pandey, 1994). The present investigation was undertaken to study the relative effects of the residue of different parts of parthenium on salvinia plants and paddy seedlings with reference to its possible use in the management of salvinia in paddy fields.

METHODS AND MATERIALS

Salvinia Plants. The plants maintained in culture as described earlier (Pandey, 1994) were used for these experiments.

Parthenium Plant Part Residue. The flowers, leaves, stem, and roots were collected from plants growing near the National Research Centre for Weed Science, Jabalpur (M.P.), India, were powdered to pass through an 80-mesh sieve, and stored until use following the procedure described in detail in Pandey et al. (1993b).

Effect of Residue of Plant Parts of Fronds and Biomass. Residues of parthenium flower (FP), leaf (LP), stem (SP), and root (RP) were each dispersed in 20 liters of tap water in plastic tubs to make 0.25%, 0.50%, 0.75%, 1.00%, and 1.25% (w/v, the convention used throughout) solutions, following the method described earlier (Pandey et al., 1993a). Tap water was used since a large quantity was required. Ten preweighed salvinia plants, each consisting of 20 fronds, were placed on the water in the tubs and were allowed to grow under natural outdoor conditions during 1992–1993. Water lost due to evapotranspiration was replenished regularly to keep the volume of water in the tubs constant. Numbers of healthy fronds and biomass were monitored throughout the period

of the experiments up to 90 days. A frond not showing wilting or drying from the marigns or desiccation was considered to be healthy.

Analysis of Phenolic Acids in Medium. The medium was sampled 72 hr after suspending the plant residue in water filtered through Whatman No. 1 filter paper, and the phenolic acids were determined using the method of Swain and Hillis (1959).

Effect of Parthenium Residue on Growth and Pigments in Paddy. Paddy (*Oryza sativa* L. var. Kranti) seeds were germinated over wet Whatman No. 1 filter paper at 25°C following the method of the International Seed Testing Association (Anonymous, 1985). Twenty seedlings, each with a 2-cm radicle, were placed on a perforated aluminum foil cover with the radicle protruding down into 100 ml of a selected medium containing FP, LP, SP, RP, water (control), or a quarter strength nutrient medium (Jain et al., 1989) in 100-ml beakers. The seedlings were allowed to grow for 10 days during which evapotranspiratory loss of liquid was replenished by adding water regularly. Thereafter, the seedlings were removed, washed with water, and root and shoot lengths and fresh weights were measured. Leaf chlorophyll *a*, *b*, and total chlorophyll were determined using the procedure of Arnon (1949). Carotenoids were assayed using the procedure described by Canal et al. (1985).

All experiments were repeated three times. The data were subjected to ANOVA and differences of means were estimated by calculating least significant differences. Values with a lethal dose showing 100% response were excluded from statistical analysis.

RESULTS AND DISCUSSION

The results (Figures 1–4) show that different parts of parthenium varied in their inhibitory activity on salvinia plants. The FP at 0.25% was stimulatory to the HFN at five or 10 days (Figure 1a). Subsequently it was inhibitory. The FP at 0.25% and 0.50% was inhibitory to the biomass, but apparently not at a significant level until 10 days (Figure 1b). The symptoms—dull green color, desiccation, browning, and finally initiation of blackening from older fronds were evident. Inhibition depended on the concentration of the residue. At and above 0.75%, FP was lethal, killing the salvinia plants. FP (Figure 2a,b) was similarly inhibitory at lower levels and was lethal at and above 0.75%. The SP and RP were not lethal even at 1.25%, the highest concentration tested. Lower levels of SP caused little enhancement in HFN, but proved to be inhibitory at higher concentrations. The inhibitory activity was highest at 1.25%. The SP at lower levels increased biomass over the plants grown in water. The RP did not inhibit HFN at the lowest concentration (0.25%) until 15 days; by contrast it increased the biomass of the treated plants. At and above 0.50% the biomass

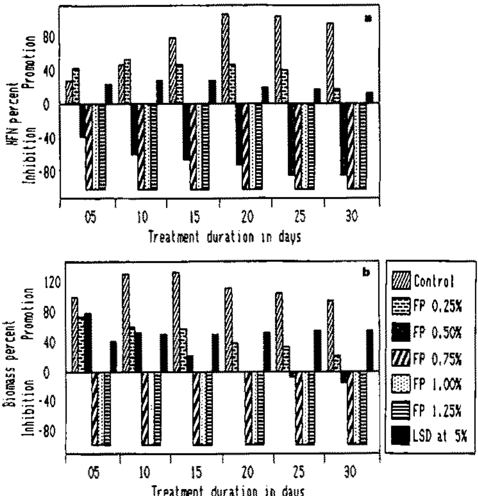

FIG. 1. Effect of parthenium flower residue (FP) on (a) number of healthy fronds (HFN) and (b) biomass of salvinia.

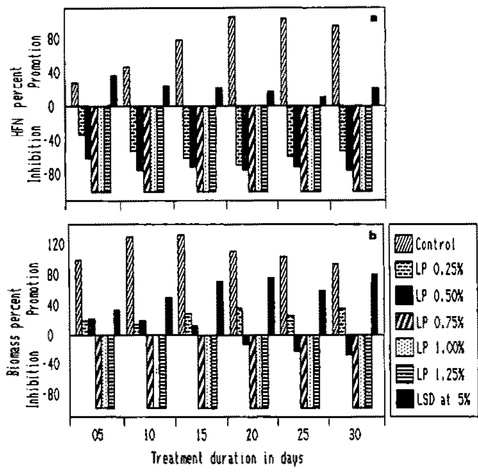

FIG. 2. Effect of parthenium leaf residue (LP) on (a) number of healthy fronds (HFN) and (b) biomass of salvinia.

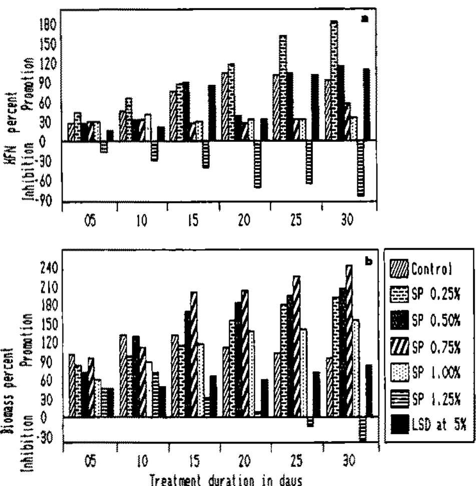

FIG. 3. Effect of parthenium stem residue (SP) on (a) number of healthy fronds (HFN) and (b) biomass of salvinia.

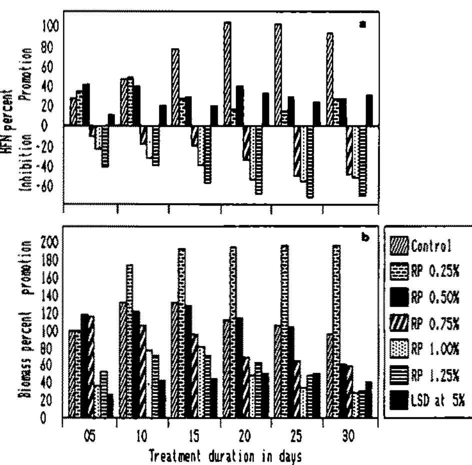

FIG. 4. Effect of parthenium root residue (RP) on (a) number of healthy fronds (HFN) and (b) biomass of salvinia.

increase was reduced considerably. Thus, the SP and RP at lower levels increased biomass over the plants grown in water. The high values of LSD obtained were probably due to elimination of the data concerning the lethal dose, which was at 100% in all cases and due to wide variation in the response of salvinia plants to parthenium residue below lethal doses.

Phenolic acids at 1% residue were maximum in the medium containing FP (180 ppm), following by LP (138 ppm), RP (85 ppm), and SP (62 ppm). Phenolic acid yield percent of residue was maximum at 1.80 ± 0.10 in FP, followed by 1.38 ± 0.08 in LP, 0.85 ± 0.02 in RP, and 0.62 ± 0.05 in SP. The inhibitory activity of the parthenium plant parts residue to salvinia is in the order: FP and LP > SP and RP. This coincides with the relatively higher levels of phenolic acids from FP and LP than SP and RP. Parthenium leaves and inflorescence have been found to contain the highest quantity of parthenin followed by the stem and roots (Kanchan and Jayachandra, 1980). The much more inhibitory activity of FP and LP than SP and RP to salvinia is probably due to the higher levels of the allelochemicals, including phenolic acids and parthenin, in the residue. Growth of paddy seedlings, as revealed by root and shoot lengths and fresh weights, was enhanced by parthenium plant residue at lower levels and inhibited at higher levels when compared with growth of plants in water (Figures 5 and 6). In some cases, the treated plants showed higher growth than those grown in the nutrient medium. This confirms the beneficial effects of the parthenium residue at lower levels, but at higher levels the growth increment

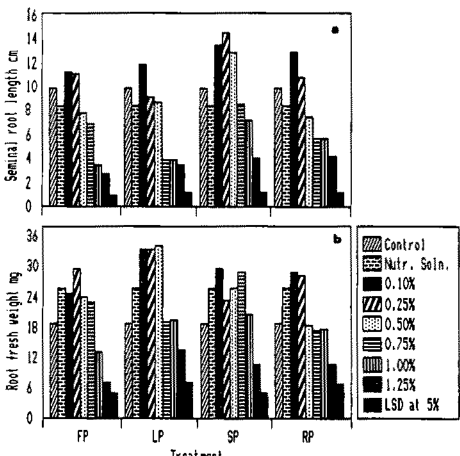

FIG. 5. Effect of parthenium plant part residue on (a) seminal root length and (b) fresh weight of paddy var. Kranti seedlings after 10 days of growth in an aquaculture test for inhibitory activity.

was inhibited and showed growth reduction over the plants grown in water. Leaves of seedlings grown in medium containing parthenium residue were a very lush green in color. The beneficial effect was also evident from enhanced chlorophyll *a*, *b*, total chlorophyll, and carotenoids in the leaves of seedlings grown in medium containing LP (Figure 7). Although the seedling growth at higher levels of LP was comparable to or a little lower than those grown in water, the pigments were considerably higher in the leaves.

The results show that at a particular concentration, FP and LP had the highest inhibitory activity due to higher levels of inhibitory allelochemicals leaching out of the residue into the medium. While FP and LP were inhibitory

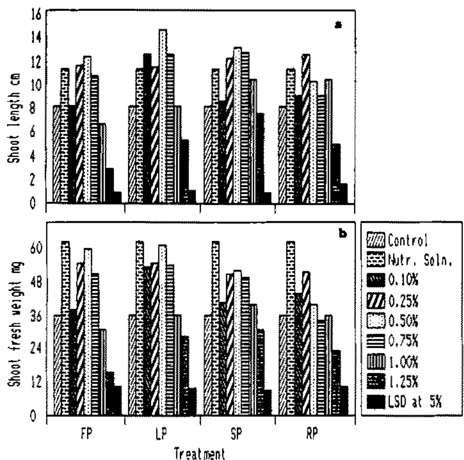

FIG. 6. Effect of parthenium plant part residue on (a) shoot length till margin of the longest leaf and (b) fresh weight of paddy var. Kranti seedlings after 10 days of growth in an aquaculture test for inhibitory activity.

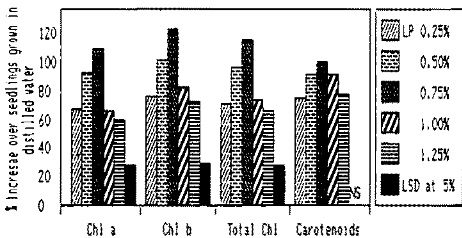

FIG. 7. Effect of parthenium leaf residue (LP) on chlorophyll *a*, *b*, total chlorophyll, and carotenoids in the leaves of paddy var. Kranti seedlings after 10 days of growth in aquaculture.

for salvinia at lower doses and lethal at higher doses, SP and RP improved growth of the treated plants at lower levels and proved to be slightly inhibitory at higher levels. By contrast, the paddy seedlings responded to FP, LP, SP, and RP more or less similarly, as lower doses usually improved the growth and higher doses were marginally inhibitory. The improvement in growth over the seedlings grown in water was, at certain levels, equal to or more than that of the seedlings grown in nutrient solution.

The FP and LP have relatively more of sesquiterpene lactones and phenolic acids. It is evident that the salvinia plants are much more susceptible to the inhibitory activity of these allelochemicals than paddy seedlings. Interestingly, the allelochemicals present in SP and RP appear almost equally inhibitory to both salvinia and paddy seedlings. This might be due to different—or differences in the composition of—allelochemicals, or both. Promotion of growth of salvinia plants at lower doses of SP and RP and of paddy seedlings by FP, LP, SP, and RP might be due to utilization of nutrients from the residue. This was probably due to a low level of allelochemicals, which was unable to inhibit growth of the treated plants. The beneficial effect of parthenium residue on paddy was consistent in pot experiments (data not presented).

Consistent with earlier findings (Pandey et al., 1993a,b), in a study (Pandey, unpublished), parthenium FP and LP at 0.25–0.50% and SP and RP at or above 1.00% were found lethal to other aquatic weeds such as *Pistia stratiotes* L., *Azolla nilotica* Decne., *Lemna pausicostata* Hegelm., *Spirodella polyrrhiza* (L.) Schleid., and to all submerged aquatic weeds, such as *Hydrilla verticillata* L.f. Royle, *Ceratophylum demersum* L., and *Najas gramineae* Del. Thus, it is established that parthenium plant residue is relatively more toxic to aquatic weeds than to test crop species (wheat and paddy). The implications of accumulation of parthenium plant residue in the environment on the population dynamics and shifts in flora of aquatic weeds in natural ecosystems need careful consideration, as accumulation of enormous quantities of the residue under natural conditions appear to be speculative for the following two reasons: (1) Picman and Picman (1984) reported that sesquiterpene lactones, parthenin and traces of coronopilin, which are found in large quantities in parthenium are toxic to its seedlings and older plants at a concentration of 0.1%. Obviously, the water-soluble plant metabolites have been suggested to play important roles in allelopathy and defense against herbivorous predators and diseases, as autotoxins in the regulation of populations, and in the timing of the germination process (Picman and Picman, 1984). (2) The toxicity of parthenium residue in the aquatic environment declines at an appreciable rate as the lethal dose of LP and FP have been found to support growth of salvinia plants freshly placed in the medium under outdoor conditions for about a month (Pandey, unpublished data). Thus, even if considerable amounts of inhibitors find their way or are washed by rain into waterbodies, building up of inhibitors to such an extent as to make

detectable changes in the occurrence and population dynamics of salvinia and of other aquatic weeds is unlikely. However, parthenium residue could be explored as a possible stimulant for paddy seedling growth and as a natural pesticide in paddy fields for the management of some aquatic weeds. The finding indicates the possibility of use of a strongly allelopathic terrestrial weed for biological control of weeds like salvinia and water hyacinth and other floating and submerged aquatic weeds.

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BISABOLENE EPOXIDES IN SEX PHEROMONE IN *Nezara viridula* (L.) (HETEROPTERA: PENTATOMIDAE): ROLE OF *cis* ISOMER AND RELATION TO SPECIFICITY OF PHEROMONE

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Abstract—The *trans*- and *cis*-(*Z*)-α-bisabolene epoxides (*trans*- and *cis*-(*Z*)-αBE) are the main components of the male sex pheromone in *Nezara viridula*. The role of the *cis* isomer and the importance of the *cis/trans* proportion for the activity and the specificity of the pheromone are not clearly elucidated and were studied here. Interindividual variation of the *cis/trans* proportion produced by males was studied by individual hexanic extracts in two strains originating from the south of France (SF) and French West Indies (FWI). The *trans* isomer composed 42–82% of bisabolene epoxides in SF males and 74–94% of bisabolene epoxides in FWI males. Means (± SD) significantly differ between SF (62.8% ± 8.4) and FWI (82.4% ± 5.9) males in spite of this interindividual variation. Different isomers of bisabolene epoxide were synthesized and their EAG activity on female antennae was compared. Racemic *trans*- and *cis*-(*Z*)-αBE elicited low EAGs, not different from the nonnatural *trans* and *cis* (*E*)-αBE that were inactive on behavior. Behavioral tests revealed that racemic *trans*- and *cis*-(*Z*)-αBE attracted 45% ($P < 0.05$) and 25% ($P < 0.05$) of females, respectively. The same levels of attraction were obtained with (−) enantiomers of *trans*- and *cis*-(*Z*)-αBE, which attracted 40% ($P < 0.05$) and 20% ($P > 0.05$) of the females, respectively. Binary blends containing 75/25, 50/50, and 25/75 proportions of *cis/trans* isomers were more attractive than *trans*-(*Z*)-αBE alone and response of females to the 25% *cis*/75% *trans* blend was significantly more important than the response to *trans*-

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isomer alone ($P < 0.05$). The importance of the *cis/trans* proportion in relation with the specificity of the male pheromone is discussed.

Key Words—*Nezara viridula*, Heteroptera, Pentatomidae, sex pheromone, *trans*- and *cis*-(*Z*)-α-bisabolene epoxides, interindividual variation, bioassay, pheromonal specificity, isomer, enantiomer.

INTRODUCTION

Attractivity of males for females in the green stink bug, *Nezara viridula*, was first demonstrated by Mitchell and Mau (1971). Pavis and Malosse (1986) isolated compounds emitted specifically by males. These male components elicit long-range behavioral sequences of females, leading them to the vicinity of males (Borges et al., 1987). The components of the male pheromone were identified by Aldrich et al. (1987) as *trans*- and *cis*-(*Z*)-α-bisabolene epoxides (*trans* and *cis* isomers of (*Z*)-2-(3′,4′-epoxy-4′ methylcyclohexyl)-6-methyl-2,5-heptadiene), (*Z*)-α-bisabolene, *n*-nonadecane, and (*E*)-nerolidol. The male pheromone was recently confirmed to be a sex pheromone, attracting only mature females in an olfactometer (Brézot et al., 1993). Bisabolene epoxides are the main components of this sex pheromone and much attention was paid to them. Females respond to synthetic *trans*-(*Z*)-α-bisabolene epoxide [*trans*-(*Z*)-αBE] (Baker et al., 1987) and to a purified fraction of male cuticular extracts corresponding to *trans*-(*Z*)-αBE (Brézot et al., 1993). The same level of attraction was obtained with this fraction and with living males (Brézot et al., 1993). Thus, bisabolene epoxides might account for most of the activity of the pheromone blend. Both *trans*- and *cis*-(*Z*)-αBE possess asymmetric carbons. The biological tests of the two pure enantiomers of *trans*-(*Z*)-αBE showed that only the (*Z*)-(1′*S*,3′*R*,4′*S*)-(−) enantiomer produced behavioral responses in females of a Brazilian strain (Baker et al., 1987). Responses of females were not affected by the presence of the inactive enantiomer, and it was concluded that (*Z*)-1′*S*,3′*R*,4′*S*)-(−) is the natural pheromone. However, the absolute configuration of natural compounds has yet not been determined.

The precise role of the *cis*-(*Z*)-αBE has still not been clearly characterized. A fraction of male cuticular extract containing *cis*-(*Z*)-αBE is not attractive for females (Brézot et al., 1993). Analysis of the pheromone of males belonging to different populations of *N. viridula* revealed geographical intraspecific variations in the *cis/trans* ratio (Aldrich et al., 1989, 1993). However, most of the *N. viridula* populations release a large excess of *trans*-(*Z*)-αBE, whereas males of sympatric Pentatomidae (Panizzi and Slansky, 1985; McPherson et al., 1979) of the *Acrosternum* genus produce blends in which the *cis* form dominates. This led Aldrich et al. (1989, 1993) to postulate that the *cis/trans* ratio is involved in the specificity of the male pheromone. However, two important points in

relation with the specificity of the pheromonal signal have not been elucidated. First, the ability of females to discriminate between various *cis/trans* blends is unknown. Second, average *cis/trans* ratios have been established from volatile collections of groups of males for *N. viridula* populations, and data on intrapopulational variations are still lacking in the absence of individual analysis.

In this paper, we report on the role of the *cis*-(Z)-αBE and on the importance of the proportion of the *trans*- and *cis*-(Z)-αBE for the chemical communication in *N. viridula*. We first investigated the individual variations of the *cis/trans* proportions in two populations by analyzing cuticular extracts of individual males and compared it to volatile collections of groups of males. Secondly, we studied the biological activity of different isomers of bisabolene epoxides for which syntheses are reported. Detection of *trans*-(*Z*)-αBE, *cis*-(*Z*)-αBE, *trans*-(*E*)-αBE and *cis*-(*E*)-αBE was studied on females by electroantennography. Behavioral responses of females to these compounds were assessed in an olfactometer. Bioassay allowed us to compare the activity of (−) enantiomer and racemic *trans*- and *cis*-(Z)-αBE and to appreciate the ability of females to discriminate between different *cis/trans* blends.

METHODS AND MATERIALS

Pheromone Synthesis and Purification. (See Figure 1: *trans*- and *cis*-(*Z*)-αBE and (*E*)-αBE). A racemic and diastereoisomeric mixture of (*Z*)-αBE and (*E*)-αBE was synthesized as reported by Tomioka and Mori (1992). A mixture of (−)-*trans*- and (−)-*cis*-(Z)-αBE was prepared from (*S*)-(−)-limonene. (*S*)-(−)-Limonene (enantiomeric purity >95% ee) was monoepoxidized to give (*S*)-(−)-limonene epoxide, which was submitted to ozonolysis. The resulting mixture of *cis*- and *trans*-4-acetyl-1, 2-epoxy-1-methylcyclohexane was separated by silica gel chromatography. The two isomers were separately submitted

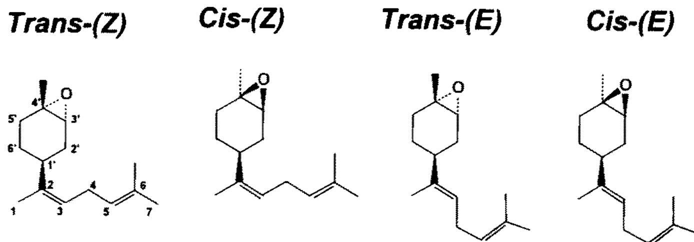


FIG. 1. Synthesized isomers of bisabolene epoxide. *trans*-(*Z*): *trans*-(*Z*)-α-bisabolene epoxide; *cis*-(*Z*): *cis*-(*Z*)-α-bisabolene epoxide; *trans*-(*E*): *trans*-(*E*)-α-bisabolene epoxide; and *cis*-(*E*): *cis*-(*E*)-α-bisabolene epoxide.

to the Wittig reaction by employing triphenyl (4-methyl-3-pentenyl) phosphonium iodide and *n*-butyllithium as reported by Tomioka and Mori (1992) to give (−)-*trans*-(*Z*)-αBE, n_D^{19} = 1.4868 and $[\alpha]_D^{19}$ = −19.9° (*c* = 0.94, CH_2Cl_2), and (−)-*cis*-(*Z*)-αBE, n_D^{21} = 1.4887 and $[\alpha]_D^{21}$ = −32.9° (*c* = 0.965, CH_2Cl_2).

After synthesis, racemic and (−) enantiomer solutions of *trans*- and *cis*-(*Z*)-αBE were purified by micropreparative capillary gas chromatography. *Trans*- and *cis*-(*E*)-αBE were contained as contaminants in the racemic solutions of *trans*- and *cis*-(Z)-αBE and were separated. Each compound was collected with a Girdel 300 gas chromatograph equipped with a fraction collector (Malosse, 1990) and a polar fused silica column (15 m × 0.53 mm ID; Carbowax, Alltech). A gas chromatograph was operated isothermally at 150°C (Ross injector: 200°C, flame ionization detector: 210°C). After micropreparative gas chromatography, collected fractions contained 82.7–96.9% of bisabolene epoxide (Table 1). Separation of isomers was not complete, and the isomeric purity of each solution, ranging from 95.6% to 100%, is given in Table 1.

Insects. Two strains of *Nezara viridula* originating from bugs collected from Avignon, in the south of France (SF) and Guadeloupe, in the French West Indies (FWI) were maintained in the laboratory. Bugs used in this study were second- and third-generation reared insects. Temperature in the rearing room was 23°C, photoperiod was 16L:8D, and the relative humidity was 75%. Bugs were reared in 1.5-liter boxes and fed on green bean sprouts (*Phaseolus vulgaris* L.) and sunflower seeds (*Helianthus annuus* L.). Emerging males and females were separated every day from the rearing boxes and maintained in different boxes. The pheromone was studied on 12- to 25-day-old virgin males. Virgin females used in biological tests were 12–20 days old.

Pheromone Extraction and Analysis. Composition of the male pheromone was assessed by volatile collections. Groups of 10–20 males (N = 2 for each strain) were contained into glass jars (V = 585 ml) and aerated at 450 ml/min for 24 hr at 23°C and in a 16L:8D photoperiod. Volatiles were trapped in cartridges containing 250 mg of Supelpak-2 (Supelco). Volatiles were washed from the adsorbent in 1.2 ml of hexane. Ten micrograms of eicosane was added as internal standard, and samples were evaporated to nearly 100 μl under a gentle stream of nitrogen before analysis. Interindividual variations of the proportion of *trans*- and *cis*-(*Z*)-αBE were studied by immersing separately each male in 1 ml of hexane for 5 min. Individual hexane extracts of SF (N = 36) and FWI males (N = 31) were then recovered and 10 μg of eicosane was added as internal standard. Each sample was evaporated to nearly 100 μl under a gentle stream of nitrogen before analysis.

Volatile collections and individual male extracts were analyzed on a Hewlett-Packard HP5890 gas chromatograph equipped with an apolar fused silica column (25 m × 0.32 mm ID, CPSil 8 CB). The oven was programmed from 60°C for 1 min to 160°C at 35°C/min and from 160°C for 1 min to 240°C at

TABLE 1. PURITY OF BISABOLENE EPOXIDES[a]

Racemic solutions				(−) Enantiomer solutions	
trans-(Z)-αBE (96.1%)	*cis*-(Z)-αBE (96.9%)	*trans*-(*E*)-αBE (82.7%)	*cis*-(*E*)-αBE (92.3%)	*trans*-(Z)-αBE (92.7%)	(−)-*cis*-(Z)-αBE (92.2%)
97.5% *trans*-(Z)-αBE	95.6% *cis*-(Z)-αBE	98.3% *trans*-(*E*)-αBE	100% *cis*-(*E*)-αBE	98.7% (−)-*trans*-(Z)-αBE	100% (−)-*cis*-(Z)-αBE
1.7% *cis*-(Z)-αBE	2.8% *trans*-(Z)-αBE	1.7% *cis*-(Z)-αBE		1.3% (−)-*trans*-(*E*)-αBE	
0.8% *trans*-(*E*)-αBE	1.6% *cis*-(*E*)-αBE				

[a] *trans*-(Z)-αBE: racemic *trans*-(Z)-α-bisabolene epoxide; *cis*-(Z)-αBE: racemic *cis*-(Z)-α-bisabolene epoxide; *trans*-(*E*)-αBE: racemic *trans*-(*E*)-α-bisabolene epoxide; *cis*-(*E*)-αBE: racemic *cis*-(*E*)-α-bisabolene epoxide; (−)-*trans*-(Z)-αBE: (−)-(1′*S*,3′*R*′,4′*S*)(Z)-α-bisabolene epoxide; (−)-*cis*-(Z)-αBE: (−)-(1′*S*,3′*S*,4′*R*)-(Z)-α-bisabolene epoxide. Numbers in parentheses represent the percentage of bisabolene epoxide isomers contained in the purified solution.

5°C/min (split-splitless injector: 225°C, flame ionization detector: 240°C). The percentage of pheromonal compounds was calculated from GC peak areas. To compare *cis*/*trans* proportions, the percentage of *trans* was calculated relative to the total amount of epoxides. Normality of the distributions of *trans* isomer percentages in the (*Z*)-αBE produced individually by males, was tested by χ^2 for each population. Then, differences between distributions of males from SF and FWI according to the *trans* isomer percentages in their pheromone were tested by Student's *t* test.

Electroantennography (EAG). Electrophysiological tests were done on FWI females ($N = 4$). Bugs were lightly anesthetized with CO_2 and placed in a polystyrene holder. Electrodes were glass capillaries filled with Roeder's solution and connected to the DC preamplifier through chloridized silver wire. The reference electrode was inserted dorsally between the head and the thorax. Once a tiny part of the distal segment of the antennae was cut, the recording electrode was gently introduced into the tip of the antennae. Electrodes were connected to a WPI 725 preamplifier and to a Tektronix 5110 oscilloscope. Humidified pure air was continually passed over the antennae (1.5 liters/min). Olfactory stimulations were delivered by 1-sec pulses of odorized air (0.5 liters/min) every 2.5 min. Racemic α-terpineol and (*E*)-dec-2-enal were used as standards. (*E*)-Dec-2-enal is a major component of the metathoracic scent gland of adult *N. viridula* (Gilby and Waterhouse, 1967). (*E*)-Dec-2-enal and racemic α-terpineol were chosen as standards because they produce medium and high EAG responses, respectively. These two compounds were tested at 100 μg between each series of bisabolene epoxides to check up antennal sensitivity throughout experiments. (*E*)-Nerolidol and *n*-nonadecane were also tested at 100 μg. Dose–response curves were realized with racemic *trans* and *cis* (*Z*)-αBE at 100 ng, 1 μg, 10 μg, and 100 μg. Racemic *trans* and *cis* (*E*)-αBE, that were available in smaller quantities, were only used at 100 ng, 1 μg, and 10 μg. Responses to bisabolene epoxide isomers were compared with a Kruskall-Wallis test (Siegel, 1956). Comparisons were done between air and doses and also between bisabolene isomers.

Olfactometry. Olfactometric tests were run in a wind tunnel (2 m long, 0.7 m high, 0.9 m wide). The attractiveness of volatiles was measured by observing the walking response of females on a Plexiglas board (110 × 12 cm) that was placed in the middle of the olfactometer (Figure 2). Three boxes (10 × 12 cm) were drawn on a paper recovering the Plexiglas board, one at the upwind extremity of the board (UW box), the second at its downwind extremity (DW box), and the third at the center of the board (CB box). A cylindrical wired cage (3.5 cm high × 4 cm diameter) was placed in the center of each of the three boxes. A filter paper, loaded with solutions of the compounds to be tested, was placed into the UW cage. The olfactometer had laminar airflow (speed: 0.05 m/sec; temperature: 23°C) and was lighted with an intensity of 175 lux. This weak

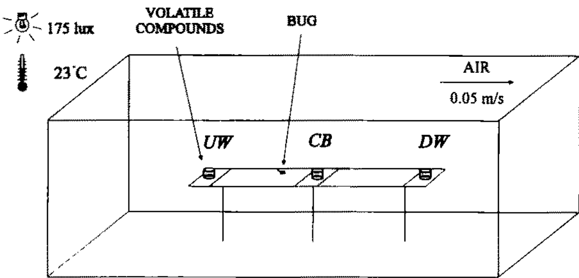


FIG. 2. Wind tunnel used for olfactometric tests. Three cylindrical wire cages were placed on three boxes UW, CB, and DW. Volatile compounds were applied on a filter paper hanging in the UW cage. Females to be tested were placed on the top of the central CB cage and their movements were recorded during 10 min.

light intensity reduced the number of bugs taking flight. Tests were run during the last 4 hr of the photophase. Special care was taken to not stress insects, in order to avoid escape behavior and to prevent the release of defensive secretions. For each test, one insect was made to walk up to the tip of a 1-m-long stem and the tip of the stem was brought into contact with the CB cage. The test began when the bug climbed on top of the CB cage, and displacements of the bug were recorded during 10 min.

Three different responses were recorded. The bugs could stay on the central box, CB, during the whole test (motionless response) or walk on the board, either reaching the source of volatiles at the UW box (source+ response) or not (source− response). Some insects directly flew off from the CB cage. These insects were replaced on the top of the CB cage for a new trial after a first or a second direct flight, but they were discarded if they flew off again at the third trial. Some bugs started walking on the board but flew off before 10 min. In this case, their responses were recorded according to their displacements before flying.

Data were interpreted in two steps. First, the increase of the displacements of the bugs in the presence of the volatiles was assessed by comparing the number of insects that stayed on the CB (motionless response) and that left CB (source+ and source− responses) for control and treatments. Second, the attractiveness of the volatiles tested was assessed by comparing the number of insects arriving on the source of volatiles (source+ response) with those not arriving (motionless and source− responses) for control or treatments. Two series of χ^2 statistical tests were performed, and a Fisher exact probability test (Siegel, 1956) was used when the number of responses was too small to use the χ^2.

A previous study (Brézot et al., 1993) allowed us to determine an optimal dose of 500 ng to test isomers of bisabolene epoxides. Females belonged to the

FWI strain and were used only once. Twenty females were tested in presence of racemic *trans*-(Z)-, *cis*-(Z)-, *trans*-(*E*)-, and *cis*-(*E*)-αBE. The attractiveness of blends of (−)-*cis*-, (−)-(1′*S*,3′*S*,4′*R*)-(Z)-αBE, and (−)-*trans*-, (−)-(1′*S*,3′*R*,4′*S*)-(Z)-αBE, was also compared. Five blends containing, respectively, 100% ($N = 30$), 75% ($N = 25$), 50% ($N = 25$), 25% ($N = 25$), and 0% ($N = 30$) of the *trans* isomer were tested at the same dose of 500 ng of total (Z)-αBE in order to avoid a dose effect.

RESULTS

Pheromone Analysis. Results of two volatile collections from males from SF and FWI are shown in Figure 3. Bisabolene epoxides are the major components emitted by males of the two populations. Percentages of *trans*-(Z)-αBE, (*E*)-nerolidol, and (Z)-α-bisabolene were nearly the same in the SF and FWI male pheromone. Differences between the two populations essentially consist in variations of percentages of *cis*-(Z)-αBE and *n*-nonadecane. Average *cis*/*trans* proportions differ between the male pheromone emitted by males of these two populations. The *trans* isomer represents 67.8% of the total epoxide produced by SF males and 84.2% of the total produced by FWI males.

Individual hexanic extracts (Figure 4) revealed a substantial variation in the *trans* isomer proportion among males. Proportion of *trans* ranged from 42% to 82% in pheromone produced by SF males. The variation was somewhat smaller in FWI males and ranged from 74% to 94%. These values are normally distributed in both populations ($P < 0.05$) and means calculated from individual analysis differed significantly between populations ($P < 0.001$). Values were

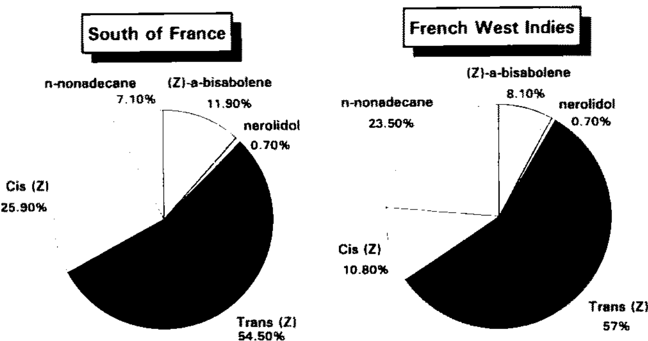


FIG. 3. Composition of the male specific volatiles from the south of France and French West Indies populations of *N. viridula* ($N = 2$). *trans*-(Z): *trans*-(Z)-a-bisabolene epoxide; *cis*-(Z): *cis*-(Z)-α-bisabolene epoxide; (Z)-a-bisabolene: (Z)-α-bisabolene and nerolidol: (*E*)-nerolidol.

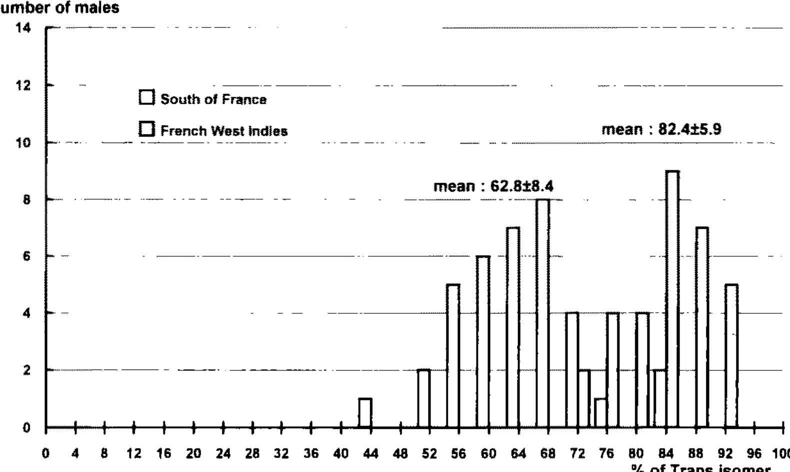


FIG. 4. Proportion of *trans* isomer in the bisabolene epoxides contained in individual extracts of males from the south of France ($N = 36$) and French West Indies ($N = 31$).

62.8 ± 8.4% (mean ± SD) in SF and 82.4 ± 5.9% in FWI. These values were similar to those of volatile collections.

Electroantennography. EAG responses of females were the same for racemic *trans-* and *cis* isomers of (Z)-αBE and (*E*)-αBE ($P > 0.05$) (Figure 5). These bisabolene epoxides produced weak responses (0.5–0.8 mV) compared to the standards (*E*)-dec-2-enal (1.1 ± 0.1 mV) and racemic α-terpineol (1.9 ± 0.2 mV). Responses were only significant compared to air, with 10 μg of *trans*-(*E*)-αBE ($P < 0.01$) and 10 μg of *cis*-(*E*)-αBE ($P < 0.05$). Two other components of the male pheromone, (*E*)-nerolidol and *n*-nonadecane, also produced low EAG responses (0.5 ± 0.1 mV and 0.6 ± 0.1 mV, respectively).

Olfactometry. In the absence of pheromonal stimulation most of the females (90% and 70%) did not leave the CB box (Figure 6). Racemic *trans*-(*E*)-αBE ($\chi^2 = 2.29$, $P > 0.05$) and racemic *cis*-(*E*)-αBE ($\chi^2 = 2.29$, $P > 0.05$) did not significantly increase the displacements of females. In return, female displacements significantly increased when racemic *cis*-(Z)-αBE ($\chi^2 = 6.14$, $P < 0.05$) or racemic *trans*-(Z)-αBE ($\chi^2 = 10.98$, $P < 0.001$) were disposed in the UW box. Twenty-five percent of the females went to UW in presence of racemic *cis*-(*Z*)-αBE, and this response was significant ($P_{\text{Fisher}} = 0.023$). On the other hand, 45% of the females of *N. viridula* arrived at the source of volatiles in presence of racemic *trans*-(Z)-αBE ($\chi^2 = 9.18$, $P < 0.01$).

Displacements of the bugs were increased by the *cis*-(−), (−)-

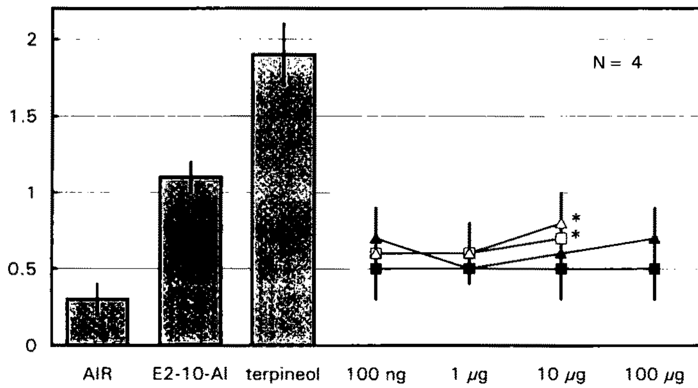
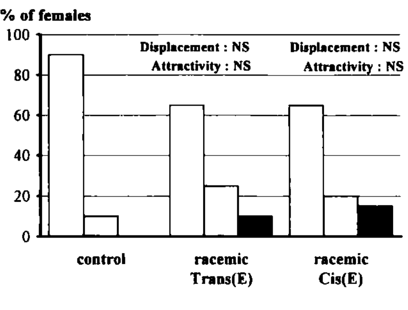


FIG. 5. Electroantennographic responses of French West Indies females ($N = 4$). Dose-response curves are shown for *trans*-(*Z*)-α-bisabolene epoxide (black triangles), *cis*-(*Z*)-α-bisabolene epoxide (black squares), *trans*-(*E*)-α-bisabolene epoxide (white triangles), and *cis*-(*E*)-α-bisabolene epoxide (white squares). E2-10-Al: (*E*)-dec-2-enal; terpineol: racemic terpineol. Vertical lines represent standard deviation. *Responses significantly different from air with $P < 0.05$.

(1′*S*,3′*S*,4′*R*)-(Z)-αBE ($\chi^2 = 4.34$, $P < 0.05$), and by the *trans*-(−), (−)-(1′*S*,3′*R*,4′*S*)-(Z)-αBE ($\chi^2 = 8.08$, $P < 0.01$), enantiomers of (Z)-αBE (Figure 7). Forty percent of the females went to UW in presence of the *trans*-(−) enantiomer ($\chi^2 = 4.02$, $P < 0.05$) compared to only 20% in the presence of the *cis*-(−) enantiomer ($\chi^2 = 0.11$, $P > 0.05$). The percentages of females reaching UW in the presence of *trans*- and *cis*-(Z)-αBE were the same for racemic and (−) enantiomer solutions. Racemic blends and (−) enantiomers of *trans*- and *cis*-(Z)-αBE exhibit the same biological activity.

Bugs tested with blends of 75/25, 50/50, and 25/75 *cis*/*trans* (−) enantiomers of (Z)-αBE increased their displacements ($\chi^2 = 7.9$, $P < 0.01$; $\chi^2 = 28.31$, $P < 0.001$ and $\chi^2 = 28.31$, $P < 0.001$ respectively) (Figure 7). Number of females reaching the source of volatiles were significantly larger for these three blends than for the control. Percentages of females walking up to the source of volatiles were 72% with the 25% *cis*/75% *trans* ($\chi^2 = 17.16$, $P < 0.001$), 60% with the 50% *cis*/50% *trans* ($\chi^2 = 11.06$, $P < 0.001$), and 48% with the 75% *cis*/25% *trans* blend ($\chi^2 = 6.27$, $P < 0.05$). Percentages of females that reached the source of volatiles were more important for blends containing both *cis*- and *trans*-(Z)-αBE than for *trans* isomer alone. The difference was significant ($\chi^2 = 5.63$, $P < 0.05$) only for the 25% *cis*/75% *trans* blend.



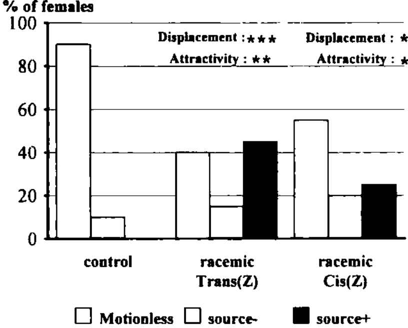


FIG. 6. Behavioral responses of French West Indies females (N = 20) to the racemic four isomers of bisabolene epoxide. Motionless: number of females staying on the central box during the whole test; Source−: number of females leaving the central box without reaching the source of volatiles; Source+: number of females reaching the source of volatiles. NS: $P > 0.05$; $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

DISCUSSION

Females are attracted by the synthetic *trans*-(Z)-αBE in our bioassay. Responses of the females (40–45%) to this diastereoisomer confirm the finding of Baker et al. (1987). Although *cis*-(Z)-αBE elicited a larger number of females walking compared to control, the number of females reaching the source of volatiles (20–25%) was lower than in the presence of *trans*-(Z)-αBE. Thus, the *cis* diastereoisomer showed lower attractivity than the *trans*-(Z)-αBE. This is consistent with the results of Brézot et al. (1993), which have tested a fraction of male extract containing this diastereoisomer. The three *cis*/*trans* blends attracted a greater number of females (48%, 60%, and 72%) than the *trans* diastereoisomer alone (40%). In particular, the blend containing 25% of *cis*-(Z)-αBE attracted 72% of the females, a number significantly larger than in the presence of *trans*-(Z)-αBE. It shows that the *cis* diastereoisomer is involved in the attractivity of the male pheromone. Pheromones are very often multicom-

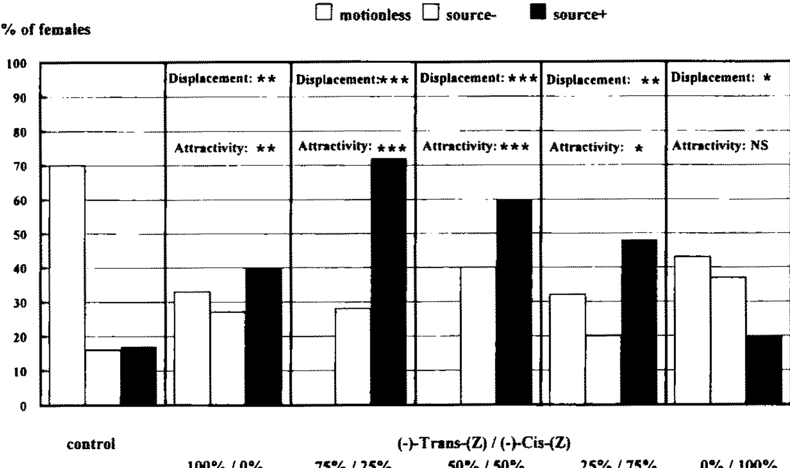


FIG. 7. Behavioral responses of French West Indies females to *trans*-(−), (−)-(1′*S*,3′*R*,4′*S*)-(*Z*)-αBE ($N = 30$), *cis*-(−), (−)-(1′*S*,3′*S*,4′*R*)-(*Z*)-αBE ($N = 30$), and to three binary blends of these (−) enantiomers ($N = 25$). Motionless: number of females staying on the central box during the whole test; Source−: number of females leaving the central box without reaching the source of volatiles; Source+: number of females reaching the source of volatiles. NS: $P > 0.05$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

ponent blends (Tamaki, 1985), with biological activity of individual components being weaker than that of the blend. In Lepidoptera, activity of blends constituted of isomers have been reported for a long time (Roelofs and Cardé, 1974; Cardé et al., 1975; Klun et al., 1973). In Heteroptera, the activity of the attractive pheromone of *Podisus maculiventris* (Aldrich, 1988) and *Campylomma verbasci* (Smith et al., 1991) is reproduced by two compounds of a five- or six-compound blend.

Racemic *trans*- and *cis*-(*Z*)-αBE showed the same biological activity as their corresponding (−) enantiomers. The presence of the (+) enantiomers in the racemic solutions did not inhibit or enhance the attractivity of the (−) enantiomers. This confirms the conclusion of Baker et al. (1987) regarding the absence of activity of the (+) enantiomer. Biological activity has been found in only one enantiomer in most of the chiral pheromonal compounds (Mori, 1984). In Heteroptera, the (+) enantiomer of α-terpineol, a component of the attractive male pheromone of *Podisus maculiventris*, is the only active enantiomer in field tests (Aldrich et al., 1984). Although the absolute configuration of the *Nezara viridula* natural pheromone has still not been chemically determined, we can suppose that FWI males produce the (−) enantiomers.

It has not been completely elucidated whether the bisabolene epoxides are responsible for all the activity of the male pheromone. Two other components of the male secretion, *n*-nonadecane and (*E*)-nerolidol, are not attractive when tested alone (Brézot et al., 1993). (*Z*)-α-Bisabolene was not available and could not be tested. The percentage of attracted females was nearly the same with the more efficient *cis*/*trans* proportion (nearly 70%) and when four and six males (nearly 65%) were used as source of pheromone in the same apparatus (Brézot et al., 1993), suggesting that *trans*- and *cis*-(*Z*)-αBE are responsible for the most important part of the pheromonal attractivity, at least in our experimental conditions.

The nonnatural *trans*- and *cis*-(*E*)-αBE do not attract females and do not increase their displacements. Behavioral tests showed that females discriminate between *E* and *Z* geometrical isomers and between *trans* and *cis* diastereoisomers. Nevertheless, this discrimination is not revealed by EAG. EAG responses to bisabolene epoxides are low and do not differ. This is surprising for a sex pheromone, since they generally elicit strong EAG responses in one sex. In Lepidoptera, male antennae are highly specialized for the detection of the female pheromone and EAG responses can be used for predicting the structure of pheromone components (Roelofs, 1984). The low number of receptors on the male antenna of *N. viridula* may explain the low amplitude of EAG responses to *trans*- and *cis*-(*Z*)-αBE. Better understanding of the detection of the male pheromone by females will necessitate the use of single sensillum recordings.

Two extraction methods were used in this work. Volatile collections are usually preferred since they reflect more exactly the composition of the emitted pheromone blend. On the other hand, extractions are easiest for analysis of individuals. Close average *cis*/*trans* proportions have been obtained from volatile collections and individual male extracts, and both methods can be used to analyze the bisabolene epoxide composition of the pheromone of *N. viridula*. Interindividual variations of *cis*/*trans* proportions produced by FWI and SF males confirmed that both populations differ in their pheromone. Although variations appear to be wider in the SF population, most of the percentages of *trans* diastereoisomer vary in a range of 20–25% of *trans*-(*Z*)-αBE in both populations. Such variation may not be uncommon in insect pheromones and has already been reported in two-compound pheromonal systems in Lepidoptera (Haynes et al., 1984; Barrer et al., 1987).

More FWI females were attracted by the 25% *cis*/75% *trans* blend that lies closest to the one emitted by the FWI males. Nevertheless, blends containing higher proportions of *cis* were still highly attractive. The total dose of the two (*Z*)-αBE isomers was 500 ng for each blend, and the slight decrease of response for blends containing 50% and 25% of *trans* isomer may be due to the decreasing amounts of *trans* in blends. Blends containing 50% and 25% of *trans*-(*Z*)-αBE attract females although no male of the FWI strain was found to produce less

than 70% of the *trans* isomer. Whether it is the proportion or the amount of *trans*-(Z)-αBE responsible for the slight difference of responses of females, it is clear that females respond to a wider range of *cis/trans* proportions than those produced by males of their strain. Selectivity of females for the *cis/trans* proportion is weak and cross-attraction between populations might be still important. However, species specificity in pheromone communication still appears possible with Pentatomidae species producing drastically different proportions. For instance, the male pheromone of *Acrosternum marginatum* and *A. hilare*, whose *cis/trans* blend contains less than 10% *trans*-(Z)-αBE (Aldrich et al., 1989), may be able to preserve specificity of long-range attraction of females. In return, despite the difference of proportion of (Z)-α-bisabolene, the *cis/trans* blend released by *N. antennata* males (nearly 80% of *trans* isomer) might be too close to that of *N. viridula* males in Japan (45–50% of *trans* isomer) (Aldrich et al., 1989, 1993) in view of our results, to prevent interspecific attraction. The existence of natural interspecific mating between *N. antennata* and *N. viridula* (Kiritani et al., 1963) supports our hypothesis.

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INTERSPECIES DIFFERENCES AND VARIABILITY WITH TIME OF PROTEIN PRECIPITATION ACTIVITY OF EXTRACTABLE TANNINS, CRUDE PROTEIN, ASH, AND DRY MATTER CONTENT OF LEAVES FROM 13 SPECIES OF NEPALESE FODDER TREES

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Abstract—Dry matter, ash, crude protein, and protein precipitation activity (PPA) of 13 Nepalese tree fodder species were monitored in dried samples prepared monthly between November 1990 and May 1991, and additionally in November 1991, covering the season when they are particularly important as fodder. Monthly levels of dry matter, ash, and crude protein were fairly stable except when there was new leaf growth, although year to year differences in dry matter were found in *Brassaiopsis hainla* (Bh), *Dendrocalamus strictus* (Ds), *Ficus roxburghii* (Fr), and *Quercus semecarpifolia* (Qs). Tannin PPA fluctuated considerably in *Artocarpus lakoocha* (Al), *Ficus glaberrima* (Fg), *F. nerrifolia* (Fn), Fr, *F. semicordata* (Fs), *Litsea polyantha* (Lp), and *Prunus cerasoides* (Pc), and to a lesser extent in Bh, *Castanopsis indica* (Ci), *C. tribuloides* (Ct), *Quercus lamellosa* (Ql), and Qs. Similar fluctuations in PPA were observed in fresh leaf samples taken weekly. Ds did not have any detectable PPA. Trends in PPA fluctuation were generally similar for trees located at similar altitudes. Fr, Pc, Al, Fn, Ql, and Ci had falling PPAs before shedding leaves. Some of the fluctuations in Fr, Fs, Fg, Pc, and Lp

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were apparently due to changes in the extractability and quantity of condensed tannins. These fluctuations in PPA may affect the nutritive value of the fodders.

Key Words—Tannins, protein precipitation, fodder trees, protein, ash, dry matter, herbivory.

INTRODUCTION

Livestock production in Nepal is constrained by a lack of feed (Giri, 1990), so it is important to improve the quantity and quality of the fodder available. Tree leaves are an important source of feed for ruminants, particularly in the dry season, with reports of the utilization of some 100 species (Panday, 1982). In a survey, Upton and Robinson (1988) found that over 70 tree species were used in one region alone. While the survey obtained indicates which species were described by local farmers as providing the best quality fodders, their species ranking varied depending on the time of the year. Upton and Robinson (1988) suggested that this was probably because species were ranked more highly during their season of utilization as they were more in the farmers' minds. There is clearly a need for a more systematic study of fodder quality using objective indicators so that superior species and varieties can be identified and grown selectively.

Many fodder trees contain tannins, which have been associated with negative effects on nutritive values (Kumar and Singh, 1984; Mangan, 1988; Leiner, 1990). Conversely, tannins in some feeds are advantageous to ruminants, as they can protect protein from degradation by microorganisms in the rumen and make it available in the lower gut (Barry et al., 1986). Tannins are, however, chemically diverse and complex; different tannins may have different effects on animals (Mangan, 1988). The mechanisms whereby tannins exert their antinutritional effects are not fully understood (Leiner, 1990), and hence there is no established method by which to assess their antinutritive effect. A measure of their ability to precipitate protein is considered to be a useful indicator of their biological activity (Hagerman and Butler, 1989). One such method involves the precipitation of protein in an agarose plate (Hagerman, 1987), and a modification of this method was used in this experiment.

The purpose of this paper is to describe screening trials conducted on 13 species of fodder tree from Nepal to provide information on how composition varied with time during the 1990–1991 dry season (known in Nepal as winter) and the start of the wet season, the period when tree fodder is particularly important to the local farmers. Additional analyses of fresh and dried material were undertaken in October and November 1991 to confirm that variability in PPA was not an artifact of sample preparation. Samples from five species in

which a high proportion of the tannin was condensed tannin (Padmini, Rossiter and Wood, unpublished data, samples analyzed using the method of Dawra et al., 1988) were also analyzed for total and extractable condensed tannins.

METHODS AND MATERIALS

Sample Preparation. Monthly samples were obtained from the species shown in Table 1. The table also indicates the altitude at which the sampled trees were growing. Sampling was started in November 1990 for all species except Pc, Ci, and Ct, which were first sampled in December. Sampling of Bh was discontinued in March as the tree had shed all its leaves by this time. For the same reason, Al sampling ended in April, and that for Lp, Fs, Fr, Ql, and Ci in May. Single samples were taken from the same trees in November 1991, except for Pc, which had no leaves at this time.

All samples were taken from a single tree of each species that was marked for identification. Branches were lopped from the bottom, middle (inside canopy), top, sunny, and shady (outside canopy) regions of the tree. A total of 1 kg of fresh leaves was removed from the branches. Two separate samples of fresh leaves were taken from the lopped branches. Each sample was bulked, mixed thoroughly, and 100 g removed for drying. The leaves were dried to a constant weight in an oven at 60°C and ground in a hand-operated sample mill to approximately 1-mm particles. Weight loss on drying was used to estimate the dry matter content of the fresh leaves sampled. The duplicate samples were

TABLE 1. FODDER TREE SPECIES ANALYZED

Species	Species code	Altitude (m)
Artocarpus lakoocha	Al	1100
Brassaiopsis hainla	Bh	1650
Castanopsis indica	Ci	1650
Castanopsis tribuloides	Ct	1650
Dendrocalamus strictus	Ds	1100
Ficus glaberrima	Fg	1100
Ficus nerrifolia	Fn	1050
Ficus roxburghii	Fr	1650
Ficus semicordata	Fs	1650
Litsea polyantha	Lp	1100
Prunus cerasoides	Pc	1650
Quercus lamellosa	Ql	2100
Quercus semecarpifolia	Qs	2200

then sent to the Natural Resources Institute (NRI) in the U.K. for analysis. Analyses were completed one to three months after sample preparation, except for the condensed tannin assays (completed up to 18 months after sampling). Additionally, in November 1991, single samples (taken as above but not duplicated) from the same trees were taken in the same manner as the initial samples for comparing composition from one year to the next. Over a six-week period, from October to the end of November 1991, single weekly samples of 500 g fresh leaf were taken from the lower reaches of different trees of Al, Bh, Ct, Fg, Fs, and Lp and analyzed for tannin PPA in Nepal. Tannins were extracted within 2 hr of sampling and analyses started immediately after extraction.

Analyses of Dry Matter, Ash, and Crude Protein. Samples were analyzed for dry matter, ash, and crude protein by the methods described in the Feeding Stuffs (Sampling and Analysis) Regulations (Anonymous, 1982). Samples were analyzed in duplicate. The duplicates obtained agreed to within 20 g/kg.

Preparation of Extracts for PPA Analysis. For each dried sample, 500 $\pm$ 10 mg was weighed in duplicate into 10-ml glass beakers, and 5 ml of aqueous acetone (700 ml acetone per liter solvent; analytical grade, BDH Chemicals) was added. The mixture was homogenized and mixed for 3 min using an Ultra-turrex (10-mm probe, 13,500 rpm; Janke and Kunkel). The mixtures were then transferred to a centrifuge tube and spun for 10 min at 2000*g*.

From the bulked sample, 100 g of fresh leaf material was removed, ground with a hand grinder, and to 5 g of the material 20 ml of 800 ml/liter (v/v) aqueous acetone was added. The mixture was thoroughly stirred with a glass rod and left covered for 10 min at ambient temperatures. It was then centrifuged in the same way as the extracted dried samples.

Tannin Analysis. The protein precipitation method used was a modification of the radial diffusion method of Hagerman (1987). Hemoglobin, 1.0 g/liter, (from bovine blood, Sigma Chemicals) in agarose was used, otherwise the plates were as described by Hagerman (1987). Values obtained were very similar to those resulting from the original Hagerman (1987) method (unpublished data). Supernatant, 15 μl, was applied to wells in two agarose plates using a Hamilton syringe, and the plates were sealed, incubated, and measured as described by Hagerman (1987). PPA values were calculated as the activity of the test extract (the diameter squared of the protein–tannin band expressed in square centimeters) per gram dry weight of sample.

Extractable and total condensed tannins were measured by the acid butanol procedure of Porter et al. (1986). For the extractable condensed tannin assay, 60 mg dried leaf sample was extracted (1 min, vortex mixer) twice with 3 ml, then once with 1 ml, of aqueous acetone (prepared as above). The supernatants were pooled and used for color development using the reaction conditions described by Porter et al. (1986). For total condensed tannins, dried leaf samples

were reacted directly with the acid butanol reaction mixture without prior extraction. Data were calculated as the absorbance (at the maximum near 550 nm) per gram dry weight of sample.

Statistical Analysis. Multifactor analysis of variance was used to assess the extent of analytical variation for PPA. The size of the analytical variation varied in proportion to the mean level of PPA. This relationship was used to derive an equation for computing a pooled estimate of standard error for the PPA data.

RESULTS

Dry Matter, Ash, and Crude Protein Contents. The dry matter contents of the fresh leaves and ash contents are given in Table 2, crude protein in Table 3. Species-to-species differences were found in these three components, but there was little variability over the period of the trial. Ds and Ct showed some drop in dry matter content in May and April, respectively; crude protein in Ct increased from December to April and decreased in May, and in Ds there was a marked increase from April to May. These changes coincided with the growth of new leaves in these species in April and May.

PPA Activity of Tannins. Data on the PPA of extractable tannins obtained from the radial diffusion assay are given in Table 3. The standard error between the replicate measurements of PPA could be represented by the best-fitting linear equation: standard error between sample duplicates = $0.41 \times$ mean activity$^{0.63}$. The large fluctuations in values noted below are much higher than the standard error between sample duplicates.

Clear species-to-species differences could be observed in some cases. For example, Qs generally had the highest tannin PPAs while Ds had none. Unlike dry matter, ash, and crude protein contents, which were generally stable, the PPA of leaf extracts varied considerably for some species.

Fluctuations in Al, Fg, Fn, and Lp appeared to show broadly similar trends. A notable exception was that the PPA of Al fell in March before leaf shedding against a rising trend in other species. All of these trees except Fn were located at the same site at an altitude of 1100 m. Fn was located at a different site but similar altitude (1050 m). All four of these species had lower PPAs in November 1991 than in November 1990. Monthly trends in Qs were similar to this group, although grown at an altitude of 2200 m.

The other species that had sharply fluctuating PPAs (Fs, Fr, and Pc) were all located at an altitude of 1650 m. Fs and Fr had trends very similar to each other. Pc did not have a high activity in December, but was otherwise similar. The trends of the fluctuations of the PPA of Fr (and the species at 1650 m)

TABLE 2. DRY MATTER AND ASH CONTENT OF FRESH LEAVES OF 13 NEPALESE FODDER TREE SPECIES

Month of sampling	Species[a]					
	Al	Bh	Ci	Ct	Ds	Fg
Dry matter (g/kg)						
Nov-90	388	290	N/A[b]	N/A	498	361
Dec	375	294	449	469	518	449
Jan-91	341	318	479	450	539	366
Feb	397	320[c]	477	460	499	369
Mar	410[c]	N/A	447	422	557	334
Apr	N/A	N/A	528[c]	319	545	358
May	N/A	N/A	N/A	287	428	357
Mean ± SD[d]	382 ± 26.4	306 ± 15.7	476 ± 32.9	401 ± 78.2	512 ± 53.7	371 ± 36.
Nov-91	419	379	462	452	545	349
Ash (g/kg dry matter)						
Nov-90	158	76	N/A	N/A	124	116
Dec	165	88	37	40	144	116
Jan-91	142	80	36	34	155	115
Feb	168	77[c]	38	44	154	133
Mar	195[c]	N/A	42	50	150	106
Apr	N/A	N/A	37[c]	50	148	123
May	N/A	N/A	N/A	46	89	95
Mean ± SD[d]	166 ± 19.2	80 ± 5.5	38 ± 2.5	44 ± 6.1	138 ± 24.2	115 ± 11.
Nov-91	185	72	35	40	146	119

[a]Key to species codes is given in Table 1.
[b]N/A: sample not analyzed.

were similar to that of Fg (and the species at 1100 m) except for the November to December period. Ql had similar trends to Fr.

Total and Extractable Condensed Tannins. Table 4 gives data on levels of apparent total and extractable condensed tannins at periods when there were particularly large changes in PPA from one month to the next. The sharp rises in PPA seen in Fr and Fs from November to December are reflected by sharp rises in extractable condensed tannin. Similarly, falls in PPA for all five species were observed from March to April, with the PPA of Fg rising from April to May. These changes are closely mirrored by changes in extractable condensed tannins. Trends in apparent total condensed tannins are also similar; they rise from November to December in Fr and Fs and fall from March to April in all species assayed. For Fg, changes in extractable condensed tannins appear to be solely due to changes in extractability, as apparent total condensed tannin actually

TABLE 2. Continued

Species[a]						
Fn	Fr	Fs	Lp	Pc	Ql	Qs
370	330	351	356	N/A	546	475
339	318	368	338	423	526	479
365	325	375	365	380	553	505
387	290	372	369	453	559	507
374[c]	360	416	367	362	576	504
N/A	309[c]	418[c]	397[c]	381	578[c]	484
284	N/A	N/A	N/A	328	N/A	510
353 ± 37.2	322 ± 23.5	383 ± 27.5	365 ± 19.2	388 ± 44.4	556 ± 19.5	495 ± 14.8
389	365	351	381	N/A	548	510
102	172	83	58	N/A	47	27
104	207	93	57	52	53	27
85	201	104	62	63	57	27
91	206	96	77	45	52	26
116[c]	212	103	78	87	54	32
N/A	222[c]	134[c]	70[c]	70	55[c]	28
102	N/A	N/A	N/A	66	N/A	30
100 ± 10.9	203 ± 17.0	102 ± 17.2	67 ± 9.2	64 ± 14.5	53 ± 3.5	28 ± 2.1
104	222	98	63	N/A	43	37

[c] Sample taken before leaf shedding.
[d] Means and standard deviations of all data from November 1990 to May 1991.

increased in April when extractable condensed tannin and PPA were low; for the other species, both changes in extractability and apparent total condensed tannin appeared to be additive, resulting in large changes in levels of extractable condensed tannins.

For Fs and Pc in March, Lp in April, and Fg in May, the extractable condensed tannins were higher than the apparent total condensed tannins, indicating that the method used gave an underestimate of the total condensed tannins.

PPA of Fresh Leaf Samples. Table 5 gives PPA results from analyses conducted on fresh leaf samples. The standard error for the analytical error of these single samples followed the relationship: standard error between analytical duplicates = $0.02 \times \text{mean activity}^{1.05}$. It was notable that the Al and Bh trees selected for this comparison had no extractable tannins, in contrast to the same

TABLE 3. CRUDE PROTEIN AND PROTEIN PRECIPITATION ACTIVITY OF LEAVES OF 13 NEPALESE FODDER TREE SPECIES

Month of sampling	Species[a]					
	Al	Bh	Ci	Ct	Ds	Fg
Crude protein (g/kg)						
Nov-90	132	165	N/A[b]	N/A	154	120
Dec	141	161	147	127	183	102
Jan-91	140	140	146	143	166	101
Feb	141	117	150	145	175	114
Mar	104	N/A	148	157	149	109
Apr	N/A	N/A	123	220	150	100
May	N/A	N/A	N/A	206	222	81
Mean ± SD[c]	131 ± 15.9	146 ± 22.1	143 ± 11.4	166 ± 37.8	171 ± 25.9	104 ± 12.7
Nov-91	135	143	130	149	174	115
Protein precipitation activity (cm^2/g dry matter)						
Nov-90	266	175	N/A	N/A	ND	614
Dec	155	137	355	564	ND	447
Jan-91	207	182	462	530	ND	293
Feb	372	210	468	545	ND	651
Mar	188	N/A	573	829	ND	633
Apr	N/A	N/A	296	609	ND	188
May	N/A	N/A	N/A	537	ND	473
Mean ± SD	238 ± 85.3	176 ± 30.0	431 ± 107.6	602 ± 114.5	ND	471 ± 178.5
Nov-91	166	211	443	448	ND	300

[a]Key to species codes is given in Table 1.
[b]N/A: sample not analyzed; ND: not detected.

species analyzed earlier. The trends for Fg, Lp, Fs, and Ct were similar but not completely synchronous.

DISCUSSION

Dry Matter, Ash, and Crude Protein Contents. Tree fodder, which annually makes up 40% of the total fodder available countrywide in Nepal (Panday, 1982), is important as a protein source as well as a source of energy. All of the fodder analyzed had high protein contents compared to straw and other fibrous feeds available to ruminants in Nepal. Crude protein levels measured were generally similar to those quoted by Panday (1982), although the value of 216 g/kg quoted for Bh is higher than that found here, and 64 g/kg for Pc is lower.

TABLE 3. Continued

Species[a]						
Fn	Fr	Fs	Lp	Pc	Ql	Qs
124	145	146	172	N/A	113	154
128	132	149	161	178	114	164
124	143	149	153	176	117	149
98	136	137	152	158	122	160
112	115	119	144	181	117	137
N/A	119	133	154	196	116	132
163	N/A	N/A	N/A	198	N/A	123
125 ± 21.7	132 ± 12.5	139 ± 11.6	156 ± 9.5	181 ± 14.6	116 ± 3.0	146 ± 15.2
126	143	144	159	N/A	133	151
371	208	337	391	N/A	475	768
172	395	719	371	190	491	682
340	147	404	349	231	565	839
411	151	632	649	408	570	1040
161	528	606	561	674	653	1007
N/A	92	145	168	124	334	600
324	N/A	N/A	N/A	149	N/A	857
296 ± 105.1	254 ± 170.7	474 ± 216.4	415 ± 169.7	296 ± 210.7	515 ± 108.9	827 ± 160.7
306	280	542	319	N/A	291	695

[c] Sample taken before leaf shedding.
[d] Means and standard deviations of all data from November 1990 to May 1991.

The dry matter contents of fresh leaves were generally consistent with the limited dry matter data quoted by Panday (1982), and ash contents were broadly similar, although Fr was quoted at 131 g/kg ash, somewhat lower than that reported here. Mahato and Subba (1988) reported even lower ash contents of 79 g/kg for Fr. There may be variations early in the growing season when the leaves are newly sprouted, as indicated by the fall in ash content in Ds in May; perhaps differences in Fr are due to similar seasonal variations.

Fluctuations in Tannin PPA. There are three possible explanations for the observed fluctuations in PPA: (1) sampling error, (2) artifacts of sample preparation, and (3) real changes in the tannins in leaves and/or changes in the makeup of the leaf sample that affects its overall tannin PPA.

Taking leaves from different parts of the tree was intended to give a sample representative of the tree as a whole. The quantity of leaf taken was, however,

TABLE 4. EXTRACTABLE AND APPARENT TOTAL CONDENSED TANNIN-CONTENT OF SELECTED LEAF SAMPLES[a]

Month of sampling	Species[b]									
	Fr		Fs		Fg		Lp		Pc	
	Extractable	Apparent total	Extractable	Apparent total	Extractable	Apparent total	Extractable	Apparent total	Extractable	Apparent total
Nov-90	155	340	246	548	N/A	N/A	N/A	N/A	N/A	N/A
Dec	405	478	559	692	N/A	N/A	N/A	N/A	N/A	N/A
Mar-91	488	496	664	637	623	634	519	679	780	693
Apr	108	226	143	334	225	742	412	325	90	366
May	N/A	N/A	N/A	N/A	687	647	N/A	N/A	N/A	N/A

[a] Values given as the absorbance at the absorption maximum near 550 nm per g dry matter.
[b] Key to species codes is given in Table 1. N/A: sample not analyzed.

TABLE 5. PROTEIN PRECIPITATION ACTIVITY (PPA) OF FRESH LEAVES FROM NEPALESE FODDER TREES[a]

	Species[b]					
Sampling date	Al	Bh	Ct	Fg	Fs	Lp
24-Oct-91	ND	N/A	N/A	332	N/A	278
30-Oct	ND	ND	288	273	319	282
03-Nov	ND	ND	273	439	319	575
11-Nov	ND	ND	440	383	542	466
18-Nov	ND	ND	306	475	527	684
24-Nov	ND	ND	136	221	203	234

[a]Values in cm^2/g dry matter. N/A: sample not analyzed; ND: not detected. Key to species codes is given in Table 1.

limited by the fear that taking excessively large samples might in itself alter the PPA of subsequent samples. The fairly low standard error of duplicated samples indicated that these samples were representative of the leaves actually taken. The fact that there were clear and consistent trends in the fluctuations rather than random variations supports the hypothesis that they were not due to sampling errors.

Tannins are chemically reactive and susceptible to change during sample preparation. The effects of drying on tannin extraction are not, however, consistent. Makkar and Singh (1991) found that *Quercus incana* leaves could be dried at 90°C for 24 hr or 60°C for 48 hr without altering the PPA; Hagerman (1988) reported that extractable PPA could be increased or decreased by lyophilizing or oven drying at 40°C, while Padmaja (1989) reported a reduction of cassava leaf PPA by drying at 60°C. Consistent trends between species might be expected if differences between sample preparation of each monthly batch gave rise to the variable loss of tannins. While it is difficult to rule out the possibility of artifacts arising, it is notable that the trends for all species were not identical. Al and Fn had a reduced PPA in March prior to leaf shedding, whereas the other species, which did not lose their leaves in March, had high PPAs compared to the other data for the particular species. Further, data from fresh leaves fluctuated in a similar way to that from dried leaves. Hence, these data were not consistent with the view that the fluctuations are artifactual.

The PPA due to condensed tannins will depend on the extractability of these tannins and also their molecular weight and composition (reviewed by Hagerman, 1989). Changes in hydrolyzable tannin content and activity will affect PPA but will not be detected by the condensed tannin assay. While the similarities in trends between PPA and extractable condensed tannins are strongly

indicative of a causal relationship for some species, there were insufficient data to explain the fluctuations in PPA in detail.

Earlier Studies of Fluctuations in Tannin Content. Seasonal changes in tannin content and activity have been noted elsewhere in the literature. Mauffette and Oechel (1989), working with *Quercus agrifolia*, found that the PPA of the new season's growth was some 60% higher than for leaves of the previous season's growth, but fell progressively over about six months to the levels of the old season's leaves. Makkar et al. (1991) observed similar trends in *Quercus ilex*, *Quercus semecarpifolia* (Qs), and *Quercus serrata*, although in *Quercus glauca* PPA was lower in young (4-day-old) leaves compared with mature (1-year-old) specimens. The relative stability of the PPA of the two *Quercus* species studied (Qs and Ql) here is broadly consistent with these observations as very young leaves did not form a major component of the samples taken. Vaithiyanathan and Singh (1989) measured the tannin PPA of leaves from 12 fodder tree species in Rajasthan, India, in the spring, summer, and winter seasons. Very considerable seasonal differences were noted for some species.

Tannin contents in a given species can vary considerably depending on the environmental conditions to which it is exposed. Waterman et al. (1984) have implicated exposure to strong sunlight with high levels of condensed tannins in *Barteria fistulosa*. Barry and Forss (1983) found that condensed tannin contents of *Lotus pedunculatus* grown in fertile and poor soils were 2–3% and 8–11% of the dry matter, respectively. Additionally, the state or treatment of the plant can have an impact on tannin levels. Regular browsing of *Acacia nigrescens* resulted in a halving of its condensed tannin content compared with lightly browsed plants, although browsing did not appear to affect condensed tannin levels in *Acacia tortilis* (du Toit et al., 1990). In contrast, van Hoven (1991) suggested that high browsing pressure led to increases in condensed tannin contents of browses, although drought conditions may have been a significant factor in causing these increases. The changes are believed to be related to plant metabolism adapting to stress conditions and may be important as a defense mechanism to herbivory (reviewed by Mueller-Harvey, 1989).

Possible Reasons for and Implications of Fluctuations in Tannin PPA. Nepal is a very mountainous country where altitude greatly affects climatic conditions. The period of the study covered the end of the wet season, the cool dry sunny winter, and the showery spring seasons in Nepal. Similar trends in trees located at similar altitudes suggest that fluctuating weather conditions may have induced the observed fluctuations in PPA by affecting tree leaf metabolism. Rainfall, temperature, and sunlight may all have been important factors causing the observed responses. Further work is required to determine the relative importance of these, and possibly other, factors.

The fluctuations in PPA could have important implications for farmers who use fodder trees as a major source of feed. Under certain weather conditions, tannin levels may rise sharply in some species and reduce the nutritional value of the fodder. There may be potential for selecting times or conditions when

tannins are less active and thus improve the nutritive value of the feed. There also may be potential to manipulate tree metabolism by simple means such as watering or shading to improve feed quality. The effect of fluctuations of tannin PPA on nutritive value has yet to be established, and the potential for improved feeding practices needs to be investigated in order to produce benefits for farmers in Nepal and elsewhere in countries where tree fodders are important.

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SEASONAL VARIATION IN PHYTOTOXICITY OF BRACKEN (*Pteridium aquilinum* L. KUHN)

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Abstract—Laboratory bioassays were used to test for the phytotoxicity of volatile compounds, fresh plant material as a seed bed, and water extracts from bracken [*Pteridium aquilinum* (L.) Kuhn] pinnules to germination and seedling growth of aspen (*Populus tremula* L.) and Scots pine (*Pinus sylvestris* L.). Fronds were sampled from two bracken populations, one in the south and one in the north of Sweden. All three bioassays showed inhibitory effects, and these varied seasonally with the most inhibitory effects occurring in May, June, and September. The peak of inhibition in May and June coincides with the start of the growing season when bracken still is immature and vulnerable to interference from other species. The increase in inhibitory effects in September appears to be due to transformation of natural products or an accumulation of inhibitory compounds that are released during decomposition following frond death. Addition of activated carbon did not remove the inhibitory effects.

Key Words—*Pteridium aquilinum*, allelopathy, phytotoxicity, volatile compounds, water extracts, regeneration failure, activated carbon, *Pinus sylvestris*, *Populus tremula*.

INTRODUCTION

Bracken [*Pteridium aquilinum* (L.) Kuhn] is one of the five most abundant plants of the world (Harper, 1977) dominating a wide variety of vegetation types (Page, 1976). It frequently is considered a serious weed in agriculture, forestry, and

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conservation of heath lands, and considerable effort has been focused on its control (Burge and Kirkwood, 1992). Bracken is effective at competing for light (Tolhurst and Turvey, 1992), nutrients (Evans et al., 1990), and moisture (Smith and Lockwood, 1990). Its competitive success is related to an invasive rhizome system (Conway, 1952), climatic and edaphic tolerance (Watt, 1955; Ader, 1990), and genetic variability (Wolf et al., 1990). Bracken contains a wide range of secondary metabolites that are considered defensive against herbivores (Lawton, 1976; Cooper-Driver, 1990; Jones and Firn, 1979) and possible allelopathic agents (Cooper-Driver, 1976; Storey, 1991). However, allelopathic studies with bracken have been difficult to interpret, sometimes giving contradictory results, which may be due to season of collection, as well as problems with the methodology for testing of allelopathy. In Sweden bracken is associated with regeneration failures. While it is sparsely distributed in the forest before clear cutting, bracken easily colonizes the cut area and tree seedlings become established with difficulty (Torkildsen, 1950). The purpose of this study was to test the hypothesis that bracken has phytotoxic properties that interfere with tree seedling germination and growth and to determine whether these properties vary seasonally. The study was based on bioassays under semisterile laboratory conditions where effects on seed germination and growth of two native tree species, aspen (*Populus tremula* L.) and Scots pine (*Pinus sylvestris* L.), were evaluated.

METHODS AND MATERIALS

To test for phytotoxic properties of bracken in Sweden, two populations were sampled, one in Mönsterås in southern Sweden, (57°00′N, 16°30′E) and one in Hössjö in northern Sweden, (63°45′N, 19°45′E). Both populations were present on barrens with dense fronds, 50–100 cm in height, on mor humus soil. Fronds were collected monthly from November 1990 to October 1991 and from May in Mönsterås and June in Hössjö to October in 1992. From October to April the sampled material consisted of dead fronds with advanced stages of senescence. Live fronds were present in material collected following emergence, i.e., in May in Mönsterås and June in Hössjö. When sampled, the fronds were put into plastic bags and stored at +2°C overnight. For all experiments only the pinnules (terminology after Thomson, 1990) were used in bioassays to evaluate the phytotoxic properties. Aspen seeds (99% viability), collected from a natural stand (65°30′N, 22°15′E), and Scots pine, collected in a plantation (95% viability, Skogsgård 56°51′N, 13°15′E) were used as test seeds. All experiments were replicated 10 times per treatment and performed in a climate chamber maintained at +20°C with 17 hr of artificial illumination per day, comparable to Swedish summer.

Volatility Bioassay. To assess whether volatile compounds were released by bracken and whether they had an influence on seed germination and root

growth of aspen, 20 g of fresh pinnules from each sample were cut into 2-cm-long pieces and placed in a 135-mm-diameter Petri dish. The bottom of a 50-mm-diameter Petri dish containing one sheet of filter paper (Munktell No. 3), 2 ml of distilled water, and 25 aspen seeds were positioned within the middle of each large dish to expose the seeds to volatile chemicals released from the bracken pinnules. The large Petri dish was sealed with parafilm. Moistened cellulose in place of the bracken pinnules was used as a control. Germination was recorded daily for seven days until no further germination took place; then the mean radicle length of all germinated seedlings was measured. Two additional treatments were conducted as above for fronds collected in June and July in Mönsterås 1991: (1) intact pinnules to test for the effects of cutting, and (2) cut pinnules plus 0.5 g of fine powdered proanalysis activated carbon in distilled water to test whether the phytotoxic properties were eliminated by adsorption of the volatile chemicals.

Seed Bed Bioassay. The phytotoxicity of bracken pinnules as a seed bed for early Scots pine seedling growth was tested. Plastic cups (60 mm diameter and 60 mm height) were filled to two thirds with washed industrial quartz sand and watered to field capacity. To prevent the radicles from penetrating the sand before harvest, 4 g of pinnules (cut in 0.5-cm-long pieces) that represented a 1.5-cm layer of pinnules were placed on top of the sand and misted with 2 ml of distilled water to maintain a high moisture level. As two separate controls, unfertilized commercial *Sphagnum* peat and pinnules powdered with 0.5 g activated carbon were placed on top of the sand. Ten pregerminated Scots pine seeds with a radicle length of 1–2 mm were placed on top of each seed bed. Two milliliters of distilled water was then added and the cups were sealed with parafilm. After seven days, radicle growth was measured and the number of upright seedlings counted. The seedlings were considered rooted when the radicle had penetrated the substrate and the hypocotyl orientated upwards from the substrate.

Water Extract Bioassay. A water extract bioassay was performed to evaluate the effects of water-soluble compounds released by bracken. Fronds were dried at +20°C for seven days and then stored at −18°C until the experiment started. Fifty grams of dry pinnules were soaked in 1 liter of distilled water and gently stirred for 96 hr at +20°C. The extract was filtered through one sheet of filter paper (Munktell No. 3). The osmotic potential in the extracts was around 51 mmol/kg and pH 6, and neither the osmotic potential (Nilsson, 1992) nor the pH affect germination of aspen or Scots pine. Two milliliters of the extract were added to a Petri dish (50 mm diameter) with one sheet of filter paper (Munktell No. 3). Either 25 aspen seeds or 25 Scots pine seeds were placed upon the filter paper. Distilled water was used as the control. Seed germination was recorded daily for seven days for aspen and 14 days for Scots pine. All germination took place within this time. At the end of the experiment, root

growth was measured on all aspen seedlings. Radicle growth was measured on a subsample of five randomly chosen Scots pine seedlings per replicate.

Analysis of Data. Two-way analysis of variance was used to evaluate differences between treatment and month of frond collection. Results were significant at the $P \leq 0.001$ level. Two-sample t test was used to evaluate differences between treatment and control for each month at $P \leq 0.01$.

RESULTS

In the volatility bioassay, aspen seed germination was significantly inhibited by fronds collected in Mönsterås in May, June, and September 1991 and June, August, and September 1992 and by fronds collected in Hössjö in September 1991 and from June to August 1992 compared with the control (Figure 1). Root growth of germinated aspen seeds was inhibited by fronds collected in Mönsterås from May until October 1992, except for fronds collected in August, which stimulated the root growth. Fronds collected in Hössjö inhibited root growth from June until September 1992 except fronds collected in August (Figure 2). Pinnules in advanced senescence and decomposing pinnules (November–April) did not inhibit seed germination of aspen. There was no significant difference in aspen seed germination whether cut or intact pinnules were used (data not presented). The addition of activated carbon did not remove the inhibitory effect (data not presented).

Radicle growth of pregerminated Scots pine seeds grown on a seed bed of bracken pinnules collected in Mönsterås and Hössjö was significantly less for all tested months, except October and March, compared with the control (Figure

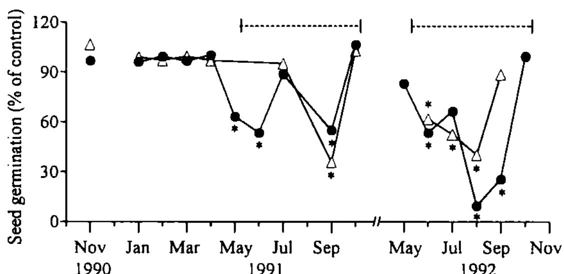

FIG. 1. Seed germination of aspen (*Populus tremula*) exposed to volatile compounds released by bracken (*Pteridium aquilinum*) pinnules, collected from two populations, Mönsterås (- ● -) and Hössjö (- △ -), 1990–1992. Data are expressed as percentage of moistened cellulose control. Length of growing season: |-----| Significant difference from the control at $P \leq 0.01$.

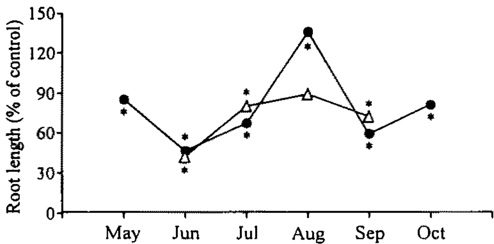

FIG. 2. Root length of aspen (*Populus tremula*) seedlings exposed to volatile compounds released from bracken (*Pteridium aquilinum*) pinnules collected from two populations, Mönsterås (- ● -) and Hössjö (- △ -), 1992. Data are expressed as percentage of moistened paper control. *Significant difference from the control at $P \leq 0.01$.

3). Bracken collected in the beginning of the growing season (May in Mönsterås and June in Hössjö) and in August and September had the most pronounced negative impact on radicle growth. The addition of activated carbon on top of the pinnules did not remove the inhibitory effect on radicle growth (data not presented). A large number of the seedlings had an abnormal development and only a small number of the seedlings were sufficiently rooted in the bracken-added treatments compared to the control (Table 1).

Water extracts made from bracken pinnules collected from the Mönsterås population in May and September significantly inhibited the seed germination of aspen and Scots pine compared to the control, while the inhibition was negligible during the rest of the year (Figure 4a and c). The rooting ability of aspen was less when grown in water extracts made from bracken pinnules col-

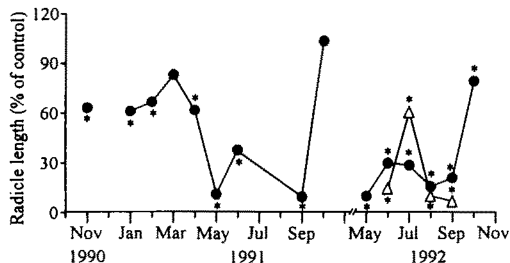

FIG. 3. Radicle length of Scots pine (*Pinus sylvestris*) seedlings grown on a seed bed of bracken (*Pteridium aquilinum*) pinnules collected from two populations, Mönsterås (- ● -) and Hössjö (- △ -), 1990–1992. Data are expressed as percentage of peat control. *Significant difference from the control at $P \leq 0.01$.

TABLE 1. SCOTS PINE (*Pinus sylvestris*) SEEDLING ($N = 10$) DEVELOPMENT WHEN GROWN ON SEED BED OF BRACKEN (*Pteridium aquilinum*) PINNULES

	No. of rooted and well-developed seedlings		
	Mönsterås (mean ± SE)	Hössjö (mean ± SE)	Control (mean ± SE)
1990			
November	3.8 ± 0.4[a]	4.4 ± 0.3[a]	7.1 ± 0.6
December			
1991			
January	3.3 ± 0.5[a]	2.2 ± 0.4[a]	6.1 ± 0.7
February	2.9 ± 0.7[a]	4.5 ± 0.7[a]	6.8 ± 0.5
Mars	3.4 ± 0.5[a]	2.8 ± 0.6[a]	7.4 ± 0.5
April	4.6 ± 0.4[a]	5.1 ± 0.7[a]	9.4 ± 0.3
May	3.2 ± 0.6[a]		9.5 ± 0.2
June	4.2 ± 0.7		5.7 ± 0.8
July	0.7 ± 0.7[a]	1.4 ± 0.6[a]	8.4 ± 0.5
August			
September	0.2 ± 0.1[a]	0.2 ± 0.1[a]	8.6 ± 0.3
October	2.2 ± 0.5[a]	2.3 ± 0.7[a]	5.3 ± 0.6
1992			
May	1.1 ± 0.8[a]		9.8 ± 0.2
June	0.7 ± 0.4[a]	0.4 ± 0.4[a]	7.6 ± 0.4
July	2.6 ± 1.1[a]	5.0 ± 1.0[a]	8.8 ± 0.3
August	0.6 ± 0.3[a]	0.0 ± 0.0[a]	8.2 ± 0.3
September	2.3 ± 1.0[a]	4.0 ± 1.2[a]	9.1 ± 0.2
October	5.4 ± 0.5[a]		9.7 ± 0.2

[a] Indicates significant difference from the control at $P \leq 0.01$.

lected in May, July, and September (Figure 4b). The radicle growth rate of Scots pine seedlings was inhibited by water extract made from bracken pinnules collected in May and September (Figure 4d). Fronds collected during the winter did not have any inhibitory effects.

DISCUSSION

This study presents evidence that volatile compounds in freshly collected bracken fronds have a significant inhibitory effect on seed germination at the beginning and end of the growing season. Root growth is inhibited during the whole growing season except for August. In contrast, Del Moral and Cates (1971) found that volatile compounds tested under laboratory conditions did not

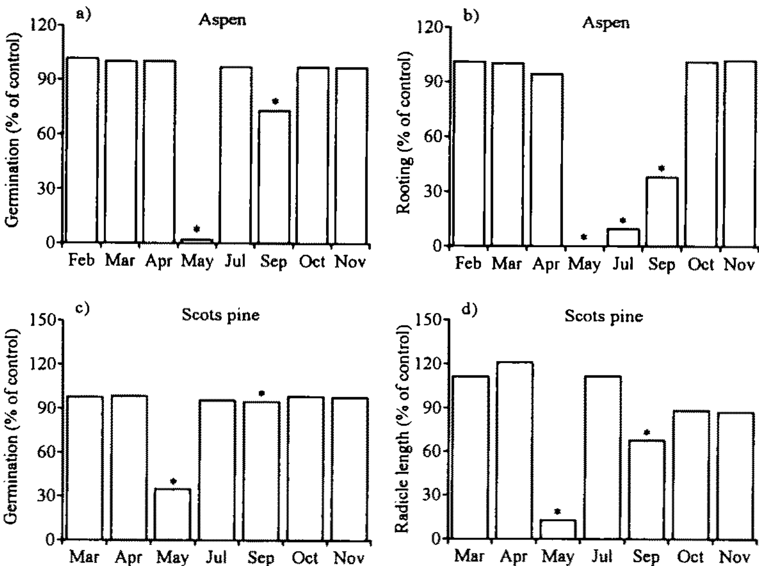

FIG. 4. Aspen (*Populus tremula*) (a) seed germination and (b) number of upright seedlings and Scots pine (*Pinus sylvestris*) (c) seed germination and (d) radicle length when germinated and grown in water extract made of bracken (*Pteridium aquilinum*) pinnules collected in Mönsterås, 1990–1992. Data are expressed as percentage of distilled water control. Samplings in July and September were made in 1992. *Significant difference from the control at $P \leq 0.01$.

have phytotoxic effects on radicle elongation of *Hordeum vulgare*. Inhibition of germination and growth of aspen by volatile compounds released from bracken pinnules was most pronounced in May, June, and September. Similar seasonal variation was observed in the seed-bed and water-extract bioassays, possibly because different chemicals found in bracken occur in different amounts seasonally. Hydrogen cyanide (Moon and Raafat, 1951; Cooper-Driver et al., 1977), phenolics (Glass and Bohm, 1969; Cooper-Driver et al., 1977), and terpenes (Alonso-Amelot et al., 1992) are found in high amounts in emerging croziers at the beginning of the growing season and decrease as the fronds mature. Concentrations of tannins, on the other hand, may increase at the end of the growing season (Moon and Pal, 1949; Cooper-Driver et al., 1977). Seasonal variation of chemical compounds by bracken has been previously hypothesized as a defense against herbivores (Lawton, 1976; Cooper-Driver et al., 1977) but could also be responsible for plant–plant interference, since these chemicals are known to be allelopathic (Rice, 1984).

When water-soluble compounds are released by bracken pinnules, their concentration depends on the extent of dilution. In the water extract, the compounds were more diluted since more water was added per unit dry weight of plant material than in the seed bed bioassay. This might explain why radicle growth of Scots pine seedlings was inhibited throughout most of the year when placed on a seed bed of bracken pinnules, while the water extract was inhibitory only in May and September.

Cutting the pinnules did not enhance the negative effects on seed germination compared with intact pinnules, and this obviously had no influence on the release of phytotoxic compounds. A possible explanation for differences between the results of Del Moral and Cates (1971) and this study is that we used more frond material per unit volume air.

Activated carbon is known to adsorb many inhibitory compounds (Yambe et al., 1992; Zackrisson and Nilsson, 1992; Mahall and Callaway, 1992) without directly affecting seed germination or seedling growth (Eliasson, 1959). By adding activated carbon to the volatile and seed bed bioassays, we attempted unsuccessfully to eliminate the inhibitory effects of bracken. It appears that the inhibitory compounds were not absorbed or were only incompletely adsorbed by the carbon, possibly indicating the presence of HCN (Guo et al., 1993) or other less lipophilic compounds.

Water extracts and leachates of litter, of green, senescent, and dead fronds, and of fronds placed on or mixed in soil previously have been shown to exert inhibitory effects on growth of seedlings of various species (e.g., Gliessman and Muller, 1972, 1978; Gliessman, 1976; Horsley, 1977; Taylor and Thomson, 1990). Conifer seeds germinated in bracken soil under field condition also have shown high mortality (Ferguson and Boyd, 1988). The results are sometimes contradictory and some test species are either unaffected or slightly stimulated by the presence of bracken, irrespective of method (Del Moral and Cates, 1971; Stewart, 1975; Nava et al., 1987). The differences could be due to either seasonal variation in the inhibition or different methodologies. Our study provides strong evidence that seasonality is important and that the peak of inhibition in May and June coincides with the start of the growing season when bracken still is immature and vulnerable to interference from other species. The increase in inhibitory effects in September appear to be due to transformation of natural products or an accumulation of inhibitory compounds that are released during decomposition following frond death.

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PHLOEM SAP COLLECTION FROM LETTUCE (*Lactuca sativa* L.): METHODOLOGY AND YIELD

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Abstract—Three methods to collect phloem sap on different lettuce lines were optimized and are described in detail. The success ratio for stylectomy of aphids was over 80% through the combination of a specially designed setup and electrical penetration graphs to monitor phloem sap ingestion. For unknown reasons on some lettuce lines stylets never showed sustained exudation. There were clear differences in stylet exudation between two aphid species on the same lettuce line. Honeydew collection in hexadecane made accurate quantitative analysis possible; samples were large and clean, but biotransformed. The EDTA chelation method produced large samples, but dilution, oxidation, and impurities from the wound surface reduced the reliability.

Key Words—Phloem sap, stylectomy, EDTA chelation, aphid-honeydew, Homoptera, Aphididae, *Lactuca sativa*, lettuce, plant resistance.

INTRODUCTION

The absolute, monogenic resistance of lettuce (*Lactuca sativa* L.) to the aphid *Nasonovia ribisnigri* (Mosley) (*Nr* gene) (Eenink et al., 1982; Reinink and Dieleman, 1989; Helden et al., 1993), is based on an early interruption of phloem sap uptake after the aphid stylets have reached the sieve tubes of the plant (Helden and Tjallingii, 1993; Helden et al., 1992). Possible resistance mechanisms are: (1) a mechanical blocking of the aphid stylets or the sieve tube

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after puncturing by the aphid, or (2) a difference in chemical composition of the phloem sap between susceptible and resistant lettuce (Helden and Tjallingii, 1993).

Phloem Sap Collection. Several collection methods of phloem sap have been described in the literature. Direct collection of phloem sap after incisions in the plant, as described for trees (Zimmerman, 1957), cucurbits (Richardson et al., 1982), and legumes (Pate et al., 1974), is not possible for lettuce because of the presence of large quantities of lactiferous ducts around even the smallest veins, which immediately exude latex at wounding. The remaining methods therefore are amputation of aphid stylets (stylectomy) (Downing and Unwin, 1977; Unwin, 1978; Fisher and Frame, 1984), collection of honeydew (Banks and Macaulay, 1964) and "facilitated exudation" through ethylene diamine tetraacetate (EDTA) chelation (King and Zeevaart, 1974; Girousse et al., 1991; Groussol et al., 1986).

The objectives of this study were: (1) the optimization of these three methods for lettuce, (2) a qualitative and quantitative comparison of the samples, and (3) comparison of the results for different lettuce lines and aphid species in relation to aphid resistance.

The detailed technical description of all three sap collection methods and their results may be useful in research on plant physiology and aphid–plant interactions in relation to host-plant resistance.

METHODS AND MATERIALS

Plants

The lettuce plants used in the experiments were of the lines Taiwan (susceptible, genotype *nrnr*), 411 (resistant, genotype *NrNr*) and two sets of isogenic lines RES (genotype *NrNr*) and SUS (*nrnr*) (Helden et al., 1993) and RES2 (*NrNr*) and SUS2 (*nrnr*). The second set is different from the first (apart from the source of the resistance gene) and was selected for partial resistance to *Myzus persicae* (Sulz.) (Reinink and Dieleman, 1989; Reinink et al., 1988). The culture of plants was as described by Helden et al. (1993). Plants were used in the 4 to 5 leaf stage (stylectomy) or in 6 to 8 leaf stage (honeydew and EDTA chelation).

Aphids. Mass culture and synchronized culture of adults of the aphids *N. ribisnigri* (biotype WN1), *M. persicae* (biotype WM1), and *Macrosiphum euphorbiae* (Thomas). (WMe1) (Reinink and Dieleman, 1989) were performed as described by Helden et al. (1993). Aphid rearing plants were *L. sativa* cv. Taiwan for *N. ribisnigri*, *Brassica napus* L. cv. Olymp for *M. persicae* and *L. sativa* cv. Snijsla for *M. euphorbiae*. For stylectomy we also tried *Aulacorthum solani* (Kltb.) and *Uroleucon sonchi* L. from a mass culture on *L. sativa* cv. Taiwan and originating from a local field population.

Stylectomy

Setup. Experiments were performed in the lab 22 ± 1°C, 60% relative humidity, and 4000 lux from HF fluorescent tubes. In some cases, the humidity was raised to over 95% using an ultrasonic humidifier. Plants were potted in small (5-cm), square pots and mounted in a specially designed setup. The abaxial side of the fourth (fully expanded) leaf of the plant was fixed over a cylindrical Perspex support in the center of the setup (Figure 1), which made it possible to turn the aphid target in every direction (arrows) while keeping it in the focus of a horizontally viewing stereomicroscope (not shown).

Electrical penetration graphs (EPG) (Tjallingii, 1988) were used to monitor the feeding behavior of the aphids. This method enables recording of different waveform patterns during penetration, which can be related to different aphid activities and stylet locations in the plant tissue (Helden and Tjallingii, 1993). Phloem sap ingestion occurs during waveform pattern E2 (Tjallingii, 1990). Six aphids were wired, and, via a choice switch, each aphid could be connected to an EPG amplifier (DC system, 1 GΩ input) (Tjallingii, 1988) coupled with a storage oscilloscope to monitor its feeding behavior. Stylets were cut the following day when aphids showed phloem sap ingestion pattern E2.

Stylectomy was performed with a high-frequency microcautery unit (48 MHz, ~25 W, Syntech) whose output was reduced to around 50% of maximum (output ~10–15 W), using a 0.2 sec pulse. Needles were prepared from 0.2-mm tungsten wire and electrolytically sharpened to produce a long (1.5 cm) tapered thin tip (Brady, 1965).

Needle Position. The aphid was approached frontally, carefully moving the tip a little beyond the labium. Just before amputation, the needle was slowly moved laterally against the narrowest part (at the second labial segment) of the proboscis (Figure 1, detail), firmly touching the proboscis, and causing a slight deflection of the needle tip and the proboscis. A foot switch activating the microcautery pulse was then pressed immediately. In some cases, it was necessary to bend the needle to reach the labium. Other needle locations on the proboscis were tried.

Phloem Sap Collection. A capillary (20-μl Microcaps, 52 mm long, outer diameter 0.97 mm, inner diameter 0.69 mm, with a tapered tip, broken at the outer diameter of 0.15 mm, and filled with silicone oil) was placed over the exuding stylets, which were positioned in the oil-filled part. The position of the upper oil meniscus was determined every 5–15 min. Volumes were estimated by the extension of the fluid column (precision 0.04 mm or 15 nl using a measuring eyepiece). An overestimation of volume and rate occurred when the oil did not completely fill the tapered end. When exudation stopped, the capillary was emptied in 100 μl of 50% methanol for sugar analysis. Samples were frozen at −80°C until analysis.

Statistics. Comparison between aphid species were made by Mann-Whitney U tests ($\alpha < 0.05$). Comparison among veins for each species were made using

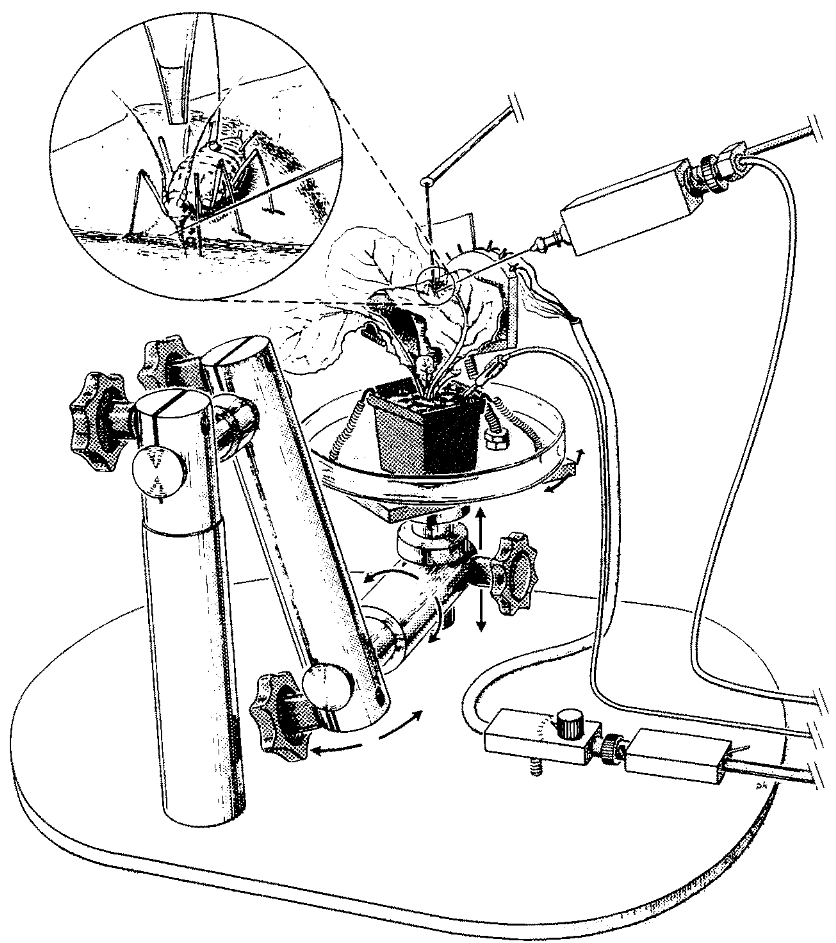

FIG. 1. Design of the stylectomy setup and microcautery unit. The plant can be turned in every direction (arrows). Six aphids can be connected over a choice switch to an EPG amplifier to record the EPG signal. Detail shows needle position and microcapillary just before amputation.

the Kruskal-Wallis test for multiple comparison ($\alpha < 0.05$). For tables and statistical tests concerning duration, sample size, and exudation rate, only values larger than zero were used and some discordant values were excluded with duration >200 min (five cases excluded), sample size >15 μl (one case), or rate >1 nl/sec, respectively (five cases).

Honeydew Collection

Honeydew was collected in the greenhouse at 22 ± 2°C and 70% relative humidity under continuous illumination. Honeydew collection cages were prepared from 5-cm Petri dishes modified after Eenink et al. (1984) (Figure 2). Five to 10 adults were placed in the bottom compartment (an empty base of a Petri dish) and left there for 24 h to allow settling and reproduction of the aphids on the leaf piece. The next day, the bottom part was replaced by a fresh one filled with 2 ml of *n*-hexadecane ($C_{16}H_{34}$, specific gravity 0.77 g/ml, melting point 18°C). At 24-hr intervals, the honeydew was collected in a capillary and stored at −80°C under *n*-hexadecane. Different aphid species were used, depending on the lettuce line (see Results). The top ring of the clip on cage was made into a second cage by closing it with nylon mesh (Figure 2, top compartment). Before and during a few collections on the lines RES, RES2, SUS, and SUS2, the top compartment was filled with 5–10 *N. ribisnigri* adults.

EDTA Chelation

Leaves were cut close to the base, and the cut surface was rinsed in water for a few seconds. Leaves were weighed and placed in 1.5 ml vials containing 0.8 ml of an EDTA solution buffered with 5 mM phosphate (pH 6). All vials were placed in a large translucent container (>95% relative humidity, 22 ± 2°C, and artificial illumination) for 18–24 hr. Different EDTA concentrations and pHs were tried. The time course of the sugar yield and the yield per gram of leaf and its relation to leaf age was determined. This method was compared to collection in darkness or collection with only a short (2-hr) EDTA exposure, after which leaves were transferred to a vial containing water only. Final collection was in 8 mM EDTA and 5 mM phosphate buffer at pH 6. Sodium metabisulfite $Na_2S_2O_5$ (5 mM) was used as an antioxidant.

Sugar Concentration. Total sugar concentration of the EDTA samples was determined using a colorimetric reaction with anthrone. After heating for 10 min at 90°C, the reaction mixture shows equal extinction per gram of sugar for different sugars (Handel, 1967) at 620 nm and therefore is a good measure of total sugar content.

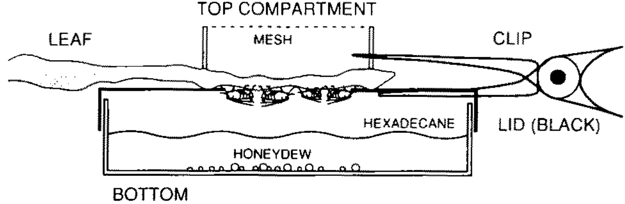

FIG. 2. Design of the honeydew collection cage.

Results of a more detailed chemical analysis of the samples are given elsewhere (Helden et al., 1994).

RESULTS

Stylectomy

A summary of the stylectomy is given in Tables 1 and 2. The results of 337 attempts were recorded, mainly on Taiwan with *N. ribisnigri* and *M. persicae*.

Needle Positioning. The optimal approach of the needle was from the front side of the aphid, amputating at the second labial segment. When the needle accidentally touched the aphid, it sometimes ended the penetration, but more

TABLE 1. OVERVIEW OF STYLECTOMY RESULTS

Result of attempt	*N*	%
Total number of attempts	337	100.0
E2 continued until microcautery	322	95.5
Succesfull amputation	284	84.3
Exudation from stump	245	72.7
Long exudation (> 1 min)	124	37.7
Samples > 1 μl	48	14.2

TABLE 2. OVERVIEW OF RESULTS PER APHID-PLANT COMBINATION[a]

	Aphid species						
	M. persicae		*N. ribisnigri*		Other		
Lettuce line	*N*	S. size	*N*	S. size	*N*	S. size	Total (*N*)
Taiwan	147	1.3 ± 0.4(54)	88	2.8 ± 0.4(47)	1		236
RES	19	##	**		27	##	46
SUS	13	##	12	##	6	##	31
RES2	0		**		0		0
SUS2	0		11	##	2	##	13
411	10	2.0 ± 1.5(2)	**		1	##	11
Total	189		111		37		337

[a] *N* = number of attempts, S. size = sample size of long exudations (> 1 min) in μl ± SE (number of samples), **Incompatible aphid-plant combination, ##No long exudation observed (sample size < 1 nl).

often disturbance was only shown in the EPG signal, which temporarily changed from waveform E2 (phloem ingestion) to E1 (stylets in sieve element, salivation but no ingestion (Prado and Tjallingii, 1994) (Table 1: E2 continued until microcautery). Usually E2 resumed after a few minutes, and the attempt could be continued. Sometimes the aphid raised and swayed its abdomen and moved its legs without a change in the EPG waveform.

Amputation. The overall success ratio for amputation was 84.3% (Table 1). Usually the proboscis was partially and the stylets completely cut, allowing the aphid to retract the severed proboscis and walk off leaving only the stylet stump. In one third of the amputations, the proboscis was also cut. The remnants could usually be removed with a fine brush; if not removable, the capillary was simply placed over them. In approximately 10% of all cases, the mandibulary stylets separated from the maxillary ones, bending away. When a pulse was fired but amputation failed it was usually attributable to inadequate contact between needle and proboscis.

Other places of the proboscis than the third segment were either more difficult to reach (proximal segments) or gave a lower success ratio of amputation (distal segments). Bending the needle with smooth curves did not affect the efficiency of the microcautery but made the positioning difficult. Stronger and/or longer pulses caused damage to the plant surface.

Exudation Success. Nearly all successful amputations were followed by some exudation from the stylet stump (245 of 284). When amputation was accidentally performed during an EPG waveform other than E2, exudation was never observed.

Duration of Exudation. Fifty percent of all exudations (124 or 245) stopped after approximately 10 secs, when a tiny droplet (diameter 0.05–0.15 mm, i.e., ± 1 nl) had been formed. When exudation continued for more than a minute, so beyond this one droplet (further referred to as long exudation), total sample volume was mostly smaller than 1 μl, with durations of 5–120 min (mean 53 min, median 30 min). Exudations producing volumes of 1–5 μl occurred irregularly (48) with durations of 30–200 minutes. One exceptionally long exudation of three days occurred.

Exudation Rate. The exudation rate (Table 3) of long exudations was very variable (range 0.05–0.8 nl/sec). Exudation rate usually decreased with time but sometimes increased temporarily, e.g., when the capillary was changed.

Miscellaneous Observations. The phloem sap flowing out of the stylet stump adhered to the wall of the capillary, quickly forming a complete disk with menisci at the oil interfaces and pushing the upper meniscus upwards when exudation continued. When the phloem sap droplets did not touch the wall of the capillary, they could be observed sinking to the lower oil-air interface. Exudation from levels below the top of the stump occurred in a few cases. Sometimes a few seconds' delay preceded the appearance of the phloem sap at

TABLE 3. DIFFERENCES BETWEEN *Nasonovia ribisnigri* AND *Myzus persicae* DURING STYLECTOMY ON TAIWAN LETTUCE[a]

Parameter	*M. persicae*			*N. ribisnigri*	
Attempts (*N*)	147			88	
Disturbed (*N*)	5			7	
Successful amputations (*N*)	135			79	
Stylets exuding (*N*)	128			68	
Exuding > 1 min (*N*)	58			48	
Stump length (μm)	154 ± 4	84	*	304 ± 10	65
Duration of exudation (min)	44.6 ± 6.2	57	*	73.5 ± 8.2	44
Sample size (μl)	0.84 ± 0.12	53	*	2.75 ± 0.38	47
Estimated speed (nl/sec)	0.33 ± 0.22	52	*	0.46 ± 0.04	43

[a]Values are mean ± standard error. Some discordant values excluded (see text). *Significant difference between species (α = 0.05, Mann-Whitney test).

the tip of the stump. Increasing the humidity did not increase exudation rate or duration. When the mandibular stylets separated from the maxillary ones, exudation seemed to stop earlier than when they remained joined.

Differences among Lettuce Lines. The success ratio for amputation or exudation did not differ among lines (Table 2). Taiwan lettuce showed long exudations quite often, while the lines RES, SUS, RES2, and SUS2 never exuded phloem sap for more than a few seconds. Line 411 (which contains the resistance gene) showed long exudations on two (out of ten) occasions.

Differences between Aphid Species. Tables 3 and 4 summarize a number of parameters to compare *N. ribisnigri* and *M. persicae* on Taiwan lettuce. Stylet stumps of *N. ribisnigri* were longer, showed higher exudation rates, and yielded larger samples. The success ratio for long exudations seemed somewhat higher for *N. ribisnigri* (47 of 88 versus 54 of 147 for *M. persicae*). *N. ribisnigri* preferred the larger veins, while *M. persicae* often probed in the lamina (Table 4).

Differences among Veins. The size, exudation rate, and duration of samples collected on different veins of Taiwan lettuce leaves showed no statistically significant differences (Table 4) but the number of replicates was low.

Honeydew

The honeydew collection method yielded 10–50 μl/cage/day. Adult aphids produced large quantities of offspring that contributed to the honeydew. On a successful aphid–plant combination, many droplets of very different sizes were collected. Honeydew droplets dropping in the oil sank to the bottom and fusion of individual droplets falling on top of one another occurred frequently. A

TABLE 4. RELATION BETWEEN PENETRATION SITE ON LEAF AND EXUDATION PARAMETERS FOR *Nasonovia ribisnigri* AND *Myzus persicae* ON TAIWAN LETTUCE[a]

Vein class	Total *N*	Duration (min)	*N*	Sample (μl)	*N*	Rate (nl/sec)	*N*
Myzus persicae							
Main vein	13	17 ± 4	5	0.6 ± 0.2	4	0.5 ± 0.1	4
Second order	38	33 ± 6	15	0.7 ± 0.1	14	0.3 ± 0.0	14
Third order	21	69 ± 20	8	1.4 ± 0.4	6	0.3 ± 0.1	6
Smallest visible vein	12	51 ± 35	5	0.8 ± 0.3	5	0.3 ± 0.1	4
Lamina	47	49 ± 14	16	0.8 ± 0.3	16	0.3 ± 0.0	16
Nasonovia ribisnigri							
Main vein	21	115 ± 22	9	4.0 ± 1.0	11	0.4 ± 0.1	10
Second order	36	75 ± 10	20	2.9 ± 0.5	21	0.5 ± 0.1	18
Third order	18	51 ± 14	11	1.5 ± 0.5	10	0.5 ± 0.1	10
Smallest visible vein	5	50 ± nd	1	3.9 ± 3.2	2	0.3 ± 0.1	2
Lamina	7	25 ± 5	2	0.7 ± 0.5	2	0.5 ± 0.2	2

[a]Values are mean ± standard error. Some discordant values excluded (see text). No significant differences among veins (Kruskal-Wallis multiple comparison, $\alpha = 0.05$).

minimum temperature of 21°C was necessary to avoid solidification of the hexadecane (melting point 18°C). Contamination by the aphids and exuviae that had dropped into the hexadecane was avoided by manual collection. Due to the affinity between the glass capillary and the aqueous honeydew, the droplets were automatically sucked into the capillary together with some oil. Other methods, such as filtration or centrifugation of the oil/honeydew suspension, were not tried, both for the risk of contamination and the need to wash the droplets from the Petri dish (dilution). Dry matter content of the collected honeydew was about 11.5%.

Differences among Aphid–Plant Combinations. Combination of aphid species and lettuce genotype gave different yields, due to differences in number of larvae produced in the cage and number of aphids dropping into the oil. On the lines RES and RES2, *N. ribisnigri* could not be used; *M. persicae* also, showed a lot of aphids dropping in the oil on these lines, as it did on the line SUS2, resulting in small yields. On these lines, *M. euphorbiae* gave better yields. No difference in aphid behavior or honeydew production was observed when *N. ribisnigri* was introduced into the adaxial top compartment (see Figure 2) on the resistant lines.

EDTA Chelation.

Optimization. The highest sugar yield was reached at 8 mM EDTA and decreased at higher concentrations (Figure 3A). A linear relationship appeared

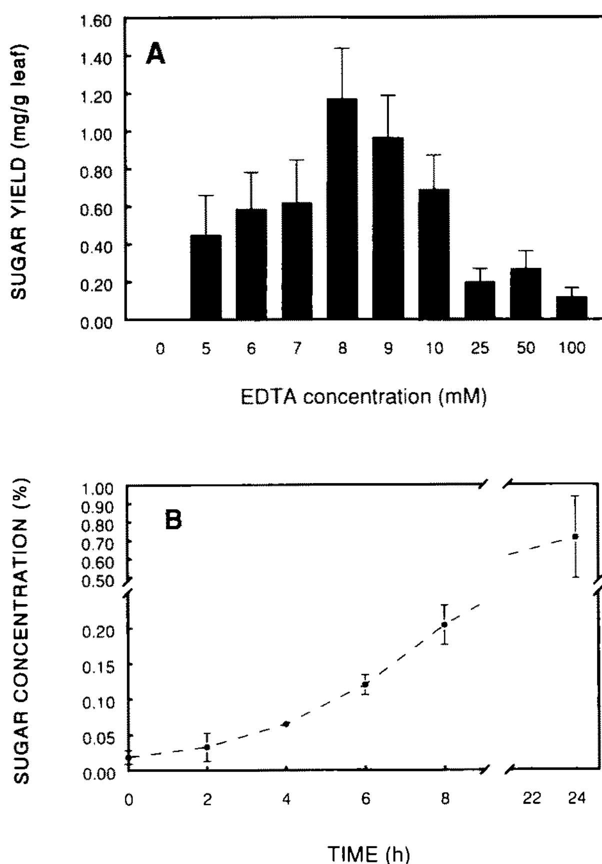

FIG. 3. Results of the EDTA chelation experiments on Taiwan lettuce. (A) Sugar yield per gram of leaf material at different EDTA concentrations. (B) Time course of sugar exudation.

between fresh-leaf weight and the total amount of sugar in the extract [sugar yield (mg) = 1.33 * leaf weight (g) − 0.06, r = 0.57)]. The young, not fully expanded leaves yielded 30–60% lower quantities of sugar per gram fresh weight compared to fully grown leaves. The time course of sugar yield showed that the rate of sugar exudation rose until the sixth hour of EDTA exposure and remained constant until the end of the collection period (Figure 3B). No relation was shown between pH and yield. When exudation was performed in the dark, yields were reduced by 50%. In experiments with a short exposure (2 hr) to

EDTA and subsequent collection in buffered water, no sugar could be found in the extract after 20 hr. Adding CO_2 (1%) to the container did not increase sugar yield. Sodium metabisulfite ($Na_2S_2O_5$) suppressed the visible browning of the extracts at 5 mM without affecting sugar yield.

Yield. EDTA chelation yielded samples of 0.2–0.6 ml/leaf. The sugar content of the samples was 0.3–0.9% (w/v, sucrose as standard) or around 1.3 (0.3–2.4) mg/g leaf. Large leaves yielded small samples with high sugar concentrations.

Differences among Lettuce Lines. Two differences were observed between resistant and susceptible plants: (1) The sugar yield (per gram of leaf) from the resistant plants was, on average, around 30% lower than on susceptible plants. (2) Leaves of resistant plants remained turgid during EDTA chelation while leaves of all susceptible plants lost turgor.

DISCUSSION

Stylectomy

Setup. The high success ratio of the stylet amputations can be attributed to the special setup with the aphid and the plant located in the center of the setup where they can be turned in every direction without moving them out of the focal point of the microscope. Disturbance of aphids during the positioning of the needle, which is considered a drawback of radiofrequency microcautery (Fisher and Frame, 1984) rarely occurred. The use of laser microcautery (Fisher and Frame, 1984) was not considered because the anatomy of lettuce makes it impossible to avoid damage to the plant. The observed behavior of some aphids moving their body and antennae (e.g., when the needle accidentally touched the aphid) was very similar to the effect of a larva walking by and touching the adult. The thin wall of the Microcaps capillary reduced refraction problems during positioning over the stylet stump. The thin tip makes it possible to move the stylet stump slightly to an optimal position with oil layers on both sides of the exuded phloem sap. The oil avoided evaporation and enabled estimation of volumes. Weighing could not be used because of the oil. Silicon oil does not interfere with the chemical analysis, and its density (0.98 g/ml) was expected to be close to that of the phloem sap. However, it appeared that the phloem sap is heavier than the oil.

Amputation. The success ratio for amputation is much higher than any result published so far using microcautery (Girousse et al., 1991; Rahbé et al., 1990; Chino et al., 1991) and comparable to the results of Kawabe et al. (1980), who used laser equipment, even though the aphids we used are smaller than the insect species they used (*Acyrtosiphon pisum* Harris, *Macrosiphum albifrons* Essig, and *Nilaparvata lugens* Stal.).

The narrowest part of the proboscis at segment 2 appeared the best place for amputation because its cuticle is less chitinized than the distal (darker) proboscis segments as used by Mentink et al. (1984). Amputation at these distal proboscis segments required higher output power from the microcautery and increased the risk of plant damage. The electrical contact between the needle and the proboscis should not be broken during the microcautery pulse. This was achieved by using needles with a long, flexible, tapered point and a firm lateral contact against the proboscis just before amputation. When only the very tip of the needle was positioned against the proboscis (Downing and Unwin, 1977), the electrical contact was often broken during the pulse, resulting in insufficient damage to the stylets. Sometimes little visible damage to the proboscis (low electrical resistance) still gave successful stylet (high resistance) amputation. The bleeding of the stylet stump below the top indicates that the maxillary stylets were sometimes cut or damaged at a lower level than the mandibulary stylets.

Differences among Aphid-Plant Combinations. The success ratio for amputation depended on the aphid species and host-plant suitability. Some species were more restless (*U. sonchii*) or had a very short proboscis when feeding (*A. solani*). Some aphid-plant combinations could not be used because of complete resistance (*N. ribisnigri* on RES and RES2) or had a low number of aphids showing E2 (e.g., *M. periscae* on SUS2 and RES2), apparently because of partial resistance (Reinink et al., 1988).

Exudation Success. The high success ratio for exudation from a successful amputation (over 90% on Taiwan) can only be achieved by the use of the EPG method to ascertain phloem feeding before amputation (Mentink et al., 1984). Without EPG recording, exudation success ratios of 0.05–55% have been reported (Winter et al., 1992; Girousse et al., 1991, Rahbé et al., 1990, Hayashi and Chino, 1986, Fisher and Frame, 1984). The extra work involved with the use of EPGs makes certain that the target is indeed ingesting phloem sap and that feeding is continued until amputation. We never observed exudation when waveform E1 (which always precedes the actual ingestion waveform E2) (Tjallingii, 1990) was present, confirming the hypothesis that no ingestion occurs during this waveform (Prado and Tjallingii, 1994). Even when the waveform E2 continued until the moment of amputation, some stylets did not produce phloem sap. It is possible that the stylets were translocated or were mechanically blocked during the amputation. The latter possibility seems to be confirmed by the short delay in the exudation that sometimes occurred after amputation, as if an obstacle had to be expelled with the first outflow.

Duration of Exudation. Many exudations stopped after about 5–20 sec, as was also reported by Girousse et al. (1991). Apparently the amputation itself or the outflow of the phloem sap caused a plant reaction, blocking the outflow very soon after amputation. This reaction could be triggered by the sudden decrease of turgor pressure in the sieve element cell (Peel, 1975) or by energy generated

during the pulse, which is transported down the stylets into the plant, either as electrical discharge or as heat. Callose formation or P-protein gelation (Girousse et al., 1991) have been mentioned but callose formation seems unlikely because of the speed of the reaction (< 10 sec). Tjallingii and Hogen Esch (1993) observed fibrous structures in the very tip of the food canal of cauterized stylets (which had shown only a short exudation), similar to P-protein bodies in the sieve elements. Long exudations apparently occur only when this short-term blocking mechanism fails completely or is incomplete. It is not clear whether these same mechanisms stop the outflow from long exudations (see hereafter).

Exudation Rate. The mean exudation rate of long exudations was 0.33 and 0.46 nl/sec for *M. persicae* and *N. ribisnigri*, respectively (range 0.2–0.8 nl/sec). Earlier reports show very variable results [Rahbé (1990): 0.1–0.3 nl/sec from *M. albifrons* Essig on lupine; Peel (1975): > 1 nl/sec from *Tuberolachnus salignus* Gmelin on willow; Tjallingii (1994): 0.09 nl/sec for *N. ribisnigri* on lettuce; Girousse et al. (1991): 0.07 nl/sec of *A. pisum* on *Medicago sativa* L.; Fisher and Frame (1984): 0.3 nl/sec for *T. salignus* and 0.03 nl/sec for *Quadrapidiotus astraeformis* Curtis, both on willow]. These differences are thought to be caused by variation in the length and diameter of the food canal in the stylet stumps (Fisher and Frame, 1984). However, the diameter of the food canal of a large aphid such as *T. salignus* (1–2 μm internal diameter) (Peel, 1975) is not very different from *M. persicae*, and diameters varied from 0.6 to 1.4 μm among 16 measured species (Forbes, 1977), which cannot explain all the variation in exudation rate. Large differences in exudation rate for the same species on different plants of the same genus [*T. salignus* on willow (Peel, 1975; Fisher and Frame, 1984), *N. ribisnigri* on lettuce (Tjallingii, 1994), and our data] show that plant quality plays an important role.

The exudation rate of long exudations was comparable to the estimated exudation rate directly after amputation (estimate based on exudation of the first droplet). This suggests an absence of blocking reactions. The slow decrease of the exudation rate over time can be caused by plant blocking reactions but also by evaporation from the air-exposed part of the stylet stump between the leaf surface and the capillary. This seems to be confirmed by Downing and Unwin (1977), who found a higher osmotic strength of the sap when collected in an oil-filled capillary (identical to our method) compared to sap collected in an oil droplet covering the whole stylet. The increase in exudation, which sometimes occurred after changing the capillary, and the fact that separation of the mandibular stylets from the maxillary ones caused exudation to stop sooner support this hypothesis and show that blocking can occur higher up the stylet stump, above the plant surface. Closing the air gap by placing the capillary on the plant surface failed because the oil flowed out of the capillary onto the leaf surface.

The mean exudation rate is much larger than the estimated feeding rate of around 25 pl/sec for *M. persicae* (Tjallingii, 1994). It shows that aphids actively

reduce the flow rate during feeding, possibly to avoid plant defense (blocking) reactions.

Differences among Lines. Unfortunately, we were unable to obtain usable stylectomy samples from the two sets of isogenic lines due to immediate blockage of the outflow. Since resistance and susceptible plants of both sets of near-isogenic lines possess this characteristic, it is possibly linked with the resistance gene, e.g., located on the same chromosome. Apart from the source of resistance gene (*L. virosa*), the two sets are genetically different. From line 411, which contains the resistance gene, some samples could be collected. Mentink et al. (1984) also reported amputated stylets producing sap (duration not specified) on a resistance lettuce line.

Differences between Aphid Species. N. ribisnigri is bigger than *M. persicae* and produced longer stylet stumps. The food canal of *N. ribisnigri* seems somewhat larger than reported for *M. persicae* [0.7 μm halfway along the stylets (Forbes, 1977)]. The diameter of the food canal [TEM pictures of *N. ribisnigri* (Tjallingii and Hogen Esch, personal communication)] varied from 0.5 × 0.8 μm (oval) near the tip of 1.1 μm (round) just above the leaf surface, and the salivary canal from 0.3 to 0.4 μm at the same locations respectively. Data are incomplete and exact comparison is difficult. This might explain the differences in exudation rate, duration, and sample size, but aphid–plant interactions can play a role. Salivation into a sieve element during E1 (Prado and Tjallingii, 1994) could prepare the sieve element for sap flow (e.g., injection of anti-coagulants) by suppressing plant defense (blocking) reactions. Efficacy could differ among species or aphid–plant combinations. *N. ribisnigri* might be better adapted to Taiwan lettuce than *M. persicae* because it was reared on this plant. This is supported by the fact that the higher outflow rate found with *N. ribisnigri* apparently did not cause exudation to stop sooner, which would be expected if plant reactions play a role in the termination of long exudations.

N. ribisnigri preferred the main veins and larger side veins, whereas *M. persicae* penetrated more often on the lamina (Table 4), presumably into minor veins. This might indicate a difference in suitability or chemical composition (Rahbé et al., 1990).

Differences among Veins. The sieve elements of different veins might react differently to penetration by stylets. However, for each aphid species, exudation rate, sample size, and duration of exudation did not differ significantly among veins (Table 4). Chemical composition can be different among different plant parts (Rahbé et al., 1990).

Summarizing, it seems that the differences observed between the two species on the same plant in exudation rate, duration, and sample size are caused by a difference in aphid–plant interactions rather than plant characteristics or stylet dimensions.

Honeydew

The collection method described here is comparable to that of Banks and Macauley (1963) and Fisher et al. (1984). Although manual collection makes the method rather time-consuming, it yields large quantities of honeydew (milliliters), making quantitative analysis of chemical compounds possible. Hygroscopic uptake or evaporation of water was prevented by the oil layer. Fisher et al. (1984) suggested a decrease of volume due to evaporation of 10% in 2 h during collection in hexadecane, but this was concluded from osmolality measurements, ignoring possible osmolality changes due to continued enzymatic activity in the droplets (Eschrich and Heyser, 1975; Helden et al., 1994). Although unlikely, it is possible that some apolar components did dissolve in the very apolar hexadecane. Banks and Macaulay (1964) reported a specific gravity of 1.040 g/ml for the honeydew. It is therefore not clear why the droplets floated just under the surface of their Castor motor oil (0.89 g/ml), but sank immediately in the lighter hexadecane (0.77 g/ml). The dry matter content of the honeydew was much lower than measured for phloem sap (Helden et al., 1994), indicating important changes made by the aphids.

Differences among Aphid–Plant Combination. The number of aphids that dropped into the oil differed among species and lettuce lines. *N. ribisnigri* does not produce honeydew on the resistant lines (Van Helden et al., 1993), but even the partial resistance to *M. persicae*, which is present in the lines RES2 and SUS2 (Reinink et al., 1988), caused an increased restlessness and therefore more aphids dropped in the oil and honeydew yield was very low.

When studying aphid resistance, finding a suitable aphid–plant combination can be difficult, and different aphid species will surely bioprocess the phloem sap differently or feed on other sieve elements.

EDTA Chelation

The method of EDTA chelation, as described by King and Zeevaart (1974), is applicable for lettuce. However, the optimal conditions for lettuce are obviously different from those for *Perilla crispa* Thunb. Light exposure of the leaves during collection almost doubled the sugar yield. A temporary exposure to EDTA was apparently not enough to establish exudation for long periods. Adding carbon dioxide, as used by Tully and Hanson (1979), to reduce evaporation from the leaves did not increase the sugar yield.

It took a few hours to reach the highest rate of exudation, which then continued for a long time with no noticeable decrease (Figure 3B) (Groussol et al., 1986). The influence of the uptake and evapotranspiration of the collection fluid through the leaf on sugar yield is unclear. This method produces large quantities of sugar with very little work involved. One plant with five or six

leaves (total mass 6–8 g) yielded around 9 mg of sugar thought to be equivalent to at least 45 μl of phloem sap (assuming 20% sucrose). Disadvantages are the strong dilution of the samples and their unknown contamination with substances from the cut surface of the petiole or the submerged part of the petiole, which showed some tissue degradation. No large-scale contamination of the samples by lactiferous ducts was observed. Latex was released immediately after excision but stopped soon and was washed off by rinsing in water. It is unknown whether major physiological changes of phloem sap occur due to the excision of the leaves.

Difference among Lines. The lower sugar yield on the resistant lines may be caused by the fact that the leaves of resistant plants were slightly smaller. In addition to their lower weight, these smaller leaves may have been physiologically younger and therefore give a lower yield per gram of leaf material. Whether the absence of turgor loss on the leaves of the plants 411, RES, and RES2 compared to Taiwan, SUS, and SUS2 has any direct relation with the mechanism of aphid resistance is not clear. However, it may have a direct relation with the lower sugar yield.

CONCLUSIONS

The three methods produce very different samples, both in quantity and in quality. Stylectomy is very laborious. Although the success ratio of amputation and exudation was exceptionally high, it yielded only very small (but presumably pure) samples, often too small for analysis. The number of cut stylets yielding a usable sample is very unpredictable, and certain plants show no long exudations, in spite of the fact that the aphids show sustained phloem sap ingestion. Honeydew collection produces relatively large samples. A good aphid–plant combination is necessary. Collection in hexadecane makes quantification of compounds possible. EDTA chelation produces large but very diluted samples with unknown contaminants. Excision might influence the phloem sap composition.

Which method is preferred depends largely on the intended use of the samples. For major compounds of the phloem sap, for which sensitive microanalysis methods are available [sugars, amino acids (Van Helden et al., 1994)] the very pure stylectomy samples are optimal. For identification of unknown minor compounds, stylectomy samples are simply too small. The other methods yield larger samples in terms of phloem sap equivalents, but their reliability is unclear. None of these methods is ideal when studying aphid resistance caused by differences in phloem sap composition as in our model. When resistance is induced by aphid attack, collection without using aphids (EDTA chelation) may not show any significant differences. Using other aphid species (honeydew or

stylectomy) might not induce the defense reaction. To gain more insight into the differences among samples from the three collection methods, a chemical comparison was performed, which will be reported in Van Helden et al. (1994).

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PHLOEM SAP COLLECTION FROM LETTUCE (*Lactuca sativa* L.): CHEMICAL COMPARISON AMONG COLLECTION METHODS

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Abstract—The chemical composition of phloem sap from lettuce, collected by three different methods, was compared. Phloem sap from stylectomy samples contained sucrose and 14 amino acids. Honeydew and EDTA chelation samples showed considerable breakdown of sucrose into fructose and glucose, several additional amino acids, and large differences in relative concentrations of amino acids, when compared to stylectomy samples. Honeydew contained considerable amounts of other oligosaccharides, and few proteins in low amounts, while EDTA showed many proteins. HPLC chromatograms showed numerous unidentified secondary plant compounds in honeydew and EDTA samples. Comparison of phloem sap samples from near-isogenic susceptible and resistant lines showed no relation of phloem sap composition with monogenic resistance to the aphid *Nasonovia ribisnigri*.

Key Words—Phloem sap, stylectomy, EDTA chelation, aphid-honeydew, *Lactuca sativa*, lettuce, sugars, amino acids, secondary plant metabolites, plant resistance.

INTRODUCTION

The absolute, monogenic and dominant resistance (*Nr* gene) of lettuce to the aphid *Nasonovia ribisnigri* is based on the interruption of phloem feeding (Hel-

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den and Tjallingii, 1993; Helden et al., 1992, 1993) probably caused by a difference in chemical composition of the phloem sieve element sap.

The composition of the phloem sap of lettuce (*Lactuca sativa* L.) can be derived from samples collected by stylectomy (Downing and Unwin, 1977), from aphid honeydew (Banks and Macaulay, 1965), and from samples collected by facilitated exudation ethylene diamine tetraacetate (EDTA) chelation (King and Zeevaart, 1974). In a previous paper, we reported on the optimization of these methods for maximum yield (Helden et al., 1994), without comparing their chemical composition. The collection of phloem sap by stylectomy failed on two sets of nearly isogenic resistant and susceptible lettuce lines [lines in one set differ only in the *Nasonovia* resistance gene (Helden et al., 1994)]. On a susceptible lettuce line Taiwan, stylectomy was successful, and samples of three collection methods are compared in this paper.

Chemical comparisons between stylectomy and EDTA chelation sap samples are available for several plant species (Weibull et al., 1990; Girousse et al., 1992). So far, only Rahbé et al. (1990) compared these three methods simultaneously, concentrating on sugars and amino acids. Although stylectomy samples are considered as the best representation of real phloem sap, this technique does not work on all plants (Helden et al., 1994) and sample size is very limited (usually <1 μl) (Fisher and Frame, 1984; Helden et al., 1994). The reliability of samples from other methods is unclear. EDTA chelation samples from excised leaves are contaminated with compounds released from the wound surface, and honeydew is a phloem sap that is biotransformed by the aphid used for collection.

The objectives of this study were: (1) qualitative comparison of three collection methods (stylectomy, EDTA chelation, and honeydew) for sugars, amino acids, proteins, and UV-absorbing secondary metabolites; and (2) comparison of phloem sap samples of nearly isogenic resistant and susceptible lines.

METHODS AND MATERIALS

Samples. In an earlier paper (Helden et al., 1994) we reported on the methodology and yield of phloem sap collection methods. Samples were collected from lettuce line Taiwan for comparison of collection methods, and two sets of near-isogenic lettuce lines: RES (genotype *NrNr*) with SUS (*nrnr*) and RES2 (*NrNr*) with SUS2 (*nrnr*), respectively, for comparison of resistant and susceptible plants (Helden et al., 1993). These two sets have distinctly different genetic backgrounds apart from the source of the resistance gene, which originates from *L. virosa* L..

Stylectomy samples (phloem sap volume 0.1–1 μl, stored in 50% MeOH or 2% sulfosalicylic acid) were available from lettuce cv. Taiwan using two aphid species, *Nasonovia ribisnigri* and *Myzus persicae* (Sulzer). Honeydew

samples were collected in hexadecane. Samples from lettuce line Taiwan were collected from the aphids *M. persicae*, *N. ribisnigri*, and *Macrosiphum euphorbiae* (Thomas). On the other lines, honeydew was collected from *M. persicae*. EDTA samples were collected in 8 mM EDTA, pH 6, yielding 0.2–0.6 ml/leaf, total sugar content 0.3–0.9% or around 1.3 mg/g of leaf. Samples were stored at −80°C (honeydew and stylectomy) or −20°C (EDTA) before analysis. All tests were done with two to four independent replicates per treatment.

Sugar analysis was performed with a high-performance anion-exchange chromatography system (Dionex CarboPac PA-1 Column, PED/PAD-Detector) as described by Gruppen et al. (1992), concentrating on sucrose, glucose, and fructose. Samples from stylectomy (±0.5 μl in 100 μl 50% MeOH) were lyophilized and redissolved in 1 ml H_2O shortly before analysis. EDTA samples were diluted 50-fold and honeydew samples 1000-fold before analysis. Injection volume was 20 μl.

Amino acid analysis was performed with an ion-exchange amino acid analyzer (Beckman). Proteins were removed by addition (EDTA samples) or dilution (stylectomy, honeydew) in 2% sulfosalicylic acid and centrifugation. Injected samples (100 μl) contained 2.5 μl stylectomy samples (several collections pooled), 5 μl honeydew or undiluted EDTA samples. Some other products (NH_3) were also detected.

Protein Analysis. Proteins in EDTA samples (5 ml) and honeydew (0.5 ml) from the lines RES and SUS were precipitated by addition of acetone up to 80% (Joosten, 1991) and centrifugation. SDS-PAGE was performed on a 12% minislab gel with a 4% stacking gel. Proteins were silver-stained according to Morrissey (1981).

Secondary Plant Chemicals. EDTA and honeydew samples were separated using a 250 × 4 mm C_{18}RP-HPLC (nucleosil 120-5 C_{18}) column (Macherey-Nagel) with a 30-mm precolumn using water–acetonitrile or water–methanol gradients or isocratic mixtures with trifluoracetic acid (TFA, 0.1%, pH 2) or tetrabutylammonium ions (added as chloride or hydrogen sulfate, 0.5%, pH 5). Flow rate was 1 ml/min and run time 35–40 min. UV detection at 200–400 nm was performed with a diode array detector (Waters 911). Injection volumes were 20 μl of undiluted EDTA samples or 10-fold diluted honeydew. Stylectomy samples were not available.

RESULTS

Sugars

The main sugar in most samples was sucrose (Figure 1A and B), with the exception of some honeydew samples. Fructose and glucose were identified in nearly all sample types from all lines.

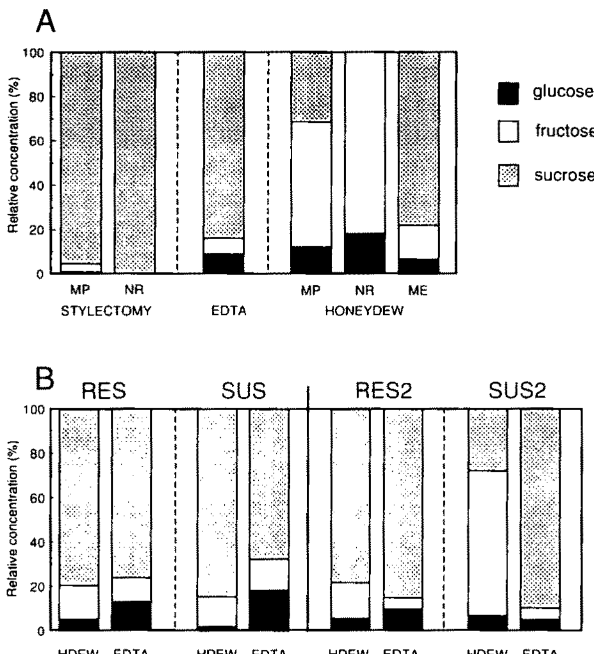

FIG. 1. Relative sucrose, fructose, and glucose concentrations of samples from different collection methods. (A) comparison on Taiwan lettuce using stylectomy of *Myzus persicae* (MP) and *Nasonovia ribisnigri* (NR), EDTA chelation, and honeydew of MP, NR, and *M. euphorbiae* (ME). (B) Comparison of two sets of isogenic lines, using *M. persicae* honeydew (HDEW) and EDTA chelation.

Differences among Collection Methods. The total sugar concentration in the stylectomy and EDTA samples was 13.9–18.0% (mean 16.2%, $N = 4$) and 0.19–0.71% (mean 0.41%, $N = 28$), respectively. In honeydew, the combined sucrose, fructose, and glucose quantity was 3.4–6.2% (mean 5.1%, $N = 24$).

In the stylectomy samples, nearly all sugar was sucrose and only traces of glucose and fructose occurred (Figure 1A, left columns), while the EDTA collected samples showed fructose and glucose in roughly equal shares of 0–15% each of the total sugar content (Figure 1A and B). Variation in profiles between replicates was larger than among lines. Honeydew samples showed little variation between replicates, but variation among aphid–plant combinations was considerable (Figure 1A, right bars; and Figure 1B). Fructose concentrations were three to eight times higher than glucose (Figure 1A and B). Large amounts of other unidentified oligosaccharides possibly representing polymerization series of glucose-containing oligosaccharides (Fisher et al., 1984; Ammeraal et al.,

1991), were found, but not quantified, amounting to as much as 50% of the total peak area of chromatogram. A small unidentified peak, probably a monosaccharide with a retention time between those of glucose and fructose, was sometimes observed.

Differences among Aphid Species. Stylectomy samples of *M. persicae* and *N. ribisnigri* showed nearly equal concentrations and profiles of sugars. Differences in sugar profiles among honeydew samples of aphid species were considerable (Figure 1A, right bars). *N. ribisnigri* honeydew from Taiwan showed no sucrose at all. The combined sucrose, fructose, and glucose quantity in honeydew from *N. ribisnigri* on Taiwan (5.3%) was higher than for *M. persicae* (3.5%) and *M. euphorbiae* (3.9%).

Differences among Lettuce Lines. Figure 1B shows the results of a comparison of the two sets of isogenic lettuce lines using EDTA samples and honeydew of *Myzus persicae*. Stylectomy samples were impossible to obtain from these lines (Helden et al., 1994). The sugar profiles of the EDTA samples of all lines were identical. The lines RES and RES2 showed total concentrations of 0.19% and 0.22%, respectively, against 0.34, 0.52, and 0.49% on lines SUS, SUS2, and Taiwan. The honeydew samples showed a high proportion of fructose on line SUS2 and on Taiwan, (Figure 1A). The combined sucrose, fructose, and glucose quantity in honeydew from isogenic lines was comparable (mean 5.2%, range 4.2–6.2%), a little higher than on Taiwan (4.2%).

Amino Acids

Differences among Collection Methods. The total concentrations of amino acids in the original samples were 54 mM for honeydew (range 32–72 mM, $N = 24$), 5.4 mM for EDTA samples (range 2.2–8.8 mM, $N = 30$), and an estimated 130 mM (range 125–168 mM, $N = 4$) for stylectomy samples. Amino acid profiles of stylectomy samples (Figure 2A) showed 14 amino acids. The main amino acids were glutamine, glutamic acid, serine, and γ-isobutyric acid (GABA). Ammonia was also present in fairly high concentrations (6 mM). Proline was not detected. No unexpected peaks were detected.

EDTA samples on Taiwan (Figure 2B) consistently showed substantial quantities of one extra amino acid (arginine, rel. conc. 2%) and trace quantities of α-aminobutyric-acid, methionine, and tryptophan. Compared to stylectomy samples, they showed an increase in the glutamine/glutamic acid ratio and lower relative quantities of serine, tyrosine, and especially GABA and ammonia.

Honeydew samples of different aphid species on Taiwan (Figure 2C) showed the same amino acids as the stylectomy samples plus traces of citrulline and arginine. Relative amounts of alanine, tyrosine, GABA and ammonia were strongly reduced and phenylalanine was somewhat reduced for all aphid species.

Differences among Aphid Species. Amino acid levels and profiles in stylectomy samples of *N. ribisnigri* and *M. persicae* on Taiwan were almost iden-

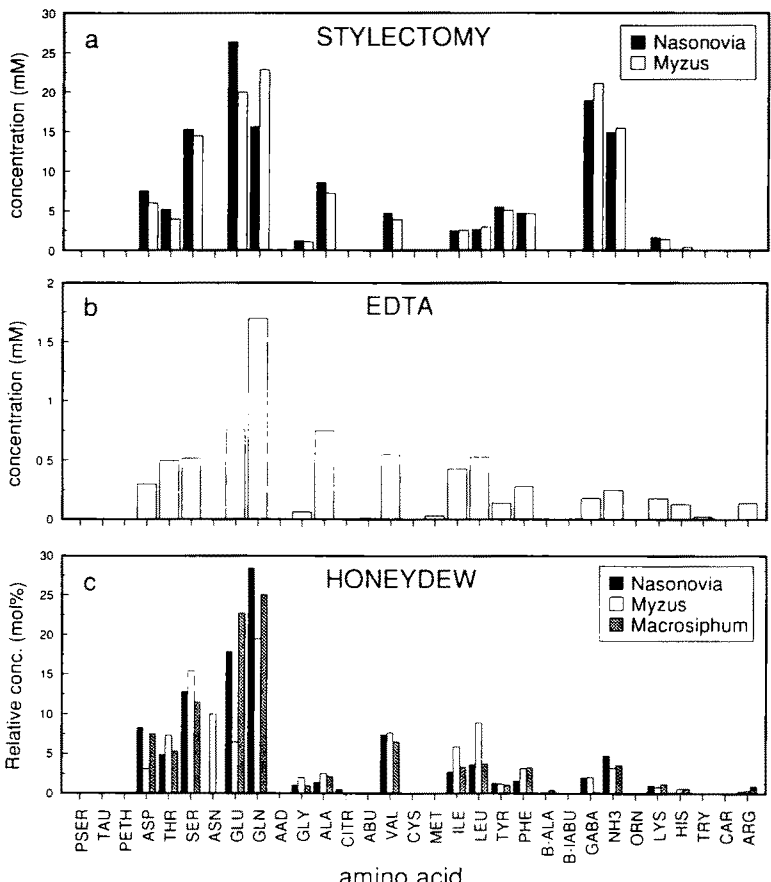

FIG. 2. Amino acid composition of phloem sap samples from Taiwan lettuce using different collection methods. (A) Absolute concentrations of stylectomy samples of *Myzus persicae* and *Nasonovia ribisnigri*. (B) Absolute concentrations of EDTA chelation samples. (C) Relative concentrations of honeydew of *M. persicae*, *N. ribisnigri*, and *M. euphorbiae*.

tical (Figure 2A). Honeydew amino acid profiles for three aphid species on Taiwan were similar (Figure 2C), although the total concentration varied from 32.4 (*N. ribisnigri*) through 46.9 (*M. persicae*) to 72.8 mM (*M. euphorbiae*). Honeydew of *M. persicae* showed asparagine (two of three replicates) and lower relative quantities of glutamic acid and higher concentrations of leucine.

Differences among Lettuce Lines. Comparison of EDTA samples of isogenic lines showed similar amino acid profiles for all lines (Figure 3) apart from

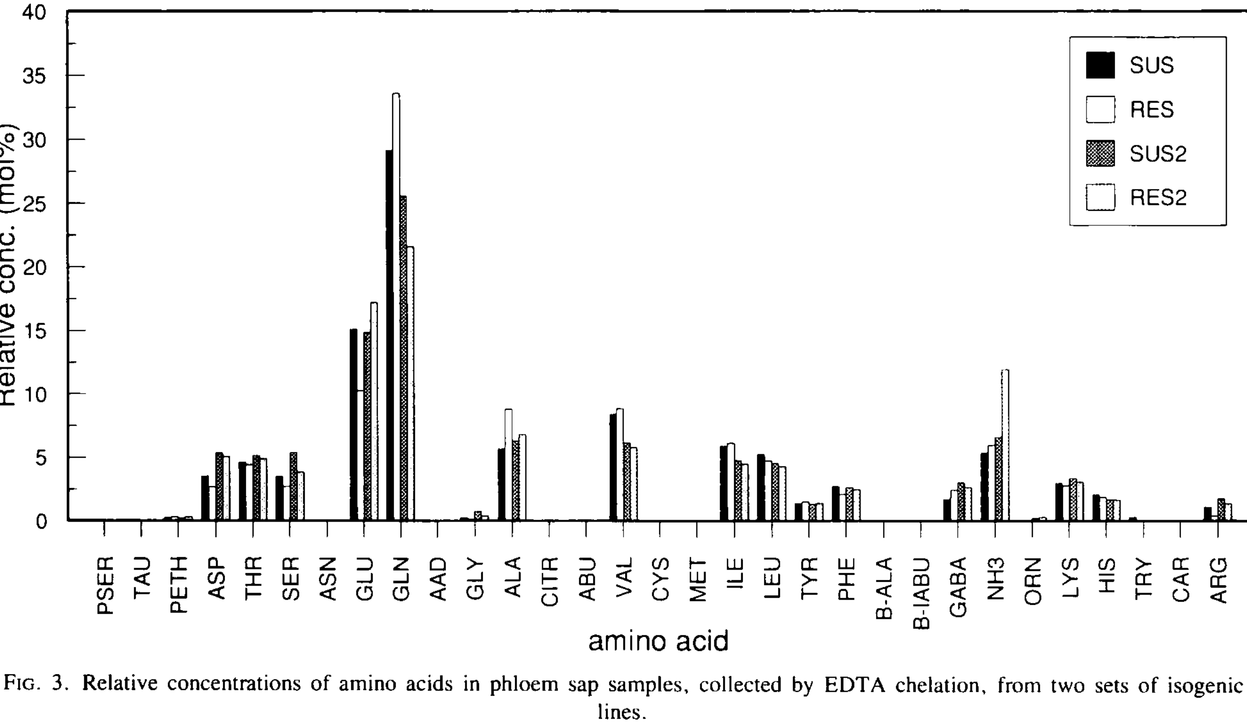

FIG. 3. Relative concentrations of amino acids in phloem sap samples, collected by EDTA chelation, from two sets of isogenic lines.

the presence of trace amounts of ornithine in RES2 and SUS2. Sometimes minor compounds were not detected (glycine, methionine, tryptophan), but no relation with resistance appeared. The lines RES, RES2, SUS, and SUS2 differed from Taiwan by the presence of traces of phospho ethanolamine. The total concentrations of amino acids in the EDTA samples were 50% lower on the resistant lines (RES 2.3 mM, RES2 3.6 mM) compared to the susceptible lines (SUS 6.3 mM, SUS2 7.1 mM, Taiwan 7.4 mM).

Proteins

EDTA and *M. persicae* honeydew samples of the lines RES and SUS showed very low amounts of protein (EDTA: 20 μg/ml, honeydew: 80 μg/ml). SDS-PAGE showed a large number (>30) of proteins in the range of 50–200 kDa in the EDTA samples. Honeydew contained only a few proteins (5–10), most of which were also found in the EDTA samples. No difference was observed between resistant and susceptible plants.

Secondary Plant Compounds

The results from our analysis of honeydew and EDTA samples showed many peaks that were not primary compounds (sugars, proteins, or amino acids) as judged by their UV spectrum and retention times. A number of them probably represented secondary plant compounds. No attempt was made to identify individual compounds, but we concentrated on differences between resistant and susceptible lines. Differences between EDTA samples and honeydew samples from Taiwan, SUS, and RES were mainly in the ratio of different peaks. No consistent differences were observed between resistant and susceptible plants.

DISCUSSION

Sugars

Stylectomy. The stylectomy samples show that sucrose is the only sugar present in the phloem sap of lettuce, as expected for most plants (Ziegler, 1975). The small quantities of fructose and glucose occurring in the stylectomy samples of *M. persicae* are probably the result of contamination of the sample by microorganisms (invertase) during collection. Invertase is not present in the phloem sap (Ziegler, 1975). The size of the stylectomy samples was difficult to determine exactly (Helden et al., 1994). Therefore, our measurements are only an estimate of the exact quantity of sugars. Our findings (16.2%) correspond well with an expected value of 15–20%.

EDTA. The near-equal amounts of fructose and glucose in the EDTA samples show the result of sucrose breakdown. Invertase activity in the sample can

occur due to microbial contamination or release of plant enzymes from the wound surface of the leaf. Although the sugar concentration in the EDTA samples is much lower than in honeydew, EDTA samples are a better representation of the phloem sugars (see below), and they are also easier to collect. Assuming 16% sugar (as sucrose) in the real phloem sap, EDTA samples are diluted approximately 40-fold.

Honeydew. Honeydew sugar composition is very different from phloem sugar due to biotransformation by the aphid, the combined activity of invertase from the aphid alimentary tract (Srivastava and Auclair, 1962b), and biotransformation. The resulting total sucrose/fructose/glucose content of the honeydew (5%) is low compared to the stylectomy samples. A higher concentration was expected (Lamb and Kinsey, 1959; Peel, 1975). Sometimes a total breakdown of sucrose in the honeydew occurred with nearly all glucose missing (e.g., honeydew of *N. ribisnigri* from Taiwan). Reports on honeydew sugars from "dry" collections nearly all show sucrose, but a total absence of sucrose has been reported as well (Auclair, 1963; Lamb and Kinsey, 1959). Enzymatic activity (invertase) in the honeydew has been reported (Srivastava and Auclair, 1962b; Rahbé et al., 1994). Fisher et al. (1984) suggested that after invertase action, a large proportion of the glucose is transformed by the aphid to glucose-containing oligomers for osmoregulation. This is in line with the observed predominance of fructose over glucose and the presence of large quantities of higher-order oligosaccharides in honeydew samples (polymerization series of five to seven peaks, retention times suggest one glucose unit extra for each peak). Identification and quantification of these oligomers is difficult, but the peak area of these compounds suggests total concentrations of up to 5% (w/v) (Ammeraal et al., 1991) or 50% of all sugars present. It is not clear whether the sucrose breakdown and glucose polymerization occur exclusively in the aphid alimentary tract or continue after excretion. The increase in osmolality of honeydew droplets as observed by Fisher et al. (1984) (see also Helden et al., 1994) suggests that invertase activity does continue. Enzymatic activity in the honeydew could also be caused by microbial contamination, which is prevented by the hexadecane cover, but might occur in the aphid alimentary tract (Grenier et al., 1994; Srivastava and Auclair, 1962a).

Differences among Lettuce Lines. The observed difference in sugar concentration between EDTA samples of resistant and susceptible plants was not reflected in the honeydew samples. This suggests that the difference is caused by variation in the efficiency of the EDTA collection method rather than a different sugar concentration in the phloem sap (also confirmed by the equally lower amino acid concentration (see below). However, a genetic coupling seems to exist, since resistant lines of both sets possess this trait (Helden et al., 1994), possibly as a leftover from the introduced part of the *L. virosa* genome (Eenink et al. 1982a,b).

Differences among Aphid Species. The differences in honeydew sugars among aphids on Taiwan are remarkably large, as are the differences between lettuce lines. This suggests an extensive difference in biotransformation of the phloem sugar by differences in invertase activity (Srivastava and Auclair, 1962b) and glucose utilization (for assimilation and by polymerization) among aphid species. Another possibility is that the amount of phloem sap ingested and, therefore, the degree of utilization is very different, which could be checked by a comparison of honeydew production.

Summary. For sugars, only stylectomy gives a good representation. EDTA samples are an acceptable substitute, although collection efficiency can differ among lines. Honeydew sugars cannot be compared to real phloem sap. Sugar composition or concentration in samples from different lettuce lines cannot explain the resistance.

Amino Acids

Stylectomy. The estimated 125 mM of amino acids in the phloem sap is in the same range as found for most other plants (Ziegler, 1975; Rahbé et al., 1990, 1994; Riens et al., 1991). The resemblance in amino acid profiles from stylectomy samples of the two different aphid species seems self-evident. Still, differences between species could have occurred due to different preparation of a sieve element prior to sap ingestion and thus stylectomy (Prado and Tjallingii, 1994) or different aphid species selecting different sieve elements for feeding (Helden et al., 1994). More sensitive methods for amino acid analysis (Riens et al., 1991) would have allowed analysis of individual stylectomy samples and better quantification of trace amounts. Differences between samples may have been reduced by the pooling of samples.

The presence of relatively large quantities of GABA in the stylectomy samples is somewhat surprising. Chino et al. (1991), Rahbé et al. (1990), and Girousse et al. (1991) claim GABA to be an artifact of the EDTA extraction method. GABA has been found in honeydew on many occasions (see Auclair, 1963). So far, the relative amount of GABA reported in stylectomy samples has always been lower than in EDTA samples. Our findings suggest that GABA is one of the major amino acids present in lettuce phloem sap, although our identification is only based on retention times. The concentration of ammonia in the stylectomy samples is higher than expected; other reports showed lower amounts (Kuo-Sell, 1989; Weiner et al., 1991). This may be explained by our collection method, which prevents evaporation. Contamination by atmospheric ammonia was prevented by the oil layers, so the ammonia is either present in the phloem sap or is a result of breakdown of nitrogenous compounds during collection and storage.

EDTA. It is not evident from our results that the amino acids found in EDTA samples are of phloem origin and not from other plant parts. The relative concentration of amino acids in the phloem sap is usually not more than a factor

of 2 different from that of the cytosol (Riens et al., 1991; Winter et al., 1991; Weiner et al., 1991). However, it seems unlikely that, except for macromolecules such as proteins, some compounds present in the phloem sap would exude (sugars) and others not. This is confirmed by concentrations of both sugars and amino acids being 50% lower in the RES and RES2 samples. The presence of arginine and traces of three other amino acids, which were not found in stylectomy or honeydew samples, suggests that contamination occurs, although amounts might have been under the detection limit in the stylectomy samples. An increase in the glutamine/glutamic acid ratio in EDTA (and honeydew) samples has been reported by several authors (Weibull et al., 1990; Girousse et al., 1991). The EDTA samples might differ from the other collection methods because the phloem sap is collected at other locations on the plant, which might have different amino acid profiles. This was shown for oat (Kuo-Sell, 1989), but not for lupin (Rahbé et al., 1990).

Honeydew. Honeydew amino acids are the result of utilization and biosynthesis of new amino acids (Sasaki et al., 1990). Total concentrations in the honeydew are 50–75% lower than in the phloem sap. The volume of the excreted honeydew is supposedly less than the actual ingested volume. Tjallingii (1994) suggests around 50%, but the difference, mainly due to evaporation, will be variable. This shows that a very large proportion is utilized by the aphid. Surprisingly, the ammonia concentration in the honeydew is lower than in the stylectomy samples, suggesting that the ammonia is of plant origin rather than a result of amino acid breakdown by the aphid (Sasaki et al., 1990).

Differences among Aphid Species. The amino acid profiles of honeydew of different aphid species showed differences in biotransformation of amino acids among species (e.g., low glutamine and glutamic acid and high leucine and isoleucine concentration for *M. persicae*). However, the differences in total concentration of amino acids were much larger.

Differences among Lines. Comparison of the EDTA samples of resistant and susceptible lettuce showed very little difference among two lines of the same set and little variation between sets, apart from traces of ornithine in the lines RES2 and SUS2 (Figure 2). Differences with Taiwan lettuce (Figure 1C) are somewhat larger. Resistance is obviously not based on amino acids.

Summary. Both honeydew and EDTA samples are not a very good representation of phloem sap amino-acids. It is difficult to judge which sampling technique is a better representation, and differences in amino acids in honeydew from different aphids are very large.

Proteins

EDTA. Based upon the sugar content, Helden et al. (1994) estimated that EDTA samples are a 40-fold dilution of the real phloem sap. The observed protein concentration of about 20 μg/ml would mean that the lettuce phloem sap contains approximately 0.8 mg/ml of protein, which is in the same range

as reported for the phloem sap of several plant species collected by incision (Ziegler, 1975) or stylectomy (Fisher et al., 1992). The number of proteins is very high, but comparable to Fisher et al. (1992), who reported several hundred different proteins in phloem sap from wheat collected by stylectomy or from broken pedicels.

It is not evident that all these proteins are normally present and mobile in the phloem sap. The loss of turgor pressure upon incision of a phloem strand (and presumably also excision of a leaf for EDTA samples or stylectomy) might cause considerable leakage of enzymes from the surrounding phloem bundle cells into the sieve elements (Eschrich and Heyser, 1975), and proteins that are normally immobile in the sieve elements and incorporated or bound to structural elements (e.g., P-protein fibrils) can be released by the wounding or EDTA action. Tjallingii and Hogen Esch (1993) observed filamentous structures, probably P proteins, blocking the food canal of amputated aphid stylets. Finally, contamination of EDTA samples can occur with proteins released from the wound surface.

Honeydew. The honeydew showed few and low amounts of proteins compared to the EDTA samples. Aphid alimentary tracts do not contain proteinases (Srivastava and Auclair, 1963) so ingested proteins are expected to pass unchanged or accumulate in the aphid (Rahbé and Febvay, 1993). It seems unlikely that nearly all proteins that were present in the EDTA samples were accumulated. If the aphid ingested phloem sap with 800 μg/ml (EDTA sample estimate) of protein and excreted it without metabolizing, the protein content of the honeydew would be higher than the value found in our experiments (80 μg/ml). Normal aphid feeding rates are a factor of 10 lower than the outflow from amputated stylets (Helden et al., 1994; Tjallingii, 1994), in which case no leaking of proteins from the companion cells to the phloem sieve element is expected (Eschrich and Heyser, 1975) and no wound reaction (P-protein gelation, responsible for the first fast wound reaction) will occur. This confirms the hypothesis that the EDTA samples contain proteins that are normally not present or not mobile in the phloem sap and are therefore not ingested by the aphid. Additional proteins, present in the honeydew but not in the EDTA samples, are produced by the aphid or its symbionts.

Summary. More experiments are necessary to judge which sampling method provides the best representation of the proteins of phloem sap (or aphid food). Honeydew might be the best sample, even better than stylectomy, although some proteins might accumulate in the aphid.

Secondary Plant Compounds

The main reason for aphids to use phloem sap as a food source is thought to be the absence or low concentrations of secondary compounds in the phloem sap as compared to other plant parts. Many different groups of secondary sub-

stances have been reported from lettuce [phenolics, flavonoids, sesquiterpene lactones, alkaloids, sterols (Gonzalez, 1977)], but nothing is known about their presence in the phloem sap.

Analysis using HPLC with a diode array detector is limited; only substances having UV absorption, present in sufficiently high amounts, and separated with our column–solvent system show up in the chromatogram. The resemblance between EDTA and honeydew samples indicates that aphids excrete most of the ingested secondary substances unchanged through the honeydew. At the same time, it confirms that EDTA samples are mainly of phloem origin; the secondary compounds originating from the phloem sap were not overrated by contamination from the wound surface by high concentrations from vacuoles of damaged cells. The effect of aphid digestion on secondary compounds is unclear although several secondary plant compounds are reported to pass unchanged (Molyneux et al., 1990). Polyphagous aphids might possess detoxification mechanisms to break down secondary compounds, thus leaving no traces in the honeydew, while monophagous species do not accept these plants. Hussain et al. (1974) reported 18 different phenolic acids present in aphid honeydew feeding on radish, five of which were found in radish seedlings together with four other phenolic acids. Therefore, they suggested that most of the phenolics present in honeydew were breakdown products. However, they used whole plants and not phloem sap samples. Compounds and concentrations in phloem sap are expected to differ widely from whole plant samples.

Difference among Lettuce Lines. We were unable to show consistent differences between lettuce lines. Therefore, a relation with resistance is not clear. Because of the limitations of the analysis method, this is not proof that secondary plant compounds are not involved in resistance.

Overall Comparison of Collection Methods

The question of which collection method is most reliable for chemical analysis cannot be answered easily and differs among chemical groups.

Stylectomy. Thus far, stylectomy has been thought to represent the real phloem sap. However, it is possible that the aphid changes the composition of the phloem sap prior to or during feeding. Auclair (1963) reports on 10–50% reduction of the amino acid content of honeydew due to crowded aphid attack. The aphid's penetration behavior, together with salivary sheath formation and salivation into the sieve element (Prado and Tjallingii, 1994), might influence the plant. Phloem sap collected by stylectomy depicts the phloem sap of an aphid-attacked sieve element. Even then, some compounds normally immobile (proteins) might be translocated due to the high outflow rate (or the sudden turgor changes), which is much higher than under normal aphid feeding circumstances. The time-consuming process and often rather uncertain yields (Helden et al., 1994; Fisher and Frame, 1984) are obvious disadvantages. Small sample

size and shortage of replicates pose problems for the chemical analysis of many compounds, especially secondary plant substances.

EDTA. EDTA-collected samples appear to be a good representation of most compounds of the phloem sap apart from proteins. What exactly happens with the proteins remains obscure. Attention should be paid to possible reactions in the collection vials (invertase, oxidation) and leakage of compounds from other cells. The quality might be improved by adding more inhibitory compounds (antioxidants, enzyme inhibitors) to the solution, although these compounds might influence the metabolism of the leaf. Leaking of compounds from the wound surface seems limited, even for secondary compounds. Washing of the surface after cutting is advisable. Cutting the petiole again after a short EDTA exposure to enhance exudation (Girousse et al., 1991) is unnecessary and will cause extra contamination from the wound surface. EDTA collection is by far the easiest method, yielding the largest quantities of phloem sap equivalents.

Honeydew. Honeydew is bioprocessed phloem sap. The profile and concentration of sugars and amino acids differs profoundly from the real phloem sap. Biotransformation occurs in the aphid and might even be continued in the honeydew. Contamination of the honeydew could also be caused by ingestion from other tissues during the cell punctures that occur during penetration towards the phloem. The number of these punctures is high (Tjallingii and Hogen Esch, 1993), and the time spent penetrating towards the phloem is a large part of feeding behavior (Helden and Tjallingii, 1993). If ingestion occurs during these punctures, it will be from vacuoles where the concentration of secondary compounds is high. To avoid possible contamination, honeydew should preferably be collected from individuals that have been feeding continuously for a long period.

Honeydew collection is much easier than stylectomy, and usually a homopteran feeding on the phloem can be found. The honeydew collection method covers the possibility of short-term or local induction of plant reactions, which then might cause differences in honeydew composition.

Comparison of Lettuce Lines. Variation in phloem sap compounds seems very limited, with only minor differences between lettuce lines. No relation with resistance has been observed so far. Comparison of phloem sap for unknown secondary compounds proved not to be a useful method to find resistance factors due to the infinite number of possibilities. The most promising option to show chemical differences related to resistance would be to develop a bioassay using honeydew (Dorschner and Kenny, 1992) or EDTA samples added to an artificial diet, guiding chemical isolation and identification techniques.

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AGGREGATION PHEROMONE OF AUSTRALIAN SAP BEETLE, *Carpophilus davidsoni* (COLEOPTERA: NITIDULIDAE)[1]

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Abstract—A male-produced aggregation pheromone was identified for the Australian sap beetle, *Carpophilus davidsoni* Dobson (Coleoptera: Nitidulidae), by bioassay-guided fractionation of volatiles collected from feeding beetles. The most abundant components were: (2*E*,4*E*,6*E*)-5-ethyl-3-methyl-2,4,6-nonatriene, (3*E*,5*E*,7*E*)-6-ethyl-4-methyl-3,5,7-decatriene, (2*E*,4*E*,6*E*,8*E*)-3,5,7-trimethyl-2,4,6,8-undecatetraene, and (2*E*,4*E*,6*E*,8*E*)-7-ethyl-3,5-dimethyl-2,4,6,8-undecatetraene. The relative abundance of these components in collections from individual males feeding on artificial diet was 100:7:9:31, respectively. Pheromone production began within several days after males were placed onto diet medium and continued for at least 20 weeks. Peak production was >3 μg total pheromone per male per day. Males in groups of 50–60 emitted less pheromone (the peak level was 0.09 μg per beetle per day), and the emissions from groups contained relatively little tetraene (proportions of the components listed above were 100:7:2:7, respectively). Three additional trienes and one additional tetraene were identified in minor amounts; the entire eight-component male-specific blend is qualitatively identical and quantitatively similar to that of the North American sibling species, *C. freemani* Dobson. A synthetic blend of the four major components on rubber septa, prepared to emit in the same proportions as from individual males, was highly attractive in the field when synergized with fermenting whole-wheat bread dough. Cross-attraction was observed in the field involving

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[1]Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standards of the products, and the use of the names by USDA implies no approval of the products to the exclusion of others that may also be suitable.

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the pheromones of *C. davidsoni*, *C. hemipterus* (L.), and *C. mutilatus* Erichson. Potential uses of the pheromones in pest management are discussed.

Key Words—Aggregation pheromone, triene, tetraene, hydrocarbon, Coleoptera, Nitidulidae, *Carpophilus davidsoni*, Australian sap beetle, trapping.

INTRODUCTION

Carpophilus davidsoni Dobson is a small (2.5 to 3.5-mm-long), reddish brown sap beetle that occurs in Australia, New Zealand, and Micronesia (Gillogly, 1962; Williams et al., 1983). In his original description, Dobson (1952) noted that the species was found infesting Australian sweet corn, figs, and peaches, and he predicted that it would grow in economic importance, given the pest status of related species. Apparently that prediction has been fulfilled; *C. davidsoni* was the most abundant nitidulid collected from major stone-fruit growing regions of southeastern Australia during 1992–1994 (James et al., in preparation). Nitidulid damage to ripening stone fruit during this period was severe. Major Australian fruit pests such as the Oriental fruit moth, *Grapholita molesta* Busck, are increasingly being managed by pheromonal disruption of mating, and the nitidulid problem has worsened as insecticides are used less (James et al., 1994). While reduced use of insecticide is desirable environmentally, new tools must be developed to manage those species inadvertently elevated to pest status by avoidance of insecticides.

Nitidulid pheromones have potential as pest management tools. Male-produced aggregation pheromones, to which both sexes respond, are known for a number of *Carpophilus* species, including two in the Australian nitidulid complex, *C. hemipterus* (L.) and *C. mutilatus* Erichson (Bartelt et al., 1990a, 1992, 1993). These pheromones were identified from North American strains of the beetles, but synthetic pheromones for these species have worked well in Australia also (James et al., 1993, 1994). In all cases, a food-related synergist is required for optimal pheromone activity; fermenting bread dough has generally been used for this purpose.

If the pheromone for *C. davidsoni* could be identified, then pheromonal approaches to nitidulid management could be developed/applied simultaneously for all of the major Australian species. In previous field tests, *C. davidsoni* responded significantly to the synergized synthetic pheromones for five other *Carpophilus* species; these were *C. hemipterus*, *C. mutilatus*, and three non-Australian species, *C. freemani* Dobson, *C. lugubris* Murray, and *C. obsoletus* Erichson (James et al., 1993, 1994). Cross-attraction suggested that the pheromone of *C. davidsoni* was similar to those of the other species, but the modest responses (2× to 10× more beetles to the pheromone-plus-bread-dough treatments than to the dough control) indicated that none of the other pheromones

were correct for *C. davidsoni*; *C. hemipterus*, for example, typically responded over 100× better to its pheromone plus dough than to dough alone.

The pheromone of *C. davidsoni* has now been analyzed, and we present here the chemical details and evidence for activity of the synthetic pheromone under field conditions.

METHODS AND MATERIALS

Beetle Culture. A laboratory culture of *C. davidsoni* was established at NCAUR, Peoria, Illinois, from beetles collected at Leeton in southern New South Wales by one of us (D.G.J.). The beetles were easily reared by the method of Dowd and Weber (1991) using a modified diet (Bartelt et al., 1993).

Volatile Collections. Volatiles from beetles feeding on artificial diet were collected on Super Q porous polymer (Alltech Associates, Deerfield, Illinois) as described previously (Bartelt et al., 1990a). The temperature during collections was 27°C, and the photoperiod was 14L:10D. Initially, collections were from groups of 50–60 male or female beetles, but most later collections were from single beetles, after it was determined that analysis of the single-beetle samples was possible.

The volatiles were flushed from the Super Q with 500 μl hexane. Nonadecane (2.5 μg) was added to each sample as an internal standard for GC. Collections from individual beetles were usually made daily, but the interval was three to seven days for two individuals monitored for 20 weeks. Collection intervals were two to four days for the beetle groups.

Bioassay-Guided Isolation of Active Compounds. Activity of volatile collections and chromatographic fractions was assessed by wind-tunnel bioassay (Bartelt et al., 1990a). Briefly, ca. 300 beetles (both sexes) were released into a wind tunnel and began to fly about after several hours without food. Two filter paper test baits to be compared (usually a chemical sample versus a control) were hung side by side in the upwind end of the wind tunnel. Beetles approached attractive baits with a casting, hovering flight and alighted readily on them. Counts of beetles landing on the baits during 3-min test periods were recorded. Tests could be initiated every 5 min for up to 4 hr with one group of beetles. Paired *t* tests were conducted on transformed data [$\log(x + 1)$] to detect treatment differences; residual error was pooled over all tests dealing with a given set of chromatographic fractions.

Active collections were partitioned by polarity on open columns of silica gel. The most active silica-gel fraction (eluted with hexane) was then subjected to HPLC on a silver-nitrate-coated silica column; the solvent was 10% toluene in hexane. Details of procedures and equipment were as in Bartelt et al. (1990a).

Chemical Analysis. All volatile collections and derived fractions were ana-

lyzed by capillary gas chromatography (GC). The Hewlett Packard 5890 gas chromatograph was equipped with a splitless injector, flame ionization detector, autosampler, and Hewlett Packard 3396A integrator. The DB-1 capillary column (J & W Scientific, Folsom, California) had a length of 15 m, internal diameter of 0.25 mm, and film thickness of 1.0 μm. Carrier gas was helium, and head pressure was 7.7 psig. Oven temperature program was 50°C to 250°C at 10°C/min. All injections were made in splitless mode. Retention indices of selected GC peaks were calculated relative to *n*-alkanes by linear interpolation (Poole and Schuette, 1984, p. 24).

Electron impact mass spectra were obtained for the sex-specific compounds in the active fractions and corresponding synthetic standards. The spectra were taken on a Hewlett Packard 5970 mass selective detector or on a Finnigan TSQ700 tandem mass spectrometer. Sample introduction was always by capillary GC.

Synthetic Compounds. Eight synthetic hydrocarbons were used in this research (Figure 1). The compounds were: (2*E*,4*E*,6*E*)-5-ethyl-3-methyl-2,4,6-octatriene **(1)**, (2*E*,4*E*,6*E*)-3,5-dimethyl-2,4,6-nonatriene **(2)**, (2*E*,4*E*,6*E*)-5-ethyl-3-methyl-2,4,6-nonatriene **(3)**, (3*E*,5*E*,7*E*)-6-ethyl-4-methyl-3,5,7-decatriene **(4)**, (3*E*,5*E*,7*E*)-5-ethyl-7-methyl-3,5,7-undecatriene **(5)**, (2*E*,4*E*,6*E*,8*E*)-3,5,7-trimethyl-2,4,6,8-undecatetraene **(6)**, (2*E*,4*E*,6*E*,8*E*)-7-ethyl-3,5-dimethyl-2,4,6,8-undecatetraene **(7)**, and (3*E*,5*E*,7*E*,9*E*)-8-ethyl-4,6-dimethyl-3,5,7,9-dodecatetraene **(8)**. These were all available from a previous project (Bartelt et al., 1990b, and references therein).

NO.	STRUCTURE	GC RET. INDEX
1		1136
2		1173
3		1224
4		1300
5		1393
6		1476
7		1515
8		1589

FIG. 1. Structures, assigned structure numbers, and GC retention indices (relative to *n*-alkanes) for the compounds used in this research.

Septa were prepared with compounds **3**, **4**, **6**, and **7** for use as baits in field traps; the general procedure was described by James et al. (1993). Small quantities of various *Z* isomers were also present unavoidably in the baits, but with other *Carpophilus* species, *Z* isomers appear not to decrease attraction to the all-*E* pheromones (see Bartelt et al., 1992). The proportions of components in the baits and the rationale for choosing these are given in the Results and Discussion section.

Field Studies. Two experiments were conducted in two unsprayed peach orchards near Leeton in southern New South Wales. Both orchards were flood-irrigated, with fruit ripening occurring during February–March. The pheromones of *C. hemipterus* and *C. mutilatus* were used in the field studies in addition to that of *C. davidsoni* [details as in James et al. (1993)]. All pheromones were loaded at a rate of 500 μg/septum. Fermenting whole-wheat bread dough was used as the pheromone synergist (≈ 15 ml per trap) (Bartelt et al., 1992). Wind-oriented funnel traps [Figure 3 in Dowd et al. (1992), the "modified design"] were used in both experiments. Traps were examined, beetles collected, and dough replaced weekly. Septa were replaced fortnightly. Trapping data were transformed to the $\log(x + 1)$ scale and subjected to analysis of variance and least significant difference procedures.

Experiment 1 was run at the end of the 1993 field season (April 27 to May 19), shortly after pheromone identification was completed, and this test allowed initial evaluation of the *C. davidsoni* pheromone. Five traps containing the pheromone of *C. davidsoni* plus dough were added to an ongoing experiment dealing with the pheromone of *C. hemipterus*. The treatments already present were *C. hemipterus* pheromone plus dough (four traps) and dough by itself (four traps). The traps were assigned randomly in a block of approximately 30 trees. Traps were separated by at least 6–12 m and hung from trees at a height of 1.5 m.

Experiment 2 was of longer duration and included most of the 1993/1994 growing season (September 22, 1993, to March 16, 1994). It further evaluated the *C. davidsoni* pheromone and gathered evidence for cross-attraction among *C. davidsoni*, *C. hemipterus*, and *C. mutilatus*. Three treatments were the pheromones of *C. davidsoni*, *C. hemipterus*, and *C. mutilatus*, all plus dough, and the fourth was dough alone (three replications per treatment). Traps were hung from trees as in experiment 1 and were 5–6 m apart; treatments were randomized.

RESULTS AND DISCUSSION

Initial Observations. In the wind tunnel, volatiles from groups of male beetles were clearly more attractive than those from groups of females or blank controls (mean landings were 9.5, 3.0, and 0.5, respectively, $n = 4$). Com-

parison of gas chromatograms from these volatile collections revealed a male-specific peak at retention index 1224; this suggested the presence of triene **3** (Figure 1), a compound encountered previously (Bartelt et al., 1990b). The identity was confirmed by mass spectrometry. Pheromone collections were continued both from groups of males and from individual males to confirm the initial result and to search for additional components.

Analysis of Pheromone from Individual Males. A combined sample from individual males (53 volatile collections, 167 total beetle-days) was active in the wind tunnel (Table 1), and most of the activity resided in the hexane fraction derived from this sample. After $AgNO_3$ HPLC of the hexane fraction, significant pheromonal activity was found in five consecutive fractions (Table 1). Analysis of these fractions by GC and GC-mass spectrometry revealed the presence of compounds **1–8** (Figure 1). The GC retentions, HPLC retentions, and mass spectra matched those of synthetic standards (see Bartelt et al., 1990b). Traces of *Z* isomers were detected, primarily related to components **3** and **7**, but no other analogous hydrocarbons were found. It is believed that degradation of the all-*E* compounds produced these isomers (Bartelt et al., 1992). The blend of male-specific hydrocarbons emitted by *C. davidsoni* was qualitatively identical to that reported previously for the North American species, *C. freemani* (Bartelt et al., 1990b).

The existence of these hydrocarbons in *C. davidsoni* made the field responses of *C. davidsoni* to other *Carpophilus* pheromones reported earlier (James et al., 1993) more understandable. The synthetic pheromone for *C. hemipterus* contained **6** and **7** (plus other compounds); that for *C. mutilatus* contained only **4** and **5**; that for *C. freemani*, **3** and **7**; that for *C. lugubris*, **6** and **7**; and that for *C. obsoletus*, only **6**. The relative weakness of responses to these may have been due to missing components and/or improper ratios. Field experiments were used to verify the pheromonal activity of the most abundant components and are presented below; preparation of field samples was guided by the following laboratory results.

GC of Crude Volatile Collections from Individual Males. The four most abundant components, **3**, **4**, **6**, and **7**, were readily observed in volatile collections from individual males feeding on artificial diet, without any prior chromatographic purification (Figure 2). In fact, the male-produced pheromone components were the only prominent beetle-related compounds in the volatile collections. Collections from virgin female beetles (e.g., Figure 2, lower panel) were virtually identical to those from diet only. (The diet volatiles probably account for the slight activity of volatile collections from feeding females, noted in initial wind-tunnel tests). The prominence of the major male components allowed analysis of single collections from individual males and from groups of males without combining samples to gain sensitivity.

TABLE 1. RESPONSES OF *C. davidsoni* IN WIND TUNNEL TO VOLATILES COLLECTED FROM FEEDING MALE BEETLES AND DERIVED CHROMATOGRAPHIC FRACTIONS[a]

Volatile collection and silica gel fractions			Silver-nitrate HPLC of silica gel hexane fraction			
Whole collection or derived fraction	Landings per test		$AgNO_3$ fraction (ml)	Landings per test		Key compounds in fractions
	Test sample	Control		Fraction	Control	
Whole volatile collection	54.5***	0.3	3–4	0.0	0.0	
			4–5	0.5	0.0	
			5–6	0.3	0.0	
Fractions			6–7	15.2***	0.0	**1, 3, 4, 5**
Hexane	45.0***	0.0	7–8	23.8***	0.0	**2, 3**
5% Ether–hexane	6.0***	0.0	8–9	17.3***	0.0	**7, 8**
10% Ether–hexane	0.0	0.0	9–10	13.5***	0.0	**6, 7**
50% Ether–hexane	1.0	0.0	10–11	8.0***	0.0	**6**
10% MeOH–ether	4.5***	0.0	11–12	0.8	0.0	
			12–13	0.3	0.0	

[a]Four paired comparisons were made between controls and test materials for each chromatographic fraction and for the whole volatile collection. The dose for the whole collection and for all fractions was 0.1 beetle-day per test. Test duration was 3 min. Differences between the test material and control that were significant at the 0.001 level (*t* tests) are indicated by ***. No other comparisons were significant at even the 0.05 level.

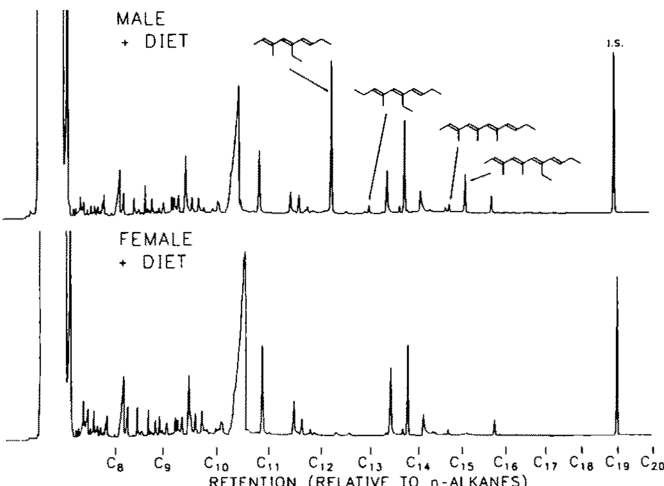

FIG. 2. Gas chromatograms of whole volatile collections from a single male and a single virgin female *C. davidsoni* feeding on artificial diet. Collection period was one day; beetles were 7 days old. Male-specific hydrocarbons are highlighted. The internal standard (I.S.) was nonadecane (2.5 μg/sample). The GC temperature program was 50–250°C at 10°C/min; run duration was 20 min. Retentions of *n*-alkane standards are noted along bottom of chromatogram. Each injection was 2 μl from a 500-μl crude volatile collection; there was no chromatographic purification or concentration prior to injection.

Amounts of Pheromone Produced. Pheromone production was dramatically higher for individual males than for beetles relatively crowded in groups (Table 2). For both the individuals and the groups, production was very low for the first two days during which the beetles were in the collectors, but even the oldest beetles tested (up to 20 weeks) still produced pheromone. Peak production usually occurred between one and two weeks after the beetles were introduced into the collectors. Most individuals tested were newly emerged, but the onset and peaking of pheromone production followed the pattern in Table 2 even when beetles were transferred from the culture two weeks after emergence.

Ratios of Components. The observed proportions of components are given in Table 3. The amount of **7** relative to **3** was fairly consistent (30.6% ± 9.0% standard deviation) among the 237 samples from single beetles for which acceptable quantitation could be obtained (total pheromone in sample >0.5 μg). By one-way ANOVA, about half of the variance that did exist in the relative amount of **7** was due to systematic beetle-to-beetle differences among the 21 individual males from which collections were taken. The amount of **7** relative to **3** was consistently lower for collections from groups of 50–60 beetles than for single

TABLE 2. PHEROMONE PRODUCTION BY INDIVIDUAL MALES AND GROUPS OF 50–60 MALES (TOTALS OVER COMPONENTS **3**, **4**, **6**, AND **7**)

Time after beetles placed in collectors[a]	Individual males		Groups of 50–60 males	
	μg/male/day ± SD	*n*	μg/male/day ± SD	*n*
0–2 days	0.22 ± 0.35	35	0.0096 ± 0.0083	3
2–4 days	1.6 ± 1.1	35	0.037 ± 0.048	2
4–7 days	2.4 ± 1.3	45	0.026 ± 0.014	5
7–10 days	3.3 ± 1.1	44	0.057 ± 0.048	5
10–14 days	2.8 ± 1.2	42	0.085 ± 0.12	8
2–3 weeks	2.5 ± 1.1	48	0.081 ± 0.056	3
3–7 weeks	2.2 ± 0.4	16		
7–11 weeks	2.0 ± 0.4	16		
11–15 weeks	1.4 ± 0.4	16		
15–20 weeks	0.7 ± 0.5	14		

[a] Each age category includes times greater than the lower limit and less than or equal to the upper limit.

TABLE 3. PROPORTIONS OF PHEROMONE COMPONENTS EMITTED FROM INDIVIDUAL MALES AND GROUPS OF 50–60 MALES[a] AND AMOUNTS OF COMPONENTS ADDED TO SEPTA FOR FIELD BAIT

Component	Individual males	Groups of 50–60 males	Septum load (μg)
Triene **1**[b]	0.9		
Triene **2**[b]	0.7		
Triene **3**	100	100	170
Triene **4**	6.5 ± 2.5	6.7 ± 3.3	13
Triene **5**[b]	1.2		
Tetraene **6**	8.9 ± 3.2	1.7 ± 1.9	52
Tetraene **7**	30.6 ± 9.0	6.6 ± 7.8	265
Tetraene **8**[b]	0.4		

[a] Amount of triene **3** was normalized to 100, and the proportional values for other components were calculated. Values for **4**, **6**, and **7** are means (±SD) for all single collections for which acceptable quantitation was obtained (i.e., total pheromone in sample >0.5 μg) (n = 237 for individual males and n = 21 for groups).

[b] Values determined from a combination of 53 volatile collections from individual males, after $AgNO_3$ HPLC; collections from groups of males were not analyzed for **1**, **2**, **5**, or **8**.

beetles (6.6% versus 30.6%, Table 3). As with the individuals, significant systematic group-to-group differences existed; two of the five groups did not produce **7** at a detectable level. The abundance of **7** relative to **3** was strongly correlated with the total amount of pheromone produced per beetle per unit time. Considering collections from both individuals and groups, the amount of **7** relative to **3** increased by 5.4% for each increase of 1 μg in daily pheromone production per beetle (simple linear regression, $t = 13.6$, 279 *df*, $P \ll 0.0001$, $R^2 = 0.40$). The relationship was still highly significant when either the individuals or the groups were considered separately.

The ratio of triene **4** to triene **3** and the ratio of tetraene **6** to tetraene **7** both remained quite stable, regardless of whether collections were from individuals or from groups. For individuals, **4** was 6.5% as abundant as **3**, and **6** was 29% as abundant as **7**. For groups, the respective values were 6.7% and 25%. The greatest distinction between the individual and group collections was in the overall ratio of trienes to tetraenes.

Under natural conditions, we suggest that beetles react to suboptimal (e.g., crowded) feeding/breeding sites by producing smaller amounts of pheromone, and furthermore, this pheromone has relatively little tetraene. Large numbers of beetles at such a site could compensate for low pheromone production per beetle. (In Table 2, for example, pheromone production per male in a group of 50–60 was roughly 1/50th of that by isolated individuals; thus total pheromone production per collector in the two situations was essentially the same.) However, the skewed ratio of components would still be detectable to responding beetles. Intuitively, a blend rich in **7** would be preferred by responding beetles because it would indicate a site with relatively little competition for food or mates. The earlier, mediocre response of *C. davidsoni* to the pheromone of *C. freemani*, which contained **7** and **3** in a 4 : 100 ratio (James et al., 1993), may have occurred because the blend signaled a suboptimal landing site in the chemical language of *C. davidsoni*. Consequently, we attempted to emulate the blend from individual male beetles when preparing baits for the field tests (Table 3).

Pheromone Baits for Field Tests. Compounds **3, 4, 6,** and **7** differ markedly in volatility; for example, **3** evaporates about five times faster from septa than **7** (R.J.B., unpublished). To achieve the desired release ratio of 3.3 : 1 for **3** and **7** (Table 3) required loading **3** and **7** in a ratio of about 0.6 : 1; load amounts for minor components **4** and **6** were chosen in a similar way. The first-order release property of septa presented a further complication; the release rate is proportional to the amount of pheromone remaining in the septum (McDonough, 1991). For **3,** it takes only 1.5 day at 27°C for the amount of pheromone remaining in the septum to decrease by one half, but for **7,** the half-life is 8.1 days (R.J.B., unpublished). Thus, proportions of emitted compounds would change considerably over the two weeks during which each septum was to be in the field. However, the emitted blend would become progressively richer in

7, moving away from, rather than approaching, the undesirable proportions observed from groups of males (Table 3).

Field Studies. The synthetic blend of *C. davidsoni* pheromone in combination with fermenting bread dough was highly attractive to this species in both field experiments (Figure 3). The effect of this pheromone, relative to the dough treatment and to the *C. hemipterus* pheromone, was somewhat weaker in experiment 2 than in experiment 1. (For example, the *C. davidsoni* pheromone plus dough treatment attracted 48× more *C. davidsoni* than the dough in experiment 2, but 377× more in experiment 1). The reason for this difference is unknown, but experiment 2 included the warmest part of the year, when depletion of the *C. davidsoni* pheromone septa would be especially rapid.

In all cases, *C. davidsoni*, *C. hemipterus*, and *C. mutilatus* responded in greatest numbers to the conspecific pheromones when these were present. This

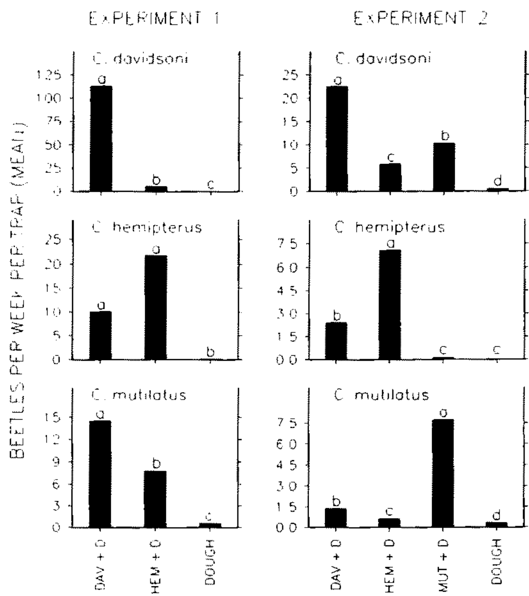

FIG. 3. Responses of three *Carpophilus* species to synthetic aggregation pheromones in Australian field tests. Experiment 1 ran from April 27 to May 19, 1993, and experiment 2 from September 22, 1993, to March 16, 1994. The abbreviations, DAV, HEM, MUT, and D, refer to the pheromones of *C. davidsoni*, *C. hemipterus*, *C. mutilatus*, and to fermenting bread dough, respectively. For each graph, the responding species is indicated above the bars. In each graph, means accompanied by the same letter are not significantly different [0.05 level, analysis of data in the log(x + 1) scale]. For experiment 1, there were 16–20 observations per mean; for experiment 2, there were 78.

preference was significant in all but one case (the response of *C. hemipterus* to the pheromones of *C. hemipterus* and *C. davidsoni* in experiment 1). Each of the three species was significantly attracted to the pheromones of the other two, except that *C. hemipterus* did not respond significantly to the pheromone of *C. mutilatus* (experiment 2). The existence of cross-attraction is not surprising because the species share pheromone components in some cases: both the *C. davidsoni* and *C. mutilatus* blends contain compound **4** (Figure 1), and both the *C. davidsoni* and *C. hemipterus* blends contain **6** and **7**. However, the attraction of *C. mutilatus* to the *C. hemipterus* pheromone can not be rationalized in this way, and slight kairomonal effects may be operating.

Use of Nitidulid Pheromones in Future Pest Management. This demonstration of an effective synthetic aggregation pheromone for *C. davidsoni* means pheromones are now available for the three nitidulid species that cause greatest concern to Australian stone-fruit growers. The potential of *C. hemipterus* and *C. mutilatus* pheromones as attractants for these species in Australia was reported previously (James et al., 1993, 1994). Synthetic pheromones have the potential to become important management tools for nitidulids in stone-fruit orchards. They could be used as the basis of a sensitive monitoring system to facilitate better timing of insecticide sprays, particularly early in the season when growers are often unaware of beetle presence. Improved spray timing would inevitably reduce insecticide inputs and the risk of residues on harvested fruit. Pheromones also provide an opportunity to develop novel control options such as mass-trapping or disruption of fruit colonization by the beetles. However, more information on nitidulid biology and ecology in stone-fruit orchards is required before these options can be explored properly. We also need to know the effective trapping radius of nitidulid pheromones together with information on optimal doses. The prospects for developing pheromones as a management tool for nitidulids in stone-fruit orchards appear to be excellent.

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DISCRIMINATION OF OILSEED RAPE VOLATILES BY HONEY BEE: NOVEL COMBINED GAS CHROMATOGRAPHIC-ELECTROPHYSIOLOGICAL BEHAVIORAL ASSAY

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Abstract—A novel technique for the simultaneous monitoring of electroantennogram (EAG) and conditioned proboscis extension (CPE) responses of honey bees to the effluent from a gas chromatograph (GC) was developed to locate biologically active components in blends of plant volatiles and to investigate odor recognition at the peripheral and behavioral levels. A six-component mixture, comprising compounds previously identified as oilseed rape floral volatiles, was used as the stimulus. Standard CPE and EAG recordings were done as a reference. EAG responses were elicited from unconditioned bees by all the components presented either in the coupled or the standard mode. Conditioned bees gave larger EAG responses than unconditioned bees, suggesting that antennal sensitivity is enhanced by conditioning. At the behavioral level, in both the standard and the coupled modes, only conditioned bees showed the proboscis extension response, with the majority of individuals responding to linalool, 2-phenylethanol, and benzyl alcohol.

Key Words—Honey bee, *Apis mellifera*, Hymenoptera, Apidae, gas chromatography, electroantennogram, conditioned proboscis extension, olfactory discrimination.

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INTRODUCTION

Volatile semiochemicals have an important role in the chemical ecology of the honey bee and facilitate flower recognition, thereby increasing foraging efficiency. Chemical analyses of volatiles from various plant species have shown the complexity of floral odors. Tens to hundreds of components have been reported, e.g., 150 in orchid flowers (Borg-Karlson and Tengö, 1986), 80 in sunflower (Etiévant et al., 1984). Moreover, the quality and quantity of these blends can fluctuate according to various factors such as plant phenology [e.g., cotton (Hedin, 1976); and sunflower (Pham-Delègue et al., 1988)] or to pedoclimatic conditions (Robacker et al., 1982). It is still not known how honey bees process the chemical information and adapt their behavior in response to such complex and variable plant volatile blends.

Odor recognition in the honey bee has been investigated using a number of approaches. Behavioral experiments with free-flying bees demonstrated conditioning processes in food source recognition (Frisch, 1919) and showed the importance of olfactory rather than visual cues (Kriston, 1973). In addition, foragers were shown to establish preferences between odorants (Koltermann, 1969). With restrained bees, the conditioned proboscis extension response (CPE) devised by Frings (1944) was used to investigate learning processes (Bitterman et al., 1983) and their neural bases (Menzel et al., 1974; Erber, 1981; Menzel, 1990). Based on this bioassay, Vareschi (1971) made an extensive study of qualitative behavioral discrimination abilities among pairs of 28 odorants, and found that in 95.5% of cases, bees were able to discriminate between learned and unlearned odors. Getz and Smith (1987) demonstrated the quantitative discrimination abilities of bees by testing binary mixtures of the same compounds combined in different proportions.

Tentative work relating behavioral responses to peripheral detection processes has also been done. Thus, Vareschi (1971) established seven reaction groups from single-cell recordings and found that components from different groups were more systematically discriminated in the behavioral assays than components from the same reaction group. More recently, Ackers and Getz (1992) repeated this work on six components belonging to three reaction groups defined by Vareschi (1971) and found a different categorization of the receptor types.

In more applied studies aimed at improving plant pollination, chemical, behavioral, and electrophysiological techniques were used to identify potentially attractive plant volatiles. Thus, Waller et al. (1974) showed that free-flying honey bees trained to the scent of alfalfa flowers used components predominant in the training scent as cues. An active fraction cueing the recognition of a complex floral sunflower extract has also been found (Pham-Delègue et al., 1986). These behavioral studies were complemented by recording the antennal

responses of honey bees to floral volatiles, using a coupled gas chromatography (GC)-electroantennogram (EAG) detector system. In a whole-flower extract containing more than 100 components, only 24 compounds elicited EAG responses from more than 80% of the bees (Thiéry et al., 1990) and of these compounds, only six had previously been found to be behaviorally active.

Electrophysiological recordings have been used widely to locate biologically active compounds in complex natural product extracts (Arn et al., 1975; Wadhams et al., 1982; Guerin et al., 1983; Thiéry et al., 1990; Henning and Teuber, 1992). However, in many studies, attempts to correlate the activity of olfactory stimuli at the antennal level with the behavioral responses have been confounded because these phenomena have been investigated separately. To locate biologically active components in blends of plant volatiles and to investigate odor recognition at the sensory and behavioral levels, we have developed a novel technique for the simultaneous monitoring of EAG and CPE responses of honey bees to the effluent from a GC column. The CPE response, which occurs naturally when a forager visits a food source, is thought to indicate behavioral recognition of complex olfactory information by the bee (Mauelshagen and Greggers, 1993). Since learning processes play an important role in floral visits by honey bees, it was decided that a bioassay based on such learning abilities should be used. Moreover, the use of restrained bees enabled the simultaneous recording of behavioral and antennal responses to the same range of stimuli. A six-component mixture, comprising components previously identified as oilseed rape floral volatiles (Tollsten and Bergström, 1988; Blight et al., 1992), was used as the stimulus to evaluate the technique. Standard CPE and EAG recordings were done as a reference.

METHODS AND MATERIALS

Chemicals

The chemicals used were linalool (97%), 2-phenylethanol (99+ %), methyl salicylate (99+ %), benzyl alcohol (99+ %), (*E*)-2-hexenal (99%), and 1-octen-3-ol (98%). Solutions of the individual components, and mixtures containing equal amounts of all six components, were made up in hexane (HPLC grade, Fisons, purified).

Insects

Although all kinds of bees can be used in this bioassay, we worked with Italian bees because of their ability to endure caged confinement. Emerging Italian worker bees, *Apis mellifera ligustica*, were collected from outdoor hives and caged in groups of approximately 50 individuals. They were fed sugar,

pollen, and water *ad libitum* and maintained in an incubator at 33°C, 50% relative humidity, until required.

Bees 14–16 days old, were starved for at least 3 hr and mounted individually in glass holders in such a way that their mouthparts and antennae remained free. For electrophysiological recordings, one antenna was fixed to the holder with water-based correction fluid (Tipp-Ex).

Coupled GC-EAG-CPE Recording

Bees tested in the coupled procedure were either unconditioned or were conditioned to the six-component mixture. The conditioning procedure (Pham-Delègue et al., 1993) was carried out independently from the coupled system. Before odor stimulation, bees were habituated to mechanical stimulation by placing them for 15 sec in an airflow of 50 ml/sec, which was delivered through a 1-cm-diameter glass tube placed 1 cm from the head of the bee. The stimulus delivery system utilized a disposable Pasteur pipet cartridge. The conditioning mixture (1 μg of each component in 10 μl hexane) was applied to a 20 × 4-mm filter paper strip, the solvent was allowed to evaporate, and the filter paper was inserted into the pipet. Vapor from the cartridge was delivered over a 6-sec period into the airstream passing continuously over the bee by means of a second airstream (2.5 ml/sec) controlled by a solenoid. Fresh cartridges were prepared immediately prior to each stimulation. Three seconds after the beginning of the odor stimulation, the free antenna was contacted with a 30% sucrose solution and the proboscis extension was rewarded by offering a drop of the sugar solution. Five conditioning trials were done for each bee, with 15-min intervals between trials. After the first trial, the conditioned response occurring during the first 3 sec of the odor stimulation was noted. A control trial, performed by stimulating the insect with hexane (10 μl), was used to check whether the odor stimulus had been learned specifically or if the bee responded to other stimuli (solvent, airflow). Only bees that gave the conditioned response at least once during the conditioning trials and that did not respond to the control stimulation were used for the coupled GC-EAG-CPE recordings.

GC Conditions. Coupled GC-EAG-CPE recordings were obtained using the GC-EAG system described previously (Blight et al., 1979). Samples (six-component mixture, 0.5 μg of each component) were separated using an AI-93 gas chromatograph equipped with a flame ionization detector (FID) and a cold on-column injector fitted with a 30-m × 0.53-mm-ID HP-1 bonded phase fused silica capillary column. The oven temperature was maintained at 40°C for 1 min and then programmed at 10°C/min to 250°C. The carrier gas was hydrogen. The effluent from the GC column was split approximately equally, with one part going to the FID of the GC and the remainder directed into the airflow passing continuously over the bee.

Electrophysiological Recordings. EAG responses were obtained using Ag–AgCl glass electrodes filled with saline solution [composition as in Maddrell (1969) but without the glucose]. The recording electrode was placed in a prepunctured hole in the tip of the fixed antenna and the indifferent electrode was inserted into a small cut in the scape. Responses of conditioned and unconditioned individuals were compared using a *t* test.

Proboscis Extension Recordings. CPE responses to the effluent from the GC column were monitored visually and were recorded simultaneously with the EAG responses. FID, EAG, and CPE responses were stored on tape (Racal Store 4). Recordings were sampled on a microcomputer with a Data Translation card (DT2821; 12 bits precision; 200 Hz/channel) and analyzed with software developed initially for spike analysis (Marion-Poll and Tobin, 1992).

Standard Proboscis Extension Testing. At the end of the GC-EAG-CPE recordings, the bees were tested (6 sec unrewarded odor stimulation) with the individual components (1 μg) presented in a random order at 15-min intervals, and with the original conditioning mixture. Bees from the conditioned group that did not give the CPE response to the conditioning stimulus at the end of the experiment was discarded from data treatment.

In this experiment, the responses from six conditioned and eight unconditioned bees were recorded.

Standard Behavioral and Electrophysiological Recordings

In parallel with the coupled recordings, responses of bees were assessed using standard behavioral and electrophysiological procedures.

Standard CPE Assay. The conditioning procedure was as described previously. After conditioning to the mixture (1 μg of each component), bees were tested with the individual components (1 μg) presented randomly and with the conditioning mixture.

Electrophysiological Recordings. EAG recordings were obtained using the technique described for the coupled experiment. Dose–response curves were obtained for each component over a concentration range of 1–100 μg. Electrophysiological preparations were stimulated using the delivery system employed for the conditioned proboscis extension assay, except that the stimulus duration was 2 sec. All samples were presented twice to each preparation (total six bees) at intervals of 2 min. The EAG signals were amplified and recorded by standard methods (Wadhams et al., 1982).

RESULTS AND DISCUSSION

When bees were tested with the six-component mixture using the coupled GC-EAG-CPE technique, EAG responses were elicited by all the GC peaks

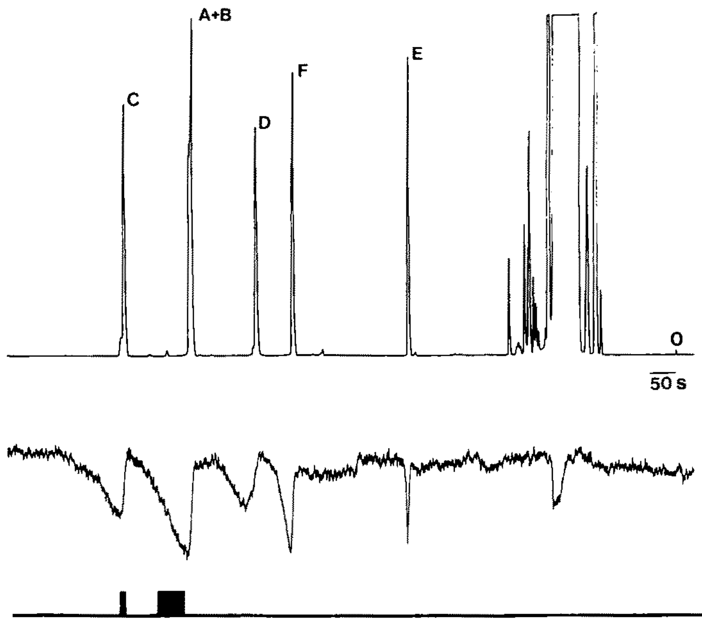

FIG. 1. Coupled GC-EAG-CPE recording on a conditioned honey bee using a six-component mixture (0.5 μg of each component injected onto GC column). Upper trace: FID response. Compounds are: A, linalool; B, 2-phenylethanol; C, methyl salicylate; D, benzyl alcohol; E, (*E*)-2-hexenal; F, 1-octen-3-ol. Middle trace: Simultaneous EAG response. Lower trace: Simultaneous CPE response.

(Figure 1). Under the GC conditions employed in this study, linalool and 2-phenyl ethanol were only partly resolved. EAG responses given by conditioned bees were larger than those elicited by the same stimulus concentration from unconditioned bees (Figure 2), although these differences were not significant. However, the means of the total EAG responses to test compounds were significantly different [conditioned ($\bar{X} \pm$ SE) = 0.37 $\pm$ 0.04 mV; unconditioned = 0.23 $\pm$ 0.02 mV; $P < 0.05$]. This suggests that antennal sensitivity is enhanced by conditioning. Similar results have been found with bees conditioned to violet and fennel odors (De Jong and Pham-Delègue, 1991), and changes in the EAG sensitivity of the parasitic wasp *Leptopilina heterotoma* were observed after an oviposition experience (Vet et al., 1990). EAG responses obtained with unconditioned bees in the standard procedure showed all six compounds to have significant activity, with linalool, 2-phenylethanol, methyl salicylate, and 1-octen-3-ol eliciting the greatest responses, particularly at high stimulus concentrations (Figure 3).

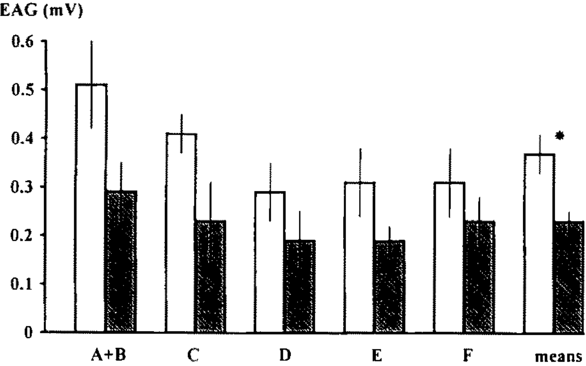

FIG. 2. EAG responses (±SE) to compounds A–F (see Figure 1) in the coupled GC-EAG-CPE assay: open columns, conditioned bees; shaded columns, unconditioned bees. *Means are significantly different at $P < 0.05$.

At the behavioral level, no proboscis extension response was obtained from any of the unconditioned bees, either in the coupled mode or when the compounds were presented in the standard CPE assay following GC stimulation. Of the six conditioned bees, only one (No. 5) did not respond to any of the individual compounds, presented either in the coupled mode or in the following standard CPE assay, although a response was still obtained to the conditioning mixture at the end of the testing protocol. The other five conditioned bees showed a close correlation between the CPE response to the components presented either in the standard CPE assay or in the coupled GC-EAG-CPE mode (Table 1). In the coupled mode, all five bees gave a response to the linalool/2-phenylethanol peak, two responded to benzyl alcohol, and one responded to methyl salicylate. When presented separately in the standard mode, linalool elicited a response in all five bees. Three of them also responded to 2-phenylethanol. Two of the bees (No. 1 and 2) showed different responses to benzyl alcohol in the coupled and standard CPE assays. This is consistent with the fact that in a previous study using the same mixture, this compound was poorly discriminated by the honey bees (Pham-Delègue et al., 1993). Overall, there was a good correlation between the responses of individual bees to the different components with the two stimulation techniques.

One hundred sixty-eight bees were subjected to the conditioning procedure prior to use in the standard CPE assay. Of these, 25% did not show any conditioned response during the conditioning trials, 28% responded to the control trial, and 24% did not respond to the conditioning mixture at the end of the testing. Therefore, the effectiveness of the conditioning in this study was poor, with only 23% of the bees being successfully conditioned to the six-component

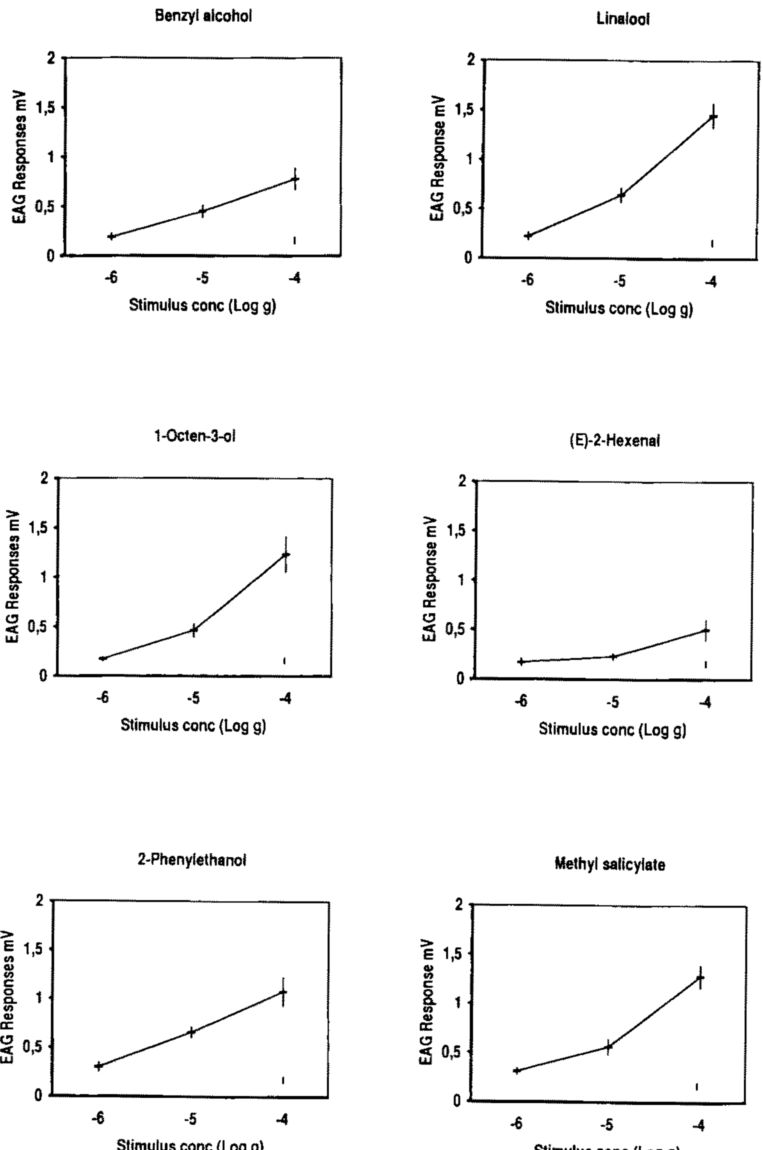

FIG. 3. EAG dose–response data for unconditioned bees stimulated with test compounds (see Figure 1) at concentrations ranging from 1 to 100 μg (means ± SE of six preparations. Responses to the solvent blank are shown in the lower right hand corner of each graph).

TABLE 1. CPE RESPONSES (CROSSES) OF SIX CONDITIONED HONEY BEES TO COMPOUNDS A–F[a] IN COUPLED BIOASSAY FOLLOWED BY STANDARD CPE ASSAY

	Responses to components										
	Coupled					Standard					
Bees	A + B	C	D	E	F	A	B	C	D	E	F
1	x	x				x		x	x		
2	x		x			x	x				
3	x					x	x				
4	x					x	x				
5											
6	x		x			x			x		
Total	5	1	2	0	0	5	3	1	2	0	0

[a]See Figure 1.

mixture. Of the 39 conditioned bees, nine did not respond to any of the compounds when they were presented individually and hence did not recognize the mixture on the basis of its individual constituents. The remaining 30 bees all showed CPE responses to one or more of the components, with the majority (10 individuals) responding to two. Linalool, methyl salicylate, and 2-phenylethanol were the most active, eliciting responses from 25, 17, and 13 bees, respectively. Despite the lower conditioning levels obtained in this study compared to those observed by Pham-Delègue et al. (1993) using the same mixture, overall response profiles of the conditioned bees were similar, with the six components being ranked in the same order of activity. These results also correlate well with the coupled GC-EAG-CPE data, with the linalool/phenylethanol peak eliciting the highest responses. Differences in the proportion of bees responding to methyl salicylate and benzyl alcohol probably relate to the small sample size.

Thus, consistent with data obtained from standard CPE procedures, the application of the coupled method confirms the occurrence of a hierarchy within the constitutive components of the conditioning mixture, with some components eliciting mixture recognition more effectively than others (Pham-Delègue et al., 1993). Moreover, presentation of the components via GC separation ensures that they are in the proportions occurring in natural plant volatile blends and also allows on-line purification, which, within the limits of column separation, permits avoiding interference from chemical impurities. However, as with the standard CPE technique, this mode of stimulation provides a sequential presentation of the components that does not allow evaluation of possible compo-

nent interactions (e.g., synergistic/antagonistic effects). To complement this work, further experiments with groups of components will be done.

In conclusion, this novel coupled system provides a useful tool for the investigation and characterization of key compounds involved in mixture recognition by honeybees. It will allow the relationship between olfactory detection and behaviorally salient cues to be assessed and the effect of learning on odor discrimination at both sensory and behavioral levels to be determined.

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SEQUESTRATION OF *Veratrum* ALKALOIDS BY SPECIALIST *Rhadinoceraea nodicornis* KONOW (HYMENOPTERA, TENTHREDINIDAE) AND ITS ECOETHOLOGICAL IMPLICATIONS

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Abstract—The larvae of the specialist sawfly *Rhadinoceraea nodicornis* Konow (Hymenoptera, Tenthredinidae) store in their hemolymph ceveratrum alkaloids originating from the host plant *Veratrum album* L. (Liliales, Melanthiaceae). The major alkaloid found in the hemolymph is 3-acetyl-zygadenine. Qualitative and quantitative data showed that the plant alkaloid 3-angeloyl-zygadenine is most probably metabolized in the larval gut to zygadenine and then acetylated. A still unidentified alkaloid with a molecular weight of 591 Da was detected in plant leaves as well as in the gut, hemolymph, and excrement of larvae. Protoveratrine A and B, on the other hand, seem to be degraded by the larvae. These findings indicate that the pathway of ceveratrum alkaloids in *R. nodicornis* larvae is fourfold: direct sequestration, metabolism followed by sequestration, excretion of intact alkaloids, and degradation. In contrast, no ceveratrum alkaloids were detected in the hemolymph and excrement of larvae of the generalist sawfly *Aglaostigma* sp. fed with *V. album* leaves. Bioassays with the ant *Myrmica rubra* L. proved that the hemolymph of *R. nodicornis* larvae is highly deterrent and toxic. In bioassays evaluating defensive efficiency against predators (ants, spiders, and bushcrickets), no larvae were eaten. Ceveratrum alkaloids were also detected in the hibernating prepupae of *R. nodicornis*. In feeding bioassays, the shrew *Crocidura russula* Hermann rarely fed upon prepupae, suggesting that this stage is also protected from predation to some degree. In field surveys, the only parasitoids recorded were two ichneumonid species that are believed to be specialized on *R. nodi-*

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cornis. Bioassays and field observations enable us to suppose that *R. nodicornis* and its enemies produce a food web of ion connectance.

Key Words—*Veratrum album*, ceveratrum alkaloids, plant–insect interactions, sequestration, hemolymph, feeding deterrence, *Rhadinoceraea nodicornis*, *Aglaostigma* sp., Hymenoptera, Tenthredinidae.

INTRODUCTION

Specialization relative to toxic plants offers the possibility of using plant secondary compounds for antipredator defense (Bernays and Graham, 1988). Alkaloids, which occur in many different plant taxa (Hegnauer, 1988), are an important class of plant toxins. Several specialist herbivores, and even a few generalists, have been reported to sequester the following alkaloids for defensive purposes: pyrrolizidine alkaloids (Aplin et al., 1968; Edgar and Culvenor, 1974; Rothschild et al., 1979; Brown, 1984; Boppré, 1986), quinolizidine alkaloids (Wink, 1992), and tropane alkaloids (Blum et al., 1981). Sequestration does not, however, seem to be a general feature of insects feeding on toxic plants (Rothschild, 1972; Bowers, 1990; Rowell-Rahier and Pasteels, 1992).

Veratrum alkaloids are a particularly toxic group of steroidal alkaloids (Fisher, 1940; Velbinger, 1947; Binns et al., 1972; Teuscher and Lindequist, 1987; Ujvary et al., 1991). Crude preparations of some of these agents have found practical application as insecticides (Crosby, 1971; Metcalf, 1977). Nevertheless, in Central Europe *Veratrum album* L. supports a herbivore community consisting of at least 29 insect species (Schaffner, in press). Insects living on *V. album* not only must handle the toxic plant compounds, but are also exposed to ants that frequently tend aphids on this plant. On *V. album*, the only specialist herbivore with free-living larvae is the sawfly *Rhadinoceraea nodicornis* Konow (Hymenoptera, Tenthredinidae). Encounters between ants and *R. nodicornis* larvae have been observed in the field. However, no predation took place on these occasions. To determine whether specialization is linked with the defense system of the larvae, we investigated the defensive ecoethology of *R. nodicornis* against natural enemies, as well as the chemical compounds involved in the defense system. We compared *R. nodicornis* with the generalist sawfly *Aglaostigma* sp., which also feeds on *V. album*.

METHODS AND MATERIALS

Host Plant and Veratrum *Alkaloids*. *V. album* (Liliales, Melanthiaceae) is a perennial monocotyledon that grows mainly on damp mountain grassland and in dwarf scrub zones of Europe and Asia. Individuals of this impressive, 50 to 175-cm-tall plant can live for several decades. At altitudes of about 1300 m,

the vegetative tips do not appear before late April. Flowering, which occurs about every four years, starts in late June. In September, the green parts dry up and the plants topple over. The vegetative period of *V. album*, therefore, last about four months.

The so-called *Veratrum* alkaloids are a group of steroidal alkaloids, which have been isolated from members of the related plant genera *Veratrum*, *Zygadenus*, and *Schoenocaulon*. The C_{27} alkamines fall into two groups: the jerveratrum group (two to three atoms of oxygen), which includes jervine, veratramine, rubijervine, and isorubijervine, and the ceveratrum group (seven to nine atoms of oxygen), which includes zygadenine, veracevine, germine, and protoverine (Kupchan et al., 1961). The jerveratrum alkaloids are found in the plant both as free alkamines and as glycoalkaloids. Ceveratrum alkaloids usually occur as esters and have never been found as glycosides. The esters of the ceveratrum group have been shown to be responsible for the acute toxicity of *Veratrum* plants (Bergmann et al., 1958; Ujvary et al., 1991). In rats, for example, the LD_{50} dose of the alkamine germine is 2 g/kg body weight, while that of the ester germerine is 3.7 mg/kg (Haas, 1938). Physiologically, ceveratrum alkaloids modify the properties of membrane sodium channels. Similar to pyrethroid insecticides and DDT, they induce repetitive firing, depolarizing afterpotentials, and/or maintained depolarizations (Honerjäger, 1982).

Powdered seed of *Schoenocaulon (Sabadilla) officinale* S. has been used against lice for several centuries. In Europe, this drug was first reported in the late 16th century (Velbinger, 1947). Interestingly, *Veratrum* alkaloids came into general use to control "gooseberry worms" (*Nematus* sp.) (Fisher, 1940), i.e., against a sawfly species. In 1945, more than 500,000 lb of powder containing ceveratrum alkaloids were used against crop pests in the United States (T.C. Allen, in Velbinger, 1947). Today, however, *Veratrum* alkaloids are rarely used.

Sawfly Life History. Field investigations on *R. nodicornis* and *Aglaostigma* sp. were carried out in the Swiss Alps (Grimsel, Berne; Col des Mosses, Vaud) and in the Jura mountains (Chasseral, Berne). The taxonomy of the latter has yet to be clarified, since rearing to adulthood did not succeed. In the field, adults of three different *Aglaostigma* species have been collected from *V. album*: *A. fulvipes* Scop., *A. pinguis* Klug, and *A. aucupariae* Klug.

The phenology and population density of *R. nodicornis* were monitored on two pastures at Chasseral. In 1991 and 1992, larvae and adults were counted repeatedly throughout the vegetative period on four plots of approximately 10 × 10 m. All larvae on plants surrounding the plots were removed in order to prevent immigration. The adults were counted early in the morning when they were sitting motionless on *V. album* plants.

To investigate the parasitoid complex and rate of parasitism of *R. nodicornis* larvae, 85 last-instar larvae (L5) were collected in a pasture at Chasseral, as well as 14 first-instar (L1), 10 third-instar (L3), 23 fourth-instar (L4) and 20

L5 larvae at Grimsel and Col des Mosses. In the laboratory, they were kept at 20°C and under long day conditions in boxes on a layer of moistened humus. The prepupae hibernated in the humus at 5°C and under short day conditions. In spring, the temperature was raised slowly to 20°C. All emerging insects were collected, and cocoons that were still intact in late July were opened and the contents inspected.

In order to determine the fecundity of *R. nodicornis*, freshly emerged females were held without their host plant to prevent oviposition and were fed with honeywater. As soon as the females died, they were dissected, and all developed and undeveloped eggs were counted.

The food intake of *R. nodicornis* larvae was assessed by keeping seven L1 larvae in separate boxes on a layer of moistened plaster of Paris–charcoal mixture. Leaf material was renewed every two days. The area of foliage consumed by each larval instar was measured by the video analysis software Java.

Insect and Plant Material. Veratrum leaves and larvae of *R. nodicornis* and of *Aglaostigma* sp. were collected at Chasseral and Grimsel. Plant samples were dried at 40°C and ground. Hemolymph was obtained from last-instar larvae by gently piercing the integument with forceps and collecting the drops of hemolymph that appeared with glass capillaries. The hemolymph was stored in EtOH at −20°C in the dark. For quantitative analyses, three groups of 15, 18, and 19 *R. nodicornis* larvae were reared from L1 to L5. The hemolymph of the L5 larvae as well as all excrement were collected. In addition, 20 larvae of *R. nodicornis* were carefully dissected. The hemolymph, as well as the integuments, guts, fat bodies, and salivary glands were stored as described above. Excrement and prepupae were dried at 40°C and then also stored at −20°C.

Alkaloid Extraction. For large-scale alkaloid extraction, 500 g of dried leaf material was used in 3 liters aq. H_2SO_4 (pH 2)–MeOH (2:8). After evaporating the MeOH, the acidic aqueous phase was washed with 3 × 600 ml diethylether. The pH of the aqueous phase was then adjusted to 9 and extracted with 3 × 600 ml CH_2Cl_2.

Evaporation of the CH_2Cl_2 extract resulted in 1.26 g of a dark yellow gum. The excrement, hemolymph, larval organs, and ground prepupae were extracted for alkaloids in the same way. For quantitative analysis, washing with diethylether was omitted in the extraction procedure, since the diethylether fraction contained traces of angeloyl-zygadenine and of a ceveratrum alkaloid with molecular mass of 591 Da.

Analysis of Alkaloid Fractions by Mass Spectrometry. Gas–liquid chromatography (GLC) has not yet been successfully applied to the analysis of original ceveratrum alkaloids, nor has it been used with derivatized ceveratrum alkaloids. Therefore, we analyzed the gum directly by mass spectrometry using electron impact ionization (EI), liquid secondary ion mass spectrometry (LSIMS), and tandem mass spectrometry (CID MIKES). Details of the mass spectrometric

analyses will be published elsewhere (Schlunegger et al., in preparation). LSIMS, tandem LSIMS, and tandem EI were recorded on a VG FISONS AUTOSPEC-Q mass spectrometer. For LSIMS, a cesium ion gun operated at 32 keV was used (matrix: dithiothreitol/dithioerythritol 5:1). Conventional mass spectra were recorded on a MAT-CH7A (direct probe inlet). Reference substances were donated by P. Honerjäger, Munich.

Purification of Alkaloids. Leaf alkaloids were isolated by preparative silica gel thin-layer chromatography (TLC) (cyclohexane–diethylamine–MeOH, 9:1:1), followed by high-performance liquid chromatography (HPLC). The TLC fractions were analyzed for alkaloids by LSIMS. Final purification was obtained by using a HPLC system from Pharmacia (LKB HPLC pump 2248, LKB low-pressure mixer, LKB variable-wavelength monitor 2142, PC software HPLC-Manager). The sample loop volume was 250 μl. Separation was performed on a Nucleosil 100-5C18 MPN reversed-phase column. The mobile phase was methanol–0.1 M ammonium acetate, pH 6.5 (60:40). The eluents were monitored at 220 and 254 nm. The components were identified by ^{1}H and ^{13}C NMR analyses. Both isolated alkaloids, 3-angeloyl-zygadenine and an unidentified alkaloid with a mass of 591 Da, have an angeloyl group that renders them UV-active.

Quantification of Zygadenine and Zygadenine Esters. Attempts to quantify zygadenine and 3-acetyl-zygadenine by GLC and HPLC were unsuccessful (Majak et al., 1992; our own experiments). Therefore, we used TLC for quantitative analysis. Following Majak et al. (1992), reference compounds and alkaloid extractions of hemolymph, prepupae, and excrement were applied to silica gel plates (Merck) and developed with freshly prepared solvent ($CHCl_3$—MeOH–25% aq. NH_3, 90:10:1). The dried plates were sprayed with conc. H_2SO_4—MeOH (1:9) and then heated in an oven at 100°C for 30 min. The spots were resuspensed in 1 ml MeOH. The fluorescence was measured in a Perkin Elmer LS-5b Luminescence spectrometer (emission wavelength 459 nm, excitation wavelength 290 nm; slit excitation 10 nm, slit emission 20 nm).

Defensive Efficiency of Sawflies Against Predators. Observations of predator–prey interactions were recorded in the laboratory. Single L3 and L4 larvae of both sawfly species were placed in a box with 20 ants (*Formica rufa* L., Formicidae; box size 10 × 10 cm), with a bushcricket (*Tettigonia cantans* Fuessly, Tettigoniidae; box size 11 × 16 cm), or with a spider (*Cupiennius salei* Keys. Ctenidae, instar IV; box diameter 6 cm), and were observed for 30 min. After being exposed to these predators for 16 hr, the state of the larvae was recorded. Ant and tettigoniid species are sympatric with the sawfly, whereas the spider species originates from Central America and is kept in our laboratory as a permanent breeding stock. In the boxes, the larvae were offered a small piece of a *Veratrum* leaf. For each sawfly and predator species, the test was repeated 12 times.

Prepupae of *R. nodicornis* were tested with 12 subadult shrews (*Crocidura russula* Hermann, Soricidae): six females (9.8–13.2 g) and six males (10.4–14.5 g). Each shrew was left one night in a box (16 × 12 cm) with water, 2 g meat and 2 g live mealworms (*Tenebrio molitor* L.). Feeding this amount ensured that the shrews remained hungry without being starved (D. Cantoni, personal communication). During the same night, they also received a cocoon containing a sawfly prepupa and a prepupa without its cocoon. The next morning, the number and state of the sawflies were examined. Some direct observations of shrews feeding on prepupae also were recorded.

Deterrent Activity of Hemolymph and Alkaloids. Hemolymph was collected from both the generalist and specialist sawfly species feeding on *V. album* and, for comparison, from another sawfly species of the genus *Rhadinoceraea* (*R. micans* Klug) as well as from two species that are known to be unpalatable to predators: *Athalia rosae* L. (Nagasaka, 1991) and *Croesus septentrionalis* L. (Boevé, 1991). The hemolymph–EtOH solution was filtered, the solvent evaporated under N_2, and the dried extract redissolved in 0.1 M sucrose in order to obtain a test solution. A complete alkaloid fraction was obtained from hemolymph of *R. nodicornis* and from *V. album* leaves as described above. These dried fractions and the alkaloids protoveratrine A and B, 3-angeloyl-zygadenine, 3-acetyl-zygadenine, and zygadenine were dissolved in a small amount of ethanol to facilitate later dissolution in water and then adjusted with 0.1 M sucrose in order to obtain the test solution. The final concentration of ethanol never exceeded 5%.

The deterrent activity was assessed by a bioassay described in Pasteels et al. (1986). Fifty *Myrmica rubra* L. workers were placed in a Petri dish (14 cm diameter) and starved for one night. Then 50 μl of a control solution (0.1 M sucrose) and 50 μl of the test solution were offered simultaneously to the ants. The initial concentration of a test solution was either 0.4 mg dry hemolymph/50 μl, or 0.4 mg alkaloids/50 μl. The amount of 0.4 mg dry hemolymph is roughly the equivalent found in a single larva. The test solution was diluted logarithmically until no significant difference in the distribution of ants on control and test solutions was observed (12 replications pooled). The number of drinking ants was recorded after 5 min; 5% ethanol in 0.1 M sucrose did not deter the ants.

Toxicity of Hemolymph of R. nodicornis. In a bioassay described previously (Pasteels et al., 1986), *M. rubra* workers were obliged to ingest the hemolymph extract of *R. nodicornis* at a concentration of 0.04 mg/50 μl (see above). During five days, 50 ants received only 1 μl of this solution per ant and per day; 50 other ants received 1 μl of the control solution. In both test groups, the ants drank all solution offered, probably because no other water source was provided during the five days and because the hemolymph extract was offered at a relatively low concentration. Mortality was recorded each morning.

RESULTS

Biology of Sawfly Species. R. nodicornis is univoltine with adults appearing at 1000 m in May, and at higher altitudes in June. Males have been observed only occasionally. At Chasseral, the sex ratio was about 1 : 7. The total number of adults during the flight period was about eight per 1000 plants. Compared to other sawfly species, the number of eggs produced per female (Table 1) is low (Benson, 1950; Schedl, 1991; Boevé, unpublished data). Usually, there is a discrepancy between the number of eggs produced and those actually laid (Benson, 1950). In fact, in captivity four *R. nodicornis* females only laid a total of 84 eggs.

Due to the oviposition behavior of the females, L1 and L2 larvae occurred both singly and in aggregations (Table 1) and were not found on plants where ants tended aphids (see below). L3 larvae spent the majority of time in the moist

TABLE 1. BIOLOGY OF *Rhadinoceraea nodicornis* AND *Aglaostigma* sp.

	R. nodicornis	*Aglaostigma* sp.
Coloration		
Adults	black with subhyaline wings	unknown
Larvae	grey with black head	greenish-white
Phenology		
Adults	males in May/June, females from May to July	
Larvae	June to early September	June to early September
Prepupae	one to several winters, in a cocoon in the soil	
Pupae	in spring	
Potential reproduction rate	42.5 ± 9.7 eggs/female (N = 22)	
Oviposition site	in leaf folds of *Veratrum* plants; singly, but often several into the same leaf fold.	not observed on *Veratrum*
Egg stage	7–12 days in the laboratory	
Larval development	five larval instars; 21–25 days in the laboratory, about one month in the field	five larval instars
Feeding specifity	on *Veratrum* spp.	polyphagous
Food intake per larva	187.0 ± 39.5 mg dry weight (N = 7)	
Maximal density of larvae	6.6 ind./10 plants	

litter layer of the soil. While on the host plant, they were observed mainly on the undersurface of the lowest leaves. In contrast, L4 and L5 larvae spent most of their time singly, in exposed positions on the youngest leaves at the top of the plants, i.e., up to 100 cm above the soil. L4 and L5 larvae were also found on plants occupied by ants, suggesting dispersal behavior by the L3 larvae. During the last two instars, *R. nodicornis* larvae consumed 90% of the total larval food intake (Table 1). After feeding was completed, the larvae molted to the nonfeeding prepupa, dropped to the ground, and entered the soil.

The mean density of L1 and L2 larvae was 36.2 ± 20.7 ($N = 4$), while that of L4 and L5 larvae was 31.0 ± 26.7 ($N = 4$) per 100 plants, suggesting that approximately 86% of L1 and L2 larvae reached the last larval instar. About 15% of L5 larvae were parasitized (see below). The low density of adults found in the field indicates that from unparasitized L5 larvae to the next generation of adults, there is a decrease in the *R. nodicornis* population density of more than 90%.

In no-choice feeding experiments, *Aglaostigma* sp. larvae fed and developed on *V. album* and on other plant species occurring in their habitat such as *Taraxacum officinale* Weber, *Rumex* sp., *Plantago lanceolata* L., and *Alchemilla vulgaris* L. (Schaffner, in press). Thus, *Aglaostigma* sp. is polyphagous. Therefore, it was not possible to estimate total larval density. The maximum density of *Aglaostigma* sp. found on *V. album* was 21 larvae/100 plants. On *V. album*, they were predominantly observed on the undersurface of the lower, i.e., mature, leaves. They were never found exposed and easily visible as were the last-instar larvae of *R. nodicornis*.

Behavioral Responses of Larvae and Adults to Attack in the Field. On the host plant, larvae of *R. nodicornis* behaved in a sluggish manner. When attacked by ants or spiders, they did not show any escape behavior; rather, they fixed themselves to a leaf with their mandibles and remained motionless. The passive behavior of the larvae enabled them, even in the presence of ants, to spend a long time feeding 80–100 cm above ground on the young, nutritious leaves of *V. album*.

In contrast, larvae of *Aglaostigma* sp. were very mobile and easily disturbed. When approached by ants, they curled up, discharged a green fluid orally and dropped off the plant. During the field survey, larvae were frequently observed dropping off the plant when we were still half a meter away from the plant. As a result of this escape behavior, larvae of *Aglaostigma* sp. had to climb up a plant repeatedly in order to resume feeding. This they did predominantly on the lower, mature leaves.

Adults of *R. nodicornis* were never observed on *V. album* plants occupied by ants. To check this observation, females were transferred onto plants inhabited by ants and aphids. In eight out of 10 cases, the adults were attacked within half a minute and seriously injured. In the laboratory, oviposition took 40–130

TABLE 2. INSECT EMERGENCE AND CONTENT OF REMAINING COCOONS AFTER HIBERNATION OF 85 *Rhadinoceraea nodicornis* LARVAE COLLECTED AT INSTAR L5 IN THE FIELD[a]

	N	% of larvae
Emerged sawfly adults	43	50.6
Emerged parasitoid adults:		
Hodostates sp.	8	9.4
Mesoleius sp.	3	3.5
Content of remaining cocoons:		
Dead sawfly adults	3	3.5
Prepupae in diapause	6	7.1
Desiccated prepupae	21	24.7
Prepupae infected by fungi	1	1.2
Total	85	100

[a]The content of the remaining cocoons was checked one month after emergence of the last sawfly adult.

sec/egg. Therefore, females would be endangered if they would try to oviposit on plants occupied by ants.

Parasitoid Complex and Rate of Parasitism of R. nodicornis *Larvae.* Collections from different localities provided two ichneumonid species that attack *R. nodicornis* larvae. At Chasseral, the total rate of parasitism on all L5 larvae collected was 12.9%, while that of emerged adults was 20.4% (Table 2). Only larvae collected at the L4 and L5 stages were parasitized. The parasitoids are undescribed ichneumonid species of the genera *Hodostates* and *Mesoleius*, neither of which has yet been recorded from other hosts (R. Hinz, personal communication). This suggests that they are specialists attacking exclusively *R. nodicornis*.

Defensive Efficiency of Sawflies against Predators. In the bioassays, *R. nodicornis* larvae were never attacked by ants or spiders (Table 3). Some ants explored the larvae with the antennae, but retreated after a while and cleaned their antennae. Others were seen to walk over the larvae without stopping. Spiders were observed to make a rapid movement in the direction of the larvae, but stopped just before biting the larva. Tettigoniids, on the other hand, bit several larvae, which subsequently died due to gut injuries, but none were swallowed (Table 3). The only occasion on which predation of *R. nodicornis* was observed during the bioassays was by shrews on prepupae. After 16 hr, shrews had eaten three prepupae, bitten two, and left seven uninjured. Four cocoons were also bitten by shrews after 16 hr, but not eaten. In all experiments, mealworms were quickly eaten.

TABLE 3. NUMBER OF LARVAE OF *Rhadinoceraea nodicornis* AND *Aglaostigma* sp. EATEN, BITTEN, OR ONLY TOUCHED DURING FIRST 30 MINUTES AND NUMBER OF LARVAE ALIVE AFTER 16 HOURS OF EXPOSURE TO ANT *Formica rufa*, SPIDER *Cupiennius salei*, AND BUSHCRICKET *Tettigonia cantans*[a]

	R. nodicornis				*Aglaostigma* sp.			
	First 30 min				First 30 min			
Predator species	Eaten	Bitten	Touched	Alive after 16 hr	Eaten	Bitten	Touched	Alive after 16 hr
Formica rufa	0	0	12	12	0	8	4	3
Tettigonia cantans	0	7	5	2	10	1	1	0
Cupiennius salei	0	0	12	12	7	0	5	1

[a]For each sawfly and predator species, the test was repeated 12 times.

In contrast to *R. nodicornis*, most larvae of *Aglaostigma* sp. were attacked by ants and spiders within the first 30 min of the experiment (Table 3). After 16 hr, the ants had killed and fed on most larvae; the spiders had digested almost all. Of all the predators used in the bioassays, tettigoniids attacked and ate the larvae most quickly. Nevertheless, feeding on *V. album* offered larvae of *Aglaostigma* sp. a certain protection, since the first attack by an ant during the bioassays was generally repulsed by the orally emitted green fluid. This fluid probably contained *Veratrum* alkaloids. Accordingly, the ants showed poisoning symptoms such as reeling and staggering. However, *Aglaostigma* sp. larvae were defenseless against subsequent attacks in the bioassays. In the field, larvae would escape from further attacks by dropping off the plant.

Fate of Veratrum *Alkaloids in Larvae of* R. nodicornis *and* Aglaostigma *sp.* Analyses by mass spectrometry and tandem mass spectrometry (CID MIKES) showed that ceveratrum alkaloids are present in the hemolymph and excrement of larvae as well as in prepupae of *R. nodicornis* (Table 4). No alkaloids were detected in the integuments, fat bodies, or salivary glands of the larvae. The qualitative composition of ceveratrum alkaloids in *V. album* and in the hemolymph and excrement of *R. nodicornis* were not identical. Protoveratrine A and B were detected in the leaves, but not in the excrement or in the hemolymph. Moreover, all three samples contained different major components: 3-angeloyl-zygadenine being a major component in the leaves, zygadenine in the excrement, and 3-acetyl-zygadenine in the hemolymph of *R. nodicornis* larvae (Table 4). In the gut, both zygadenine and 3-acetyl-zygadenine were detected, but not 3-angeloyl-zygadenine. LSIMS spectra of the different samples will be provided elsewhere (Schlunegger et al., in preparation).

A signal at m/z 592 (m/z 591 protonated) was detected upon LSIMS in

TABLE 4. OCCURRENCE OF CEVERATRUM ALKALOIDS IN *Veratrum album* AND IN *Rhadinoceraea nodicornis*[a]

Alkaloids	*V. album* leaves[c]	*R. nodicornis* Larvae Gut[d]	Hemolymph[e]	Excrement[e]	Prepupae[d]
3-Angeloyl-zygadenine	1.77 ± 0.19			(+)[f]	
3-Acetyl-zygadenine		0.98 (2.9 μg)	0.70 ± 0.29[b] (14 μg)	(+)[f]	
Zygadenine		1.33 (3.9 μg)	0.48 ± 0.43[b] (9.6 μg)	0.24 ± 0.16 (20.1 μg)	0.90 (7.6 μg)
Ceveratrum alkaloid, 591 Da (not UV-active)	+ + + +	+ +	+ + +	+ + + +	+
Ceveratrum alkaloid, 591 Da (UV-active)	+ +				
Protoveratrine A	+				
Protoveratrine B	+				

[a] All data in μg/mg dry weight ± SE; except for [b] given in μg/μl. Plus marks indicate proportion of total alkaloid extract: from + + + + (the major component) to + (minor component). Values in parentheses: total amount per larva or prepupa.
[c] Three samples of 2 g of dried leaf material were extracted and analyzed.
[d] Twenty individuals were combined and analyzed in bulk.
[e] Three lots of 15, 18, and 19 larvae were combined and analyzed.
[f] Traces of these alkaloids were occasionally detected upon LSIMS.

Veratrum foliage, as well as in the hemolymph and excrement of *R. nodicornis* larvae. Tandem mass spectrometric analyses (electron impact ionization) of these ions provided identical spectra of the signal at *m/z* 591 in *Veratrum* leaves and in the hemolymph of *R. nodicornis*. However, LSIMS analyses of HPLC fractions revealed another ceveratrum alkaloid with a mass of 591 Da in the leaves that was not detectable in the hemolymph. Having a labile angeloyl group, this UV-active compound was not detectable by EI. Hence, the signal at *m/z* 592 (*m/z* 591 protonated by LSIMS) in the *Veratrum* leaf extract is composed of at least two ceveratrum alkaloids. The UV-active alkaloid seems to be metabolized by *R. nodicornis* larvae, while the non-UV-active compound is sequestered in the hemolymph in its original form.

Prepupae of *R. nodicornis* contain zygadenine and traces of a non-UV-active alkaloid with a mass of 591 Da. 3-Acetyl-zygadenine, the major com-

ponent in the larval hemolymph, was detected only occasionally and in very low concentration in the prepupae.

No ceveratrum alkaloids were detected upon LSIMS in the larvae and excrement of *Aglaostigma* sp. In the excrement extract, a signal at *m/z* 546 (*m/z* 545 protonated by LSIMS) was detected and shown to be identical with the corresponding signal in the excrement of *R. nodicornis*. As the CID MIKE spectrum of the signal at *m/z* 546 did not show characteristic fragmentation patterns of either ceveratrum alkaloids (e.g., no *m/z* 112 fragment) or of jerveratrum alkaloids (e.g., no *m/z* 114 fragment), we conclude that the ion with a mass of 545 Da is not a *Veratrum* alkaloid.

Quantitatively, 3-acetyl-zygadenine is the major alkaloid in the hemolymph of *R. nodicornis* larvae. With a hemolymph volume estimated at 20 μl/larva, one individual stores 14 μg of 3-acetyl-zygadenine in its hemolymph. The total amount of zygadenine and 3-acetyl-zygadenine found in the hemolymph (24 μg) was similar to the amount of zygadenine measured in the excrement collected during the total life of a larva (20 μg). The total amount of alkaloids in the different samples was not determined, since these extracts probably contained substances other than *Veratrum* alkaloids (see above).

Deterrent Activity of Hemolymph and Alkaloids. In Table 5, the deterrent activities of the hemolymph of different sawfly species and that of several ceveratrum alkaloids are given. At the lowest concentration tested, the hemolymph of *Aglaostigma* sp. did not act as a deterrent against ants. In contrast, the hemolymph of *R. nodicornis* was highly deterrent and had to be diluted 10^4 times more than the hemolymph of *Aglaostigma* sp. before it was rendered ineffective. The hemolymph of *R. micans*, whose larvae feed on the toxic *Iris pseudacorus* L. (Lorenz and Kraus, 1957), as well as the hemolymph of *Athalia rosae* and *Croesus septentrionalis*, whose larvae are unpalatable to predators (Nagasaka, 1991; Boevé, 1991), also deterred ants, but to a much lesser degree than that of *R. nodicornis*.

The alkaloid extracts of *R. nodicornis* hemolymph and of *V. album* leaves had a similar deterrent effect (see Table 5). On the other hand, the concentration at which less than 10% of the workers drank the alkaloid extract of the hemolymph was 10 times lower compared to the solution containing the whole hemolymph extract of *R. nodicornis*. The pure alkaloids tested can be divided in three groups with a decreasing deterrent effect on ants: first the tetraesters protoveratrine A and B, then the monoesters 3-angeloyl-zygadenine and 3-acetyl-zygadenine, and finally zygadenine.

Toxicity of Hemolymph of R. nodicornis. During a five-day period, ants receiving sucrose solution showed a daily mortality of 2, 9, 4, 4, and 3 ants on each successive day. In contrast, a solution of sucrose plus hemolymph of *R. nodicornis* resulted in a mortality of 14, 28, 5, 2, and 1 ants, respectively. Thus, 11 of 50 ants died after receiving sucrose solution for two days, whereas during

TABLE 5. DETERRENT ACTIVITY OF HEMOLYMPH OF SAWFLY LARVAE AND ALKALOIDS AGAINST ANT *Myrmica rubra*[a]

	Concentration (mg/50 μl solution)[b]				
Test objects	4×10^{-1}	4×10^{-2}	4×10^{-3}	4×10^{-4}	4×10^{-5}
Aglaostigma sp., H	50 (186)				
Rhadinoceraea nodicornis, H	0* (82)	11* (132)	28* (157)	43* (210)	51 (186)
Rhadinoceraea micans, H	15* (62)	41* (148)	52 (199)		
Athalia rosae, H	1* (121)	22* (154)	54 (140)		
Croesus septentrionalis, H	34* (144)	46 (112)			
Rhadinoceraea nodicornis, HA		2* (116)	30* (82)	53 (174)	
Veratrum album, LA		0* (86)	41* (139)	53 (168)	
Protoveratrine A&B (1 : 1)			5* (82)	37* (104)	45 (124)
3-Angeloyl-zygadenine		8* (107)	40* (139)	55 (143)	
3-Acetyl-zygadenine		7* (68)	42* (126)	51 (128)	
Zygadenine		18* (119)	53 (127)		

[a] Percentages of ants drinking a solution containing a biological extract or alkaloids, compared to a control. Number of ants in parantheses. $*P < 0.05$, Wilcoxon matched-pairs signed-ranks test. H = hemolymph extract; HA = extract of hemolymph alkaloids; LA = extract of leaf alkaloids.
[b] Milligrams of dry extract when sawfly or plant is tested.

the same period 42 of 50 ants died after ingesting individually about 1.6 ng dry hemolymph of *R. nodicornis* ($P < 0.001$, Fisher exact probability test).

DISCUSSION

R. nodicornis is the first insect species known to us that is able to sequester ceveratrum alkaloids. In the larvae, the fate of the alkaloids is fourfold: sequestration of either original (non-UV-active ceveratrum alkaloid with a mass of 591 Da) or metabolized alkaloids (3-acetyl-zygadenine), degradation (protoveratrine A and B), and direct excretion (Table 4). Thus, the mechanisms of alkaloid metabolism in the gut of the larvae are complex. A general degradation mech-

anism for ceveratrum alkaloids seems to be the splitting of the angeloyl group, since both plant alkaloids 3-angeloyl-zygadenine and the UV-active ceveratrum alkaloid with a mass of 591 Da, which also has an angeloyl group, were not detectable in the larvae or in their excrement. In the case of 3-angeloyl-zygadenine, splitting of the angeloyl group leads to the alkamine zygadenine. The acetylation of zygadenine is probably the fundamental aspect of the sequestration mechanism of *R. nodicornis*, since 3-acetyl-zygadenine is more lipophilic than zygadenine and may therefore more easily pass through the gut epithelium. Moreover, 3-acetyl-zygadenine is more deterrent to ants than zygadenine (Table 5).

Of the tested ceveratrum alkaloids, the tetraesters protoveratrine A and B showed the most deterrent effect against ants, followed by the monoesters 3-acetyl-zygadenine and 3-angeloyl-zygadenine. Our results on the deterrency coincide with results on the toxicity of ceveratrum alkaloids found by Bergmann et al. (1958) and Ujvary et al. (1991). These authors showed that the more ester groups ceveratrum alkaloids contain, the more toxic they are. In this context, it is surprising that the tetraesters protoveratrine A and B are degraded, rather than sequestered, by *R. nodicornis* larvae. One may speculate that the toxicity of protoveratrine A and B exceed the tolerance limit of *R. nodicornis* larvae.

In contrast to the specialist *R. nodicornis*, the polyphagous larvae of *Aglaostigma* sp. seem to degrade all ingested ceveratrum alkaloids. Neither original alkaloids nor typical fragments were detectable in the excrement. Nevertheless, feeding on *V. album* offers the polyphagous larvae some degree of protection when emitting orally a deterrent fluid, which probably contains ceveratrum alkaloids.

In our experiments, ant and spiders did not bite *R. nodicornis* larvae. As ceveratrum alkaloids were not detectable in the thin integument of the larvae, the predators must perceive deterrent volatiles. It may be possible that nonpolar ceveratrum alkaloids can pass through the integument and may be moderately volatile. Tettigoniids bit the larvae, but then refrained from eating them, probably due to direct contact with the deterrent hemolymph. In natural, the larvae are most frequently confronted by spiders and by ants, which tend aphids on *V. album*. Hence, the chemical defense of the larvae may serve predominantly as a protection against these two predator groups. We did not bioassay the reaction of birds towards *R. nodicornis* larvae, since birds were never observed searching for insects half a meter or more above the ground on *Veratrum* plants. Furthermore, the absence of aposematic coloration of the larvae (Table 1) suggests that visually searching predators may not be of primary importance.

Unlike *Aglaostigma* sp., injuries to the integument and loss of hemolymph do not lead to the death of *R. nodicornis* larvae. In comparison to numerous sawfly species examined by us, *R. nodicornis* larvae are particularly fragile and are easily wounded. Such an easy release of hemolymph occurs sporadically in

the subfamilies Blennocampinae and Selandriinae. For example, it is known from *Rhadinoceraea micans* Klug, *Phymatocera aterrima* Klug (J.-L. Boevé, personal observation), *Strongylogaster lineata* Christ, and *Aneugmenus* spp. (Heads and Lawton, 1985). In all these sawfly species, easy release of hemolymph upon wounding is linked with deterrency or toxicity.

The sequestration of ceveratrum alkaloids imparts a highly potent defense to *R. nodicornis* larvae. Even a 1000-fold dilution was deterrent against ants. When the hemolymph of other sawfly species was tested, the threshold values of feeding deterrency to ants were achieved when the extract was diluted 10- to 100-fold (Table 5). Using the same test, Pasteels et al. (1988) determined threshold values for several defensive compounds of chrysomelid beetles. These values are comparable with those found for the sawflies *R. micans*, *Athalia rosae*, and *Croesus septentrionalis*. All these chrysomelid and sawfly species have been shown to be well defended against ants. Hence, *R. nodicornis* larvae store in their hemolymph more ceveratrum alkaloids than needed for an effective defense against ants. This may indicate that the highly potent defense is not primarily directed at ants, but at other biotic factors such as predators not investigated here, parasitoids and fungi, or at abiotic factors.

An alternative explanation for the highly potent defense in *R. nodicornis* larvae is that the more alkaloids sequestered during the larval stage, the better the survival chance during the prepupal stage. In fact, changes in qualitative (i.e., zygadenine instead of 3-acetyl-zygadenine) and quantitative alkaloid content (Table 4) could reduce the defensive efficiency of prepupae to a critical level. The reduced chemical defense during the prepupal stage coincides with the large fall in the population of *R. nodicornis* observed between autumn and spring. Therefore, one may assume that predation could be a major mortality factor for prepupae. However, bioassays showed some defense of *R. nodicornis* prepupae against shrews. Since *Veratrum* alkaloids are also known to have antimicrobial effects (Teuscher and Lindequist, 1987), we suspect that abiotic factors could be mainly responsible for prepupal mortality. Climatic conditions, such as low humidity, generally play an important role in the prepupal mortality of sawflies (Benson, 1950).

From a taxonomic point of view, it is remarkable that all species of the genus *Rhadinoceraea* known to us are specialists feeding on toxic plants. In Europe, *R. micans* and *R. reitteri* Konow live on *Iris* spp. and *R. ventralis* Panzer on the alkaloid-containing *Clematis* spp. (Lorenz and Kraus, 1957). Known hosts of North American species are *Veratrum* spp. for *R. aldrichi* MacGillivray, *R. insularis* Kincaid, and *R. nubilipennis* Norton; *Zygadenus* spp. for *R. zigadenusae* Smith; and *Calochortus* sp. for *R. nigra* Rohwer (Smith and McDearman, 1990). Smith and McDearman (1990) suggest that the alkaloid phytochemistry of the plant tribe Veratreae, which includes *Zygadenus* and *Veratrum*, may be involved with sawfly speciation in *Rhadinoceraea*. Our findings

point in the same direction in that they imply a strong relationship between *R. nodicornis* and *V. album*. We suspect that sequestration is a common feature in the genus *Rhadinoceraea*, presumably tracing back to a common ancestor that evolved the mechanism of sequestration. The deterrent effect of the hemolymph of *R. micans* larvae (Table 5) collected from *Iris pseudacorus* supports this hypothesis.

Our data suggest that the specialist *R. nodicornis* and its enemies produce a food web with a low number of connecting links. On *V. album*, there is no competition for food between folivorous herbivores (Schaffner, in press). Field observations and bioassays confirmed that predation on *R. nodicornis* larvae should be very low. Moreover, the two parasitoids recorded are probably monophagous. Price et al. (1980) predict that specialized herbivores feeding on toxic plants and their enemies may produce food webs of low connectance and that such food webs should be relatively stable. However, the degree of stability of a food web depends also on the stability of the environment (Price et al., 1980). Since *V. album* plants can live for several decades, and the pastures are maintained in a pseudostable state, the small food web built by *R. nodicornis* and its enemies meets the requirements for stability. The low reproductive potential of *R. nodicornis* may be interpreted as an indication for stable conditions. Further work will be necessary to determine the reasons for the high prepupal mortality and to confirm the stability of the food web produced by *R. nodicornis* and its enemies.

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OLFACTORY RECEPTION OF POTENTIAL PHEROMONES AND PLANT ODORS BY TARNISHED PLANT BUG, *Lygus lineolaris* (HEMIPTERA: MIRIDAE)

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Abstract—Olfactory reception of potential pheromones and host-plant odors by male and female tarnished plant bugs (TPBs), *Lygus lineolaris* (Hemiptera: Miridae), was investigated by utilizing electroantennogram (EAG) techniques. In general, EAGs were similar between the sexes. Among 31 compounds of seven chemical groups tested, insect-produced butyrates and host-plant-containing green leaf volatiles (GLVs) were the most active. Hexyl butyrate and (*E*)-2-hexenyl butyrate elicited greater EAGs in males than in females. Females responded with significantly greater EAGs to alcohol and aldehyde GLVs than to their acetate derivatives. Among GLVs, sexual dimorphism was also observed in response to (*E*)-2-hexenol and (*E*)-2-hexenal. Females were more sensitive to the monoterpene geraniol than were males. While nonanal was the most stimulatory compound tested, no sexual differences in EAGs to this compound were observed. These studies reveal olfactory receptors on TPB antennae responsive to insect and host-plant volatiles that are likely to play a role in host finding and sexual attraction.

Key Words—*Lygus lineolaris*, olfaction, pheromone, host odor, electroantennogram, EAG, butyrates, green odor, host finding, sex attraction.

INTRODUCTION

The tarnished plant bug (TPB), *Lygus lineolaris* (Palisot de Beauvois), is a serious pest recorded on 169 plant species belonging to 36 families in the Mis-

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sissippi River delta of Mississippi, Louisiana, and Arkansas (Snodgrass et al., 1984). TPBs proliferate in spring on a variety of weeds with successive generations often migrating to cotton and other economically important crops (Womack and Schuster, 1987; Fleischer and Gaylor, 1987) where they cause damage by feeding on growing terminals and fruiting buds (Scales and Furr, 1968). Insecticide application on cotton to contain TPB populations also reduces numbers of parasites and predators, which allows other cotton pests, such as the tobacco budworm, *Heliothis virescens* (Fabr.), to reach economic damage levels (Gueldner and Parrott, 1978).

Synthetic attractants for *Lygus* bugs could play an important role in management programs of these polyphagous pests. The attraction of TPB males to traps baited with virgin females was first demonstrated in the field by Scales in 1968, and later by Blumenthal (1978) and Slaymaker and Tugwell (1984). Attraction of males by conspecific and congeneric females was studied by Graham (1987) in four *Lygus* spp.: *L. lineolaris*, *L. hesperus*, *L. desertinus*, and *L. elisus*. In sexual attraction studies of *L. hesperus*, removal of antennal flagella of males or isolating them from female odors eliminated attraction of males to females (Graham, 1988). Female TPBs contain equivalent amounts of *n*-hexyl butyrate and (*E*)-2-hexenyl butyrate, but males contain much less of the unsaturated ester (Gueldner and Parrott, 1978). Field responses of TPBs to these and other esters were not statistically significant (Hedin et al., 1985). Another study of airborne extracts demonstrated sexual dimorphism in which the ratio of (*E*)-2-hexenol to 1-hexanol appears to be greater than the relative differences in the concentrations of esters in males and females (Aldrich et al., 1988).

The neural basis of reception of plant odors and pheromone components by Hemiptera is poorly known. Olfactory responsiveness of a single neuron of *Triatoma infestans* to human breath was the first report on bug olfaction (Mayer, 1968). Electrophysiological studies on *Oncopeltus fasciatus* revealed that adult milkweed bugs have olfactory receptors on their antennae that respond to host plant odors (Pantle and Feir, 1976). The purpose of this study was to use electroantennograms (EAGs) to investigate peripheral olfactory responsiveness of adult *L. lineolaris* to potent pheromones and plant odors. This investigation was further intended to provide a basis for studies of single-receptor neuron responses of TPBs.

METHODS AND MATERIALS

Insects. Adults of *Lygus lineolaris* were obtained from a laboratory colony maintained at the USDA, Southern Insect Management Laboratory, Stoneville, Mississippi. Upon arrival, insects were segregated by sex, placed in separate plastic cups, and fed broccoli florets. Insects were held in an incubator pro-

grammed at 25°C temperature and a photoregime of 14 hr of light and 10 hr of darkness. The sexes were separated for at least two days prior to use.

Olfactory Stimuli. Odorants used as olfactory stimuli, their sources and purities are listed in Table 1. Test compounds were selected based on their presence in *L. lineolaris* or its host plants (see Table 1 for references). Stimulus dilutions in nanograde hexane were delivered from glass odor cartridges (80 mm long × 5 mm ID) as 1-μl aliquots on Whatman No. 1 filter paper (7 mm × 18 mm). These odor cartridges were oriented towards the preparation from 1 cm. Odor molecules evaporating from the filter paper were carried over the preparation by dry, charcoal-filtered, hydrocarbon-free air. Stimulus duration was 1 sec during the first series of experiments, and 0.5 sec for dose-response studies. Interstimulus time intervals of 2–3 min allowed for recovery of the EAG. The atmosphere around the preparation was continuously exhausted. Because of the variation in volatility of test compounds, only relative comparisons can be made between the odorous stimuli except for closely related compounds.

Electrophysiological Recording System. Electroantennogram (EAG) techniques utilized in these studies were a modification of a previous technique used by Schneider (1957a) and are described in detail elsewhere (Payne, 1970; Dickens and Payne, 1977; Dickens, 1984). In general, Ag–AgCl capillary electrodes filled with *Drosophila* Ringer were used. Intact bugs were immobilized on a cork block using adhesive tape. The recording electrode was inserted into the distal region of the terminal antennal segment, while the indifferent electrode was positioned into the scape. The electrodes were placed into the antenna after puncturing with an electrolytically sharpened tungsten wire. The signal was amplified 100× by a Grass P-16 microelectrode DC amplifier and viewed on a Tektronix 5111A storage oscilloscope. EAGs were recorded on a Houston Instruments strip-chart recorder.

Experimental Protocol. In order to elucidate the selectivity and sensitivity of the antennal receptors of the TPB for potential pheromones and plant odors, two series of experiments were performed. In the first series of experiments, the general responsiveness of the antennal receptors to the individual odorants was measured by recording EAGs to volatiles emanating from a 100-μg stimulus load of each. Presentation of each odorant was randomly ordered for each preparation.

In the second series of experiments, nine odorants were selected for closer examination based on data obtained in initial experiments. Dose–response curves were constructed from EAGs elicited by serial dilutions of each compound (0.001–100 μg/μl). Serial dilutions were presented in order from the lowest to the highest dose. Six replicates for each sex (12 insects total, six males and six females) were recorded for both experimental series. Control stimulations (using filter paper impregnated with 1 μl of the hexane solvent) were made at the

TABLE 1. SOURCE, PURITY, AND BIOLOGICAL PRESENCE OF ODOROUS STIMULI USED IN ELECTROPHYSIOLOGICAL EXPERIMENTS

Compound	Source of supply[a]	Chemical purity (%)	Identified from insect (I) or host plant (P)[b]
Butyrates			
Ethyl butyrate	A	>99	I 1, 2
(*E*)-2-Hexenyl butyrate	B	>99	I 1, 2
Butyl butyrate	C	>99	I 1, 2
Hexyl butyrate	C	>99	I 1, 2
Green leaf volatiles			
(*E*)-2-Hexenal	A	>99	I 2 P 2, 4, 5 in cotton buds
(*Z*)-3-Hexenol	A	>99	P 2, 4, 5 in buds I 2
(*E*)-2-Hexenyl acetate	A	>99	I 1, 2
Hexyl acetate	A	>99	I 1, 2
Benzenoids	A		
Phenylacetaldehyde	A	>99	I 2, 5 P 2, 3 in mustard and corn silks
Benzaldehyde	A	>98	P 4, 5 in buds
Monoterpenes			
(±)-linalool	A	>99	P 4, 5 in buds
(−)-linalool	D	>99	
Geraniol	E	71	P 4, 5 in buds
Nerol	E	64.2	P, 5 in buds
(±)-α-Pinene	A	>99	P 3, 4, 5 cotton bud oil and golden rod
(−)-Limonene	A	>99	
(+)-Limonene	A	>99	P 3, 4, 5 cotton bud oil, croton and golden rod
Myrcene	A	85	P 4, 5 bud oil
Aliphatic alcohols			
1-Heptanol	A	90–95	
1-Octanol	A	85–90	P 5 bud
1-Nonanol	A	80–90	P 5 bud
1-Decanol	A	>99	
Aliphatic aldehydes			
Heptanal	A	70–75	P 4, 5 buds
Octanal	A	75–80	
Nonanal	A	80–85	P 4, 5 buds
Decanal	A	95	P 5 buds
Other compounds			
Ethyl hexanoate	A	>99	I 1, 2
Ethyl (*E*)-2-hexenoate	A	>99	I 1, 2
Ethyl myristate	A	>99	I 1
Butylacetate	A	>99	I 1, 2

[a] A, Aldrich Chem. Co., Milwaukee, Wisconsin; B, Bedoukian Research Inc., Danbury, Connecticut; C, synthesized by Jan Kochansky, USDA, ARS, INHL, Beltsville, Maryland; D, K&K Laboratories, Inc., Cleveland, Ohio; E, Pfaltz & Bauer, Inc., Stamford, Connecticut.

[b] 1 = Aldrich et al. (1988); 2 = Gueldner and Parrot (1978); 3 = Gueldner and Parrot (1981); 4 = Hedin et al. (1973); 5 = Hedin et al. (1976).

beginning and at the end of each preparation. The mean response to the control was subtracted from each EAG.

1-Hexanol (100-μg stimulus load) was used as a standard for normalizing all responses, so that responses within an individual and among individuals (Payne, 1975) could be compared. Stimulation with the standard preceded and followed every two stimulations. Millivole responses were converted into percentages of the mean of the two nearest responses to the standard (Dickens, 1978, 1981). Maximal EAG depolarizations, which usually occurred during the initial 500 msec of the stimulation period, were measured from strip-chart recordings. Hyperpolarizations, as observed for some chemicals, were treated as zero responses for statistical purposes. The magnitude of the EAG depolarization was considered to be a measure of the relative number of responding acceptors (receptor sites) (Kaissling, 1971, 1974; Payne, 1975; Dickens and Payne, 1977).

Definitions of threshold and saturation were modified from earlier definitions (Dickens, 1981, 1984). The threshold was considered to be the lowest dose at which the mean response increased, while the saturation level was taken as the highest dose at which the mean response was equal to or less than the succeeding dose.

Statistical Analyses. EAGs were compared statistically using the analysis of variance procedure and Duncan's multiple-range mean separation test (Duncan, 1955). Sexual differences between points on dose-response curves were compared for significant differences in sexes using the t test for two means (Ostle, 1969).

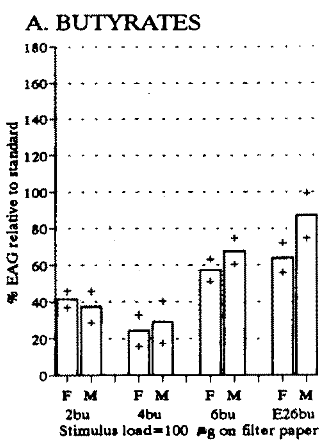
RESULTS

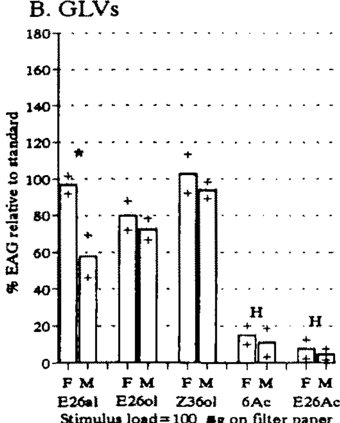
Mean responses of *L. lineolaris* to the 1-hexanol standard were not significantly different between males (-1.74 ± -0.147 mV) and females (-1.61 ± 0.173 mV) for 29 males and 31 females, respectively.

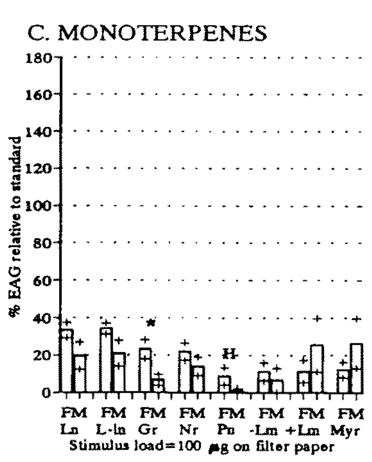
Selectivity

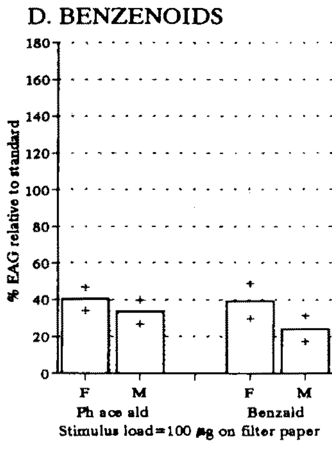
In general, results indicated significant differences in the size of acceptor populations for the various compounds tested. Although slight differences between the sexes in EAGs to each of the odorants were noted, in only a few cases were the differences significant.

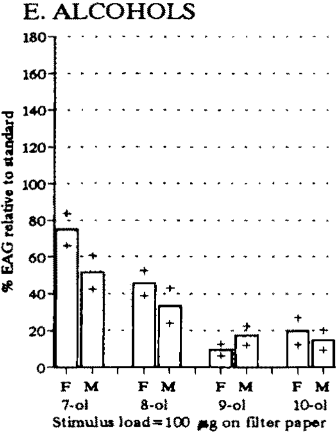
Response to Butyrates. EAGs elicited by the hexyl butyrates were significantly greater than those elicited by either ethyl or butyl butyrate (Figure 1A) in both the sexes. Male responses to (E)-2-hexenyl butyrate and hexyl butyrate were equivalent and were greater than female responses to these esters. However, no significant differences were found between responses of either sex to esters at the 100-μg stimulus load.

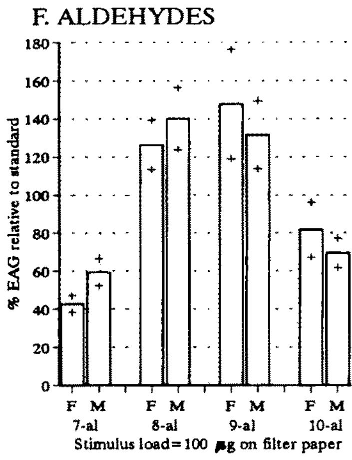

A. BUTYRATES
% EAG relative to standard
180
160
140
120
100
80
60
40
20
0
F M
2bu
F M
4bu
F M
6bu
F M
E26bu
Stimulus load=100 µg on filter paper


B. GLVs
% EAG relative to standard
180
160
140
120
100
80
60
40
20
0
*
H
H
F M
E26al
F M
E26ol
F M
Z36ol
F M
6Ac
F M
E26Ac
Stimulus load=100 µg on filter paper


C. MONOTERPENES
% EAG relative to standard
180
160
140
120
100
80
60
40
20
0
*
H
FM
Ln
FM
L-ln
FM
Gr
FM
Nr
FM
Pn
FM
-Lm
FM
+Lm
FM
Myr
Stimulus load=100 µg on filter paper


D. BENZENOIDS
% EAG relative to standard
180
160
140
120
100
80
60
40
20
0
F
M
Ph ace ald
F
M
Benzald
Stimulus load=100 µg on filter paper


E. ALCOHOLS
% EAG relative to standard
180
160
140
120
100
80
60
40
20
0
F M
7-ol
F M
8-ol
F M
9-ol
F M
10-ol
Stimulus load=100 µg on filter paper


F. ALDEHYDES
% EAG relative to standard
180
160
140
120
100
80
60
40
20
0
F M
7-al
F M
8-al
F M
9-al
F M
10-al
Stimulus load=100 µg on filter paper

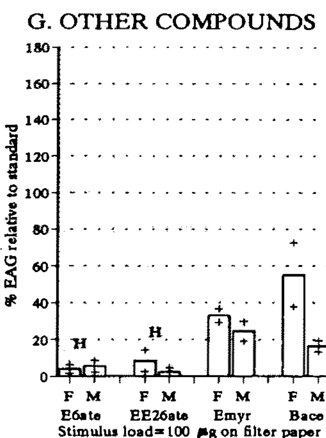


FIG. 1. Mean EAGs of male and female *Lygus lineolaris* to 100-μg stimulus loads of selected insect and plant odorants: (A) butyrates (2bu = ethyl butyrate; 4bu = butyl butyrate; 6bu = hexyl butyrate; E26bu = (*E*)-2-hexenyl butyrate); (B) GLVs (leaf volatiles [E26al = (*E*)-2-hexenal; E26ol = (*E*)-2-hexenol; Z36ol = (*Z*)-3-hexenol; 6Ac = hexyl acetate; E26Ac = (*E*)-2-hexenyl acetate]; (C) monoterpenes [Ln = (±)-linalool; L-ln = (−)-linalool; Gr = geraniol; Nr = nerol; Pn = (±)-α-pinene]; (D) benzenoids (Ph ace ald = phenylacetaldehyde; benzald = benzaldehyde); (E) alcohols (7-ol = heptanol; 8-ol = octanol; 9-ol = nonanol; 10-ol = decanol); (F) aldehydes (7-al = heptanal; 8-al = octanal; 9-al = nonanal; 10-al = decanal); (G) other compounds [E6ate = ethyl hexanoate; EE26ate = ethyl (*E*)-2-hexenoate; Emyr = ethyl myristate; Bace = butyl acetate]. A plus sign above and below bars represents ±SE; *Significant sexual difference, $P < 0.05$, t test for two means. H indicates odorant often produced hyperpolarization of EAG.

Response to Green Leaf Volatiles (GLVs). Mean EAGs from females to six carbon aldehydes and alcohols were significantly greater than the EAGs elicited by corresponding six carbon acetates. EAGs elicited by (*E*)-2-hexenal were significantly greater for females than males (Figure 1B). Although differences in responses between the sexes were not significant, the response elicited by (*Z*)-3-hexenol was the highest among five compounds tested in this group.

Response to Monoterpenes. Although there are no significant differences in EAGs by both sexes to individual monoterpenes, a relatively greater response was observed in females to oxygenated monoterpenes [(−)-linalool, racemic (±)-linalool, and the geometric isomers geraniol and nerol] than to monoterpene hydrocarbons. EAGs elicited by geraniol in females were significantly greater than those elicited in males (Figure 1C). Occasionally hyperpolarizations of the EAGs were observed for stimulation with monoterpene hydrocarbons.

Response to Benzenoids. Although no significant differences in EAGs were noted between the sexes and among the individual benzenoids (Figure 1D), EAGs recorded from females to both aromatic compounds were greater than those recorded from males.

Response to Aliphatic Alcohols and Aldehydes. EAGs to primary alcohols decreased with increasing carbon chain length (Figure 1E). EAGs elicited by aldehydes of increasing chain length increased to a maximum at eight or nine carbons before declining with the 10-carbon compound for both sexes (Figure 1F).

Response to Other Compounds. Butyl acetate and ethyl myristate elicited depolarizing EAGs. Ethyl hexanoate and ethyl (*E*)-2-hexenoate elicited hyperpolarizations. Butyl acetate elicited maximal EAGs among compounds in this group (Figure 1G). However, no significant sexual differences were found.

Sensitivity

In general, after reaching threshold, responses to the compounds tested increased with increasing stimulus loads until saturation. Dose–response curves for EAGs of males and females to 1-hexanol were almost identical (Figure 2A). Saturation was not reached by either sex even at the 1000-μg dose.

For convenience the odorants tested in dose–response studies were grouped into two categories: insect-produced compounds and plant volatiles.

Insect-Produced Compounds. In general, the dose–response curves constructed from EAGs elicited by compounds present in insects were similar in shape. To the compounds (*E*)-2-hexenyl butyrate and hexyl butyrate, male *Lygus* receptors responded similarly in reaching threshold and saturation levels at the same doses tested. Statistically significant differences were observed between the sexes at the 10-μg dose, where the responses reached saturation level for both sexes (Figure 2B and C). EAGs elicited by butyl acetate in both females and males at the intermediate doses tested (0.1 and 1.0 μg) were significantly different (Figure 2D). However, responses at higher doses tested were nearly the same, and the curves showed an increasing trend in both the sexes.

Plant Volatiles. Some of the plant volatiles were also reported in small amounts in *Lygus* adults (Table 1). Generally, the green leaf volatiles, (*Z*)-3-hexenol, (*E*)-2-hexenol, and (*E*)-2-hexenal, elicited greater EAGs than any other odors tested in both sexes. Male EAGs to (*E*)-2-hexenol and (*E*)-2-hexenal were significantly greater than those of females at the 100-μg and 0.01-μg doses tested, respectively (Figure 2F and G). Dose–response curves constructed from the EAGs to (*Z*)-3-hexenol in both the sexes were identical (Figure 2E).

The oxygenated monoterpene, geraniol, which elicited significantly greater response by females than males in selectivity studies, also elicited significantly greater EAGs by females in dose–response studies. Females reached threshold

earlier (0.01-μg dose) than males (0.1 μg), and responded with significantly greater EAGs to 10-μg and 100-μg doses (Figure 2H). Dose–response curves to increasing stimulus loads of phenylacetaldehyde increased after reaching the threshold in both sexes (Figure 2I). This aromatic compound was reported both in bugs and as well in host plants (see references in Table 1).

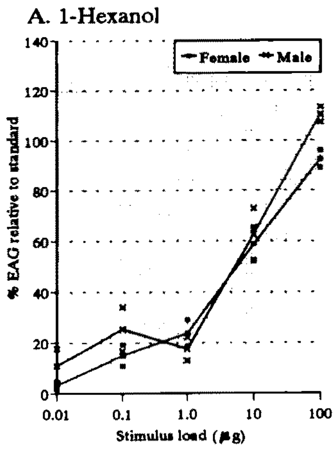
Nonanal, a known plant volatile that was recently identified from another *Lygus* spp. (Borden, personal communication), elicited greater EAGs than any other compound tested. Females responded greatly to this odorant in both experimental series. Dose–response curves show similarity in reaching the thresholds in both sexes, while male antennae appeared to have reached saturation at the 10-μg dose. However, the differences in EAGs between the sexes were statistically insignificant.

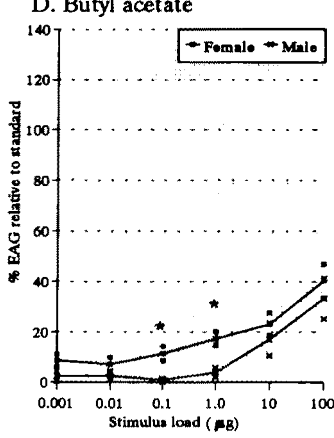
DISCUSSION

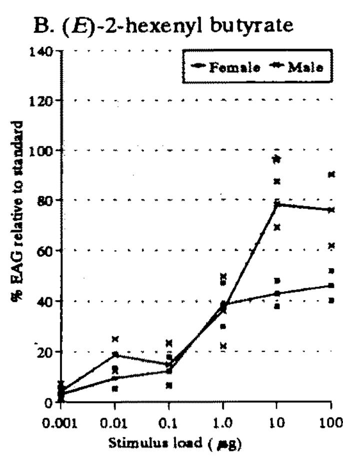
Selectivity

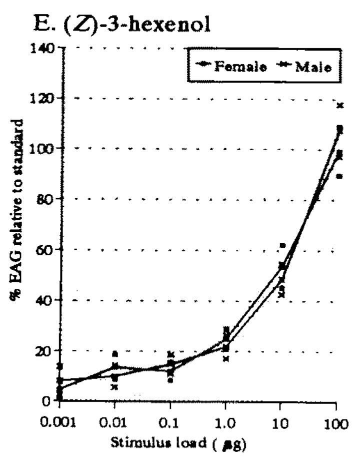
The similarity in EAGs of male and female *L. lineolaris* to plant odors may be explained by their common habitat, where they may utilize the same chemical cues in locating the host plants on which they feed and mate (Li et al., 1992). Similar results were obtained in previous studies with other plant-feeding insects (Fein et al., 1982; Dickens, 1984; Wellso et al., 1984; Light et al., 1988; Hansson et al., 1989).

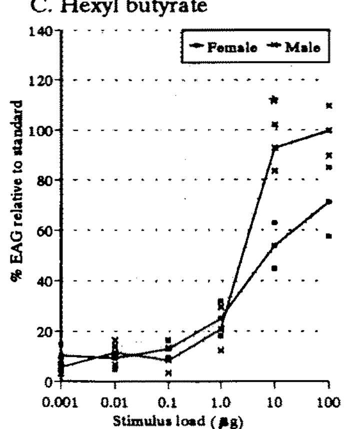
In general, EAGs to butyl esters for males and females increased with increasing chain length of the parent acid moiety. The acceptor populations for (*E*)-2-hexenyl butyrate and hexyl butyrate in males were greater than in females. This correlates with the fact that females contain relatively much more (*E*)-2-hexenyl butyrate in their scent glands than do males (Gueldner and Parrott, 1978; Aldrich et al., 1988). Gueldner and Parrott (1978) reported that most of the compounds detected in their studies from TPB were esters. They also suggested (*E*)-2-hexenyl butyrate could be an important component of the sex pheromone based on its greater abundance in females than in males. They observed (*E*)-2-hexenyl butyrate and hexyl butyrate in male and female TPBs at 10 : 1 and 1 : 1 ratios. The sexual dimorphism observed in EAGs of *L. lineolaris* males and females to (*E*)-2-hexenyl butyrate and hexyl butyrate, and their presence in females, may indicate the involvement of these compounds in the process of pheromonal attraction. However, a comparative study of *L. lineolaris*, *L. elisus*, and *L. hesperus* revealed that the ratio of (*E*)-2-hexenyl butyrate to hexyl butyrate is not sexually dimorphic in *L. hesperus*, whereas in *L. elisus* this ester dimorphism is similar to that for *L. lineolaris* (Aldrich et al., 1988). Games and Staddon (1973) demonstrated sexual dimorphism of chemicals in *Oncopeltus fasciatus* (Dallas): male scent consisted of acetates, while female scent was

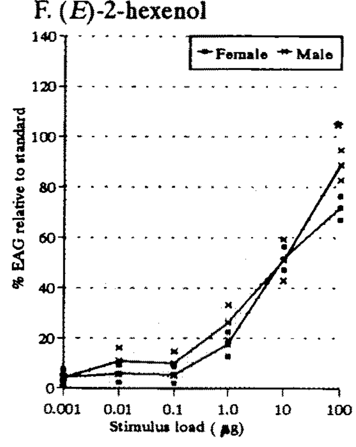

A. 1-Hexanol
Female
Male
% EAG relative to standard
Stimulus load (µg)
0
20
40
60
80
100
120
140
0.01
0.1
1.0
10
100


D. Butyl acetate
Female
Male
% EAG relative to standard
Stimulus load (µg)
0
20
40
60
80
100
120
140
0.001
0.01
0.1
1.0
10
100


B. (E)-2-hexenyl butyrate
Female
Male
% EAG relative to standard
Stimulus load (µg)
0
20
40
60
80
100
120
140
0.001
0.01
0.1
1.0
10
100


E. (Z)-3-hexenol
Female
Male
% EAG relative to standard
Stimulus load (µg)
0
20
40
60
80
100
120
140
0.001
0.01
0.1
1.0
10
100


C. Hexyl butyrate
Female
Male
% EAG relative to standard
Stimulus load (µg)
0
20
40
60
80
100
120
140
0.001
0.01
0.1
1.0
10
100


F. (E)-2-hexenol
Female
Male
% EAG relative to standard
Stimulus load (µg)
0
20
40
60
80
100
120
140
0.001
0.01
0.1
1.0
10
100

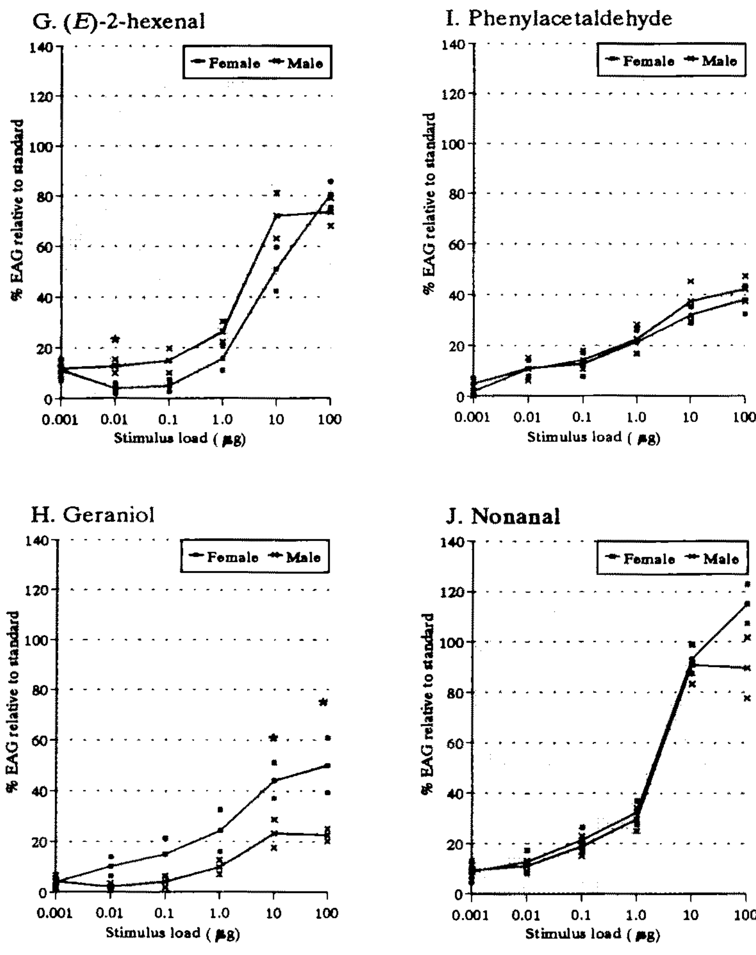


FIG. 2. Dose–response curves constructed from EAGs of male and female *Lygus lineolaris* to serial stimulus loads of selected insect and plant odorants: (A) 1-hexanol, (B) (*E*)-2-hexenyl butyrate, (C) hexyl butyrate, (D) butyl acetate, (E) (*Z*)-3-hexenol, (F) (*E*)-2-hexenol, (G) (*E*)-2-hexenal, (H) geraniol, (I) phenylacetaldehyde, (J) nonanal. Markers above and below points on dose–response curves represent ± SE (x = males; □ = females). *Significant sexual difference, $P < 0.05$, t test for two means.

mostly aldehydes. They attributed these differences to the tubular glands of the scent gland complex, which were larger in males than in females. Similar sexual dimorphism was noted in the giant water bug *Lethocerus indicus* for (*E*)-2-hexenyl butyrate (Devakul and Maarse, 1964).

Greater EAGs of females to six-carbon aldehyde and alcohols, i.e., GLVs, may be attributed to their role in host orientation (Visser et al., 1979, Visser and Avé, 1978; Visser, 1983). (*E*)-2-Hexenal, (*Z*)-3-hexenol, and (*E*)-2-hexenol were reported in cotton buds (Hedin et al., 1973, 1976). (*E*)-2-Hexenal and (*Z*)-3-hexenol were also identified in TPB (Gueldner and Parrott, 1978). EAGs recorded in response to these compounds are comparable with those obtained for several other insect species (Visser, 1983) including the oak leaf weevils, *Rhychaenus quercus* L. (Kozlowski and Visser, 1981), which were most responsive to both the six-carbon alcohols and aldehydes.

In general, *Lygus* bugs had more acceptors responsive to the oxygenated monoterpenes than to the monoterpene hydrocarbons. Greater responsiveness at the EAG level to oxygenated monoterpenes versus monoterpene hydrocarbons has been observed in several phytophagous insects [Visser (1979) in *Leptinotarsa decemlineata*; Kozlowski and Visser (1981) in *R. quercus*; Guerin and Visser (1980) in *Psila rosae*; and Dickens (1984) in *Anthonomus grandis*]

Benzenoids elicited significant EAGs in both female and male TPBs. Benzaldehyde was reported in cotton buds (Hedin et al., 1973, 1976). Phenyl acetaldehyde was found in TPB and mustard plants (Gueldner and Parrott, 1978, 1981). Cantelo and Jacobson (1979) reported phenylacetaldehyde in corn silk and found it to be attractive to TPBs in the field.

A general decrease in EAG response with increasing chain length for aliphatic alcohols and increase in EAG responsiveness to the increasing chain length of aliphatic aldehydes through nine carbons was found in both the sexes. The fact that EAGs elicited by aldehydes were sometimes greater than for the corresponding alcohols may be due to their higher volatility. However, volatility of both alcohols and aldehydes decrease with increasing carbon chain length. Thus, decreasing EAGs to the primary alcohols with increasing chain length corresponds with the decreasing volatility of these compounds. The increasing EAGs to aldehydes of increasing chain length may only be explained by a greater affinity of antennal receptors for these molecules since fewer molecules evaporating from the filter paper would be available for interaction with the acceptors. Nonanal was reported from cotton (Hedin et al., 1973, 1976), and more recently was identified by coupled gas chromatography (GC) -electroantennographic analysis and coupled GC-mass spectrometry as a potential semiochemical in volatiles captured from female TPBs feeding on bean pods, *Phaseolus vulgaris* L. (Pierce, Gries, Wardle and Borden, personal communication). Greater EAGs to this compound and its presence in both plants and bugs indicates that this compound may be of significance in both pheromonal attraction and host finding.

A similar increase in response to seven- to nine-carbon aldehydes was reported in both the honeybee, *Apis mellifera* (Dickens et al., 1986), and the wax moth, *Galleria mellonella* (Payne and Finn, 1977), in which nonanal functioned as a component of its male-produced sex attractant.

Hyperpolarizations were frequently noted while testing the compounds (*E*)-2-hexenyl acetate, hexyl acetate, ethyl hexanoate, ethyl (*E*)-2-hexenoate and (±)-α-pinene. Positive polarity receptor potentials defined as hyperpolarizations by Boeckh et al. (1965) and Kaissling (1971) had been observed previously by Schneider (1957a,b). They considered this type of waveform to be due to the inhibition (i.e., decreased action potential frequency) in receptor neurons when stimulated with certain acid compounds. Light et al. (1988) also recorded hyperpolarizations in medfly antennae when stimulated with some short-chain carboxylic acids. Ethyl hexanoate, ethyl (*E*)-2-hexenoate, and ethyl myristate were identified by Gueldner and Parrott (1978) in *Lygus* bugs. None of these compounds were attractive when presented individually in field tests (Hedin et al., 1985).

Sensitivity

In general, dose–response curves constructed from EAGs of male and female to the compounds revealed males to be more sensitive than the females to many odorants tested. Different thresholds and saturation levels observed for the various odorants in electrophysiological studies might be indicative of the role of each compound in host selection and pheromonal attraction. As reported in previous studies (Dickens et al., 1984), a low threshold for a given compound might indicate the ability of the insect to detect the compound in low concentration at greater distances from its source.

Insect-Produced Compounds. Dose–response curves constructed from EAGs to serial stimulus loads of the esters hexyl butyrate and (*E*)-2-hexenyl butyrate revealed males to be more responsive than females to both odorants (Figure 2B and C). The similar shapes of the dose–response curves for both odorants indicate similar receptor mechanisms for each. Reports by Gueldner and Parrott (1978) and Aldrich et al. (1988) on these ester compounds showed both compounds to be present in both sexes. Butyl acetate, which was also identified in TPB by Gueldner and Parrott (1978), elicited significantly greater responses in female antennae (Figure 2D). The acceptor population for this compound is smaller than the previously mentioned six-carbon esters.

Plant Volatiles. Among all the compounds tested, nonanal elicited the greatest EAGs from both male and female antennae, and dose–response curves for both sexes revealed a relatively low threshold (stimulus load = 0.1 μg) for this compound (Figures 1F and 2J). However, the responses between the sexes were not significantly different. Nonanal was identified as a host plant (cotton)

volatile by Hedin et al. (1973, 1976). Our results indicate that TPBs have the ability to detect this odorant in low concentration, perhaps at greater distances from its source.

While significant EAGs were elicited by all of the GLVs (Visser, 1979) tested, the alcohol and aldehyde derivatives were more active than the acetates (Figure 1B). We realize that what is being compared in our experiments is the number of molecules evaporating from the filter paper within the odor cartridge and reaching the preparation, which will differ for odorants of different volatilities and binding characteristics of the compound to the filter paper. Based on boiling points, the order of volatility of the GLVs should be: 1-hexanal (bp = 128.6°C) > 1-hexanol (bp = 157.5°C) > hexyl acetate (171.5°C). Thus the greater activity of the aldehyde and alcohol derivatives observed may be explained by differences in volatilities among the derivatives. However, it seems unusual that while the boiling points of the alcohol and aldehyde differ by nearly 30°C, similar EAGs are elicited; yet insignificant or hyperpolarizing EAGs are elicited by the acetates, even though the difference in boiling points for the alcohol and acetate is only 14°C. (*Z*)-3-Hexenol was reported as the body constituent of male and female TPBs (Gueldner and Parrott, 1978). (*E*)-2-Hexenol in cotton and (*E*)-2-hexenal in both cotton and TPB were identified (see Table 1). These compounds may play a role in host-plant finding (Visser and Avé, 1978) or enhancement of pheromone responses (Dickens, 1989; Dickens et al., 1990) as shown for other insects.

Female TPBs were more sensitive and responsive to the oxygenated monoterpene geraniol than were males. Geraniol was reported in cotton buds (Hedin et al., 1973, 1976). Since this compound was not detected in *Lygus* bugs of either sex, and a greater number of acceptors in female antennae responded to this compound, it may be hypothesized that females may use this odor in locating food sources or oviposition sites.

Although previous behavioral experiments in the field indicated phenylacetaldehyde as a *Lygus* attractant (Cantelo and Jacobson, 1979), only small EAGs were recorded in response to this compound. This indicates a small but behaviorally significant population of receptors for this odorant.

The EAG studies presented here demonstrate TPBs to have receptors for a wide range of insect-produced odors and host-plant volatiles. In the case of hexyl butyrate, (*E*)-2-hexenyl butyrate, and geraniol sexually dimorphic responses were noted. Green leaf volatiles and nonanal were the most active odorants tested in both sexes. These results and concurrent morphological studies provide a basis for single-sensillum recordings already in progress.

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SEX PHEROMONE OF CRANBERRY FRUITWORM, *Acrobasis vaccinii* RILEY (LEPIDOPTERA: PYRALIDAE)

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Abstract—The following compounds and (approximate ratios) were identified in sex pheromone gland extracts of female *Acrobasis vaccinii* Riley by comparison of gas chromatography–mass spectrometric traces with those of synthetic standards: (*E,Z*)-, (*Z,E*)-, (*Z,Z*), and (*E,E*)-8,10-pentadecadien-1-ol acetates (100:1:2:12), a dodecen-1-ol acetate (8), (*Z*)-8-, (*Z*)-9-, and (*E*)-9-pentadecen-1-ol acetates (3:23:4), two heptadecen-1-ol acetates (4:4), tetradecyl, pentadecyl, hexadecyl, and heptadecyl acetates (3:15:10:8), dodecan-1-ol (6), tetradecan-1-ol (5), and hexadecan-1-ol (23). The amount of (*E,Z*)-8,10-pentadecadien-1-ol acetate (*E*8,*Z*10–15:Ac) in the extract was about 0.5 ng/female. Electroantennographic analysis of gas chromatographic fractions of female sex pheromone gland extract showed that the fraction containing *E*8,*Z*10–15:Ac elicited the greatest response. Alone, *E*8,*Z*10–15:Ac failed to elicit upwind flight of males in flight-tunnel tests, and traps baited with it did not catch males in field experiments. When *E*8,*Z*10–15:Ac was combined with (*E*)-9-pentadecen-1-ol acetate (100:4), male upwind flight response in flight-tunnel tests was equivalent to those obtained with extract of female sex pheromone glands (synthetic, 62%; natural, 51%), but the percent of males flying upwind that contacted the source was lower (synthetic, 47%; natural, 88%). The lower percent of source contact elicited by the

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synthetic pheromone could be a result of the difference in isomer ratios of 8,10-15:Ac in the natural and synthetic pheromone or could indicate that the synthetic pheromone is incomplete. Traps baited with the 100:4 combination caught large numbers of males in field experiments.

Key Words—Cranberry fruitworm, Lepidoptera, Pyralidae, *Acrobasis vaccinii*, sex pheromone, (*E,Z*)-8,10-pentadecadien-1-ol acetate, (*E*)-9-pentadecen-1-ol acetate.

INTRODUCTION

The cranberry fruitworm, *Acrobasis vaccinii* Riley, occurs throughout the United States and Canada wherever its host plants occur (Heinrich, 1956; Crowley, 1954). Its host plants include huckleberries, dangle-berries, beach plums, and apples (Beckwith, 1941), in addition to the crops on which it is an important economic pest: blueberries and cranberries. It is considered the most or one of the most important pests on cranberries (Tomlinson, 1960; Eastwood, 1859) and has been known to destroy entire crops (Beckwith, 1941). *A. vaccinii* overwinters as a diapausing larva in a hibernaculum and is univoltine. A larva typically consumes five to eight berries (Brodel and Roberts, 1984).

The purpose of this research was to determine the structure of components of the sex pheromone of *A. vaccinii* and to develop a lure that could be used in pest management programs. Two components of the sex pheromone were identified, which, when formulated in gray elastomeric septa at a dose of 0.3–1 mg, provided a lure that could be used for this purpose.

METHODS AND MATERIALS

Insects. Each year in the springs of 1986 and 1987, 80–90 *A. vaccinii* hibernacula were collected from the soil of blueberry fields in Michigan and shipped to Yakima in moist peat moss. In 1989 and later, *A. vaccinii* larvae were collected from infested cranberries from Massachusetts bogs and placed on hardware cloth over a tray of wet sand. Upon leaving the berries, mature larvae fell through the cloth and formed hibernacula in the sand. The hibernacula were conditioned at 21°C for two weeks, 15°C for two weeks, 10°C for three weeks, and 4°C for four weeks. Then they were placed in moist peat moss and shipped to Yakima where they were placed on a 5- to 6-cm layer of moist peat moss in covered clear plastic shoe boxes and held in a rearing room at a temperature of 5°C, a relative humidity of 55–60%, and a 12:12 hr light–dark cycle. In 1989 and 1990, 300 hibernacula were obtained and in 1991, 3000 hibernacula were obtained. As adults were needed, boxes were removed and placed in another facility at a temperature of 55°C and a 13:11 hr light–dark cycle for a week and then at 21°C and a 15:9 hr light–dark cycle. Emergent

adults were collected daily at least 1 hr before the dark period and placed in individual vials. Usually, about 70% emergence of adults from hibernacula was obtained.

Collection of Pheromone. Female moths (2–3 days old) in their vials were collected 8 hr after the beginning of scotophase when they appeared to be calling, and placed in a refrigerator to inactivate them. They were removed individually, and their terminal abdominal segments containing the sex pheromone gland were severed and steeped for 15–60 min in ca. 200 μl hexane. Then the hexane solution was removed with a syringe and kept in a freezer until analysis.

Liquid Chromatography of Sex Pheromone Gland Extract. The column, tapered at one end, was 8 mm in diameter and about 20 cm long. The tapered end was plugged with glass wool and filled with about 0.5 cm sodium sulfate, 0.6 g silica gel (ca. 7.5 cm), and topped with about 1.0 cm sodium sulfate. The volume of the column was 1.2 ml. The column was washed with 4.8 ml dichloromethane–hexane (4 : 10), followed by 3.6 ml hexane. The sex pheromone gland extract (100 female equivalents) in 200 μl hexane was added to the column, which was eluted with three 1.2-ml portions of hexane, four 1.2-ml portions of dichloromethane–hexane (3. : 10), and finally with five 1.2 ml portions of dichloromethane. The esters eluted in the dichloromethane–hexane fractions, and the alcohols eluted in the dichloromethane fractions.

Gas Chromatography-Mass Spectrometry (GC-MS). A Hewlett-Packard (Avondale, Pennsylvania) gas chromatograph (model 5790) with a quadrupole mass spectrometer (model 5970) was equipped with either a DB-1 or DB-Wax capillary column, 60 m × 0.25 mm ID (J&W Scientific, Folsom, California). For different analyses, both total ion abundance and single ion monitoring (SIM) were used.

Gas Chromatography-Electroantennography (GC-EAG). Fractions of extracts of sex pheromone glands were collected from a GC equipped with megabore capillary columns (15 m × 0.54 mm ID) (J&W Scientific). One series of collections were made with a DB-1 liquid phase and the other with a DB-Wax liquid phase. Fractions were collected in 3-mm-ID, U-shaped, glass traps cooled by Dry Ice–acetone and then dissolved in dichloromethane and deposited on the inside of glass tubes for EAG determinations.

Electroantennogram (EAG) Determinations. EAG determinations of model compound profiles (Roelofs, 1984) were made with a previously described apparatus and procedure (McDonough et al., 1980) except that 60-μg charges were used. Duplicate determinations were made for each compound. If the determinations were not close, a third determination was made. The saturated compounds were obtained from commercial sources, and the monoenes were synthesized by the procedure of Voerman (1988). The conjugated dienes were synthesized by the procedures of McDonough et al. (1982), Ujvary et al. (1985), and McDonough and Smithhisler (1989), and the isomeric mixtures were produced as earlier reported (McDonough et al., 1993). All compounds were at

least 98% pure by capillary gas chromatography and the monoenes contained 1% or less of their geometric isomers. The monoenes tested were the 12-, 13-, 14-, 15-, and 16-carbon acetates, and the 12-, 14-, 15-, and 16-carbon alcohols. The dienes tested were the stereoisomeric mixtures of the positional isomers (6,8-, 7,9-, 8,10-, 9,11-, and 10,12-) of pentadecadien-1-ol acetate. (*E*,*Z*)-8,10-pentadecadien-1-ol acetate was of 92.6% isomeric purity.

Flight-Tunnel Tests. A flight tunnel, constructed as described by Miller and Roelofs (1978) and maintained at 21°C, was used to study pheromone elicited flight. Males were released in groups of three. The test compounds were formulated either on dental rolls or in gray elastomeric septa (formulation number 1888, size 6 mm × 10 mm, West Co. Phoenixville, Pennsylvania) (Brown and McDonough, 1986).

Field Tests. Candidate lures in 100 μl dichloromethane were impregnated into gray elastomeric septa; dichloromethane alone was added to control septa. Pherocon 1C sticky traps (Trece Corp., Salinas, California) were used. Traps were suspended from a movable metal arm (an 18- × 23-cm shelf bracket) attached by a metal hose clamp to a 120-cm-long wooden broom handle driven into the ground. This arrangement allowed the traps to be positioned at the top of the plant canopy, where most flight activity was believed to occur. There were 24 traps per test deployed at approximately 10-m intervals in either an 8 × 3 or a 6 × 4 arrangement, or 30 traps per test deployed in a 6 × 5 arrangement. The position of each trap in the experimental plot was drawn randomly. There were four replicates of each test composition, and the trap data were transformed by $(x + 0.5)^{0.5}$ and compared by Duncan's (1955) multiple-range test ($P = 0.05$). Lures were tested in blueberry fields in Michigan and in cranberry bogs in Massachusetts.

RESULTS AND DISCUSSION

Identification of Major Sex Pheromone Component. In 1986, extracts of 20 female sex pheromone glands were collected in fractions from a gas chromatograph equipped alternately with a polar (DB-Wax) and a nonpolar (DB-1) column, and the EAG responses elicited by the fractions from each GC column were determined. The data are summarized in Table 1. On the DB-Wax column, the fraction with I_x (Kovats, 1965) values of 2347–2419 elicited the greatest response, and on the DB-1 column, the fractions with I_x values of 1834–1930 elicited the greatest response. The two most active fractions from the EAG analysis were recovered, combined, and analyzed by GC-MS (DB-1 column). Based on the mass spectra, most of the compounds found within the active I_x range were GC column bleed, phthalates, or hydrocarbons, but in addition, a pentadecadien-1-ol acetate was identified (M^+, 266; M^+-60, 206; $CH_3CO_2H_2^+$, 61) with a retention index of 1912. The difference between the retention index

TABLE 1. EAG RESPONSES RELATIVE TO FRACTION ELICITING GREATEST RESPONSE (= 100) OF MALE *A. vaccinii* TO GAS CHROMATOGRAPHIC FRACTIONS OF FEMALE SEX PHEROMONE GLAND EXTRACTS[a]

	DB-Wax		DB-1	
Fraction	I_t range	Relative EAG response	I_t range	Relative EAG response
1	0-1910	0.9	0-1613	3.4
2	1910-2064	4.6	1613-1730	9.4
3	2064-2176	12.5	1730-1834	12.0
4	2176-2268	16.1	1834-1930	100
5	2268-2347	23.4	1930-2029	31.4
6	2347-2419	100	2029-2200	31.3
7	2419-2560	43.2		

[a] I_t is the retention index (Kovats, 1965).

values of the fractions eliciting the greatest EAG responses on the polar and nonpolar columns and the easy detectability of the M^+ ion of the pentadecadien-1-ol acetate indicated that the double bonds were conjugated.

The EAG responses to pentadecen-1-ol acetates were determined as an aid in determining double bond positions in the diene (Roelofs, 1984) (Figure 1). The strong response to the *E*8 isomer suggested this as one of the double-bond positions, but there was no distinction between the 6- or the 10-isomers as indicators of the other double-bond position.

Because of limited availability of insects and the small amount of the pentadecadien-1-ol acetate per insect (ca. 0.5 ng), the double-bond positions of the pentadecadien-1-ol acetate could not be determined by ozonolysis. There-

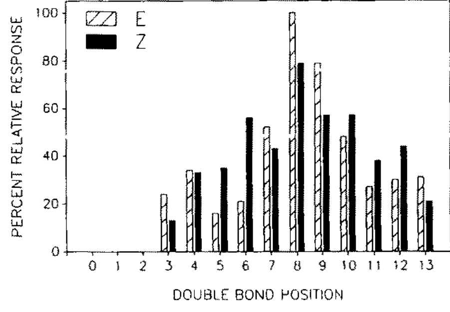

FIG. 1. EAG responses of male *A. vaccinii* to pentadecen-1-ol acetates.

fore, all of the stereoisomers of the pentadecadien-1-ol acetates with double bonds in the 6,8, 7,9, 8,10, 9,11, and 10,12 positions were synthesized and compared to the natural compound. The retention indexes of these isomers on the polar and nonpolar GC columns are given in Table 2. GC-MS analysis of sex pheromone gland extract from the 1987 batch of insects showed the presence of two pentadecadien-1-ol acetates in a ratio of 100:6. The major compound had a retention index of 1912.2 on DB-1 and 2385.7 on DB-Wax and the minor compound had a retention index of 1936.8 on DB-1 and 2422.5 on DB-Wax. The best fit of these indexes with those of Table 2 corresponded to the *E*8,*Z*10 and *E*8,*E*10 isomers for the major and minor compounds, respectively.

When EAG responses elicited by the stereoisomers mixtures of the positional isomers of the pentadecadien-1-ol acetates (6,8-, 7,9-, 8,10-, 9,11-, and 10,12-) were compared, the 8,10-pentadecadien-1-ol acetates elicited responses 20–40% stronger than the other stereoisomeric mixtures. The response to the isomeric mixture of 8,10-pentadecadien-1-ol acetates was ca. 80% of that to (*E*)-8-pentadecen-1-ol acetate. Purified (*E*,*Z*)-8,10-pentadecadien-1-ol acetate (92.6% *EZ*) elicited ca. 10% greater response than (*E*)-8-pentadecen-1-ol acetate.

Traps baited with (*E*,*Z*)-8,10- and (*E*,*E*-8,10-pentadecadien-1-ol acetates in a 100:6 ratio at a 1-mg dose per gray septum were tested in infested blueberry fields during the 1987 flight season, but no fruitworms were caught in 10 traps, therefore indicating that there was probably one or more essential components missing.

TABLE 2. RETENTION INDEX (KOVATS, 1965), I_k, VALUES OF SYNTHESIZED PENTADECADIEN-1-OL ACETATES ON POLAR (DB-WAX) AND NONPOLAR (DB-1) GC COLUMNS

GC column and double-bond configuration	I_k values at given bond positions				
	6, 8-	7, 9-	8, 10-	9, 11-	10, 12-
DB-1					
ZE	1899.2	1902.4	1905.4	1909.7	1916.2
EZ	1902.3	1906.4	1911.7	1918.1	1924.6
ZZ	1922.4	1923.9	1926.7	1929.7	1931.7
EE	1937.8	1937.8	1936.6	1935.0	1935.3
DB-Wax					
ZE	2368.9	2373.3	2377.8	2381.0	2395.9
EZ	2375.2	2381.7	2388.1	2395.4	2406.2
ZZ	2400.4	2402.4	2405.9	2409.1	2415.1
EE	2425.5	2426.6	2425.8	2419.7	2426.7

Identification of Minor Sex Pheromone Component. In EAG studies, none of the other series of compounds (12-, 13-, 14-, and 16-carbon acetates and 12-, 14-, 15-, and 16-carbon alcohols) elicited intense responses compared to 15-carbon acetates. Other compounds, eliciting moderately intense EAG responses with responses compared to *E*8–15:Ac (100%), were: *Z*6–13:Ac (74%), *E*8–14:Ac (62%), and *E*8–13:Ac (59%).

To gain information about other possible pheromone components, fractions of sex pheromone gland extract were collected from the gas chromatograph (DB-1 column) and assayed in the flight tunnel. These tests are summarized in Table 3. The results were not entirely consistent because fraction 3 (which contained the pentadecadien-1-ol acetates), and fractions 1 + 3 did not elicit response, but responses were only obtained from other test mixtures in which fraction 3 was present. Because the pentadecadien-1-ol acetates alone did not elicit responses, these results indicated that one of the other components necessary for pheromonal activity was probably in fraction 3.

In preparation for a mass spectral analysis search for minor components, sex pheromone gland extract (100 female equivalents) was chromatographed on a small silica gel column and the alcohol and ester fractions were combined. The recovery of nanogram quantities of model compounds by this procedure was 45%. As an aid to the determination of retention indexes, hydrocarbon standards were added to the combined fractions, which were concentrated and then analyzed by GC-MS. The previously found compounds, *E*8, *Z*10–15:Ac

TABLE 3. RESPONSES OF MALE *A. vaccinii* IN FLIGHT-TUNNEL TESTS TO GAS CHROMATOGRAPHIC FRACTIONS OF FEMALE SEX PHEROMONE GLAND EXTRACTS[a]

GC fraction	I_r range (DB-1)	Response (%) in flight tunnel
1	1650–1750	0
2	1750–1850	0
3	1850–1950	0
4	1950–2050	0
5	2050–2150	0
1 + 2 + 3 + 4 + 5		50
1 + 3		0
2 + 3		33
4 + 3		33
5 + 3		33
2 + 4		0

[a] I_r is the retention index (Kovats, 1965). Six males were used in each test. The pentadecadien-1-ol acetates occurred in fraction 3.

and *E*8,*E*10–15:Ac, were again detected. In addition 15:Ac (M-60 = 210; M-60-28 = 182; $CH_3CO_2H_2$ = 61; I_x = 1890.4 vs. I_x = 1890.3 for the standard) and *Z*9–15:Ac (M-60 = 208; M-60-28 = 180; $CH_3CO_2H_2$ = 61; I_x = 1871.4 vs. 1871.5 for the standard) were detected in ratios of 15% and 23%, respectively, relative to *E*8,*Z*10–15:Ac.

The four identified components of the sex pheromone gland were formulated in the ratios found in the extract at a dose of 1 mg/septum of *E*8,*Z*10–15:Ac to test their ability to evoke trap catch of males. Ten traps baited with the lures were tested in cranberry bogs known to be infested with *A. vaccinii*, but again no males were caught.

When more hibernacula were available, the first part of the experiment of Table 3 was repeated, and fraction 3 (15 female equivalent) elicited 33% upwind flight and 30% contacting the source (*N* = 27). The combined fractions 1–5 gave 57% upwind flight and 57% contacting the source (*N* = 35). Fractions 1, 2, 4, and 5 elicited no responses. These results reinforced the previous conclusion that the components necessary for upwind flight to the pheromone source were in fraction 3.

To search further for minor pheromone component(s), an extract of 489 female sex pheromone glands was collected in two fractions from a gas chromatograph equipped with a DB-Wax column. The first fraction covered retention index values of 2158–2337 and included the pentadecen-1-ol acetates. The second fraction covered the retention index values of 2340–2355 and included the pentadecadien-1-ol acetates. Each of these fractions was individually analyzed by GC-MS (DB-1 column) in the single ion monitor mode. For the diene fraction, the ions monitored were 266, 206, and 61. Besides *E*8,*Z*10- and *E*8,*E*10-15:Ac, *Z*8,*E*10- and *Z*8,*Z*10-15:Ac were detected. The ratios were: 100:1:2:12 (*EZ*:*ZE*:*ZZ*:*EE*). For the monoene fraction, the ions monitored were 208, 180, and 61. Besides *Z*9–15:Ac, *Z*8- and *E*9–15:Ac were detected and the ratios were 100:14:18 (*Z*9:*Z*8:*E*9).

Flight-tunnel tests were conducted with the newly discovered components. The isomeric purity of *E*8,*Z*10–15:Ac in these tests was 92.6% (1.8% *ZE*, 1.8% *EE*, 3.8% *ZZ*). When *E*8,*Z*10–15:Ac was tested in combination with *E*8,*E*10–15:Ac, 15:Ac, *Z*9–15:Ac, *Z*8–15:Ac, and *E*9–15:Ac in a ratio of 100:12:15:23:3:4, respectively, at a dose of 12 ng *E*8,*Z*10–15:Ac on a dental roll, 76% of males flew upwind and 38% contacted the source (*N* = 21). When all of the components except *E*9–15:Ac and *E*8,*Z*10–15:Ac were removed, the response was 62% upwind flight and 31% contacting the source (*N* = 16). When *E*9–15:Ac alone was removed, there was no response (*N* = 24). Comparison of sex pheromone gland extract and synthetic pheromone showed that the synthetic stimulated upwind flight as well as the extract, but the percent contacting the source as a percent of those flying upwind was not as high for the synthetic: for synthetic pheromone, 62% of males flew upwind and 47% of

those contacted the source (N = 159); for sex pheromone gland extract, 51% of males flew upwind and 88% of those contacted the source (N = 65). The difference in source contact could be the result of a still missing component or of the higher percentage of the *ZE* and *ZZ* isomers of *E*8,*Z*10–15:Ac in the synthetic pheromone.

Other Compounds in Female Sex Pheromone Gland Extract. During the course of these studies, other pheromonelike compounds were detected in the extract of the female sex pheromone gland. To further identify such compounds, five fractions, covering retention index values of 1350–1550, 1550–1750, 1750–1950, 1950–2150, and 2150–2350, were collected from a GC equipped with a DB-1 column. Each fraction was individually analyzed by GC-MS (DB-1 column; total ion monitor mode). The compounds found in these and previous analyses are summarized in Table 4. The monoene and saturated acetates were identified by the M-60, M-60-28, and 61 m/z values and by retention index, and the alcohols were identified by the M-18 and M-18-28 m/z values and by retention index.

Field Tests. In 1992 traps baited with *E*8,*Z*10–15:Ac and *E*9–15:Ac in gray septa were tested in cranberry bogs to determine the most effective ratio of the pheromone components (Table 5). In the first test, the most effective ratios were 100:2 to 100:8, although the 100:12 ratio only separated out sta-

TABLE 4. SURVEY OF COMPOUNDS FOUND BY GC-MS ANALYSES AND RETENTION INDEX

Compound	Relative amount
*E*8, *Z*10–15:Ac	100
*Z*8, *E*10–15:Ac	1
*E*8, *E*10–15:Ac	12
*Z*8, *Z*10–15:Ac	2
*Z*9–15:Ac	23
*Z*8–15:Ac	3
*E*9–15:Ac	4
Δ*X*–17:Ac	4
Δ*Y*–17:Ac	4
Δ*Z*–12:Ac	8
14:Ac	3
15:Ac	15
16:Ac	10
17:Ac	8
12:OH	6
14:OH	5
16:OH	23

TABLE 5. FIELD TESTS OF RATIOS AND DOSAGES OF (*E*, *Z*)-8,10-PENTADECADIEN-1-OL ACETATE (I) AND (*E*)-9-PENTADECEN-1-OL ACETATE (II)[a]

1992 tests		1993 tests	
Composition	Total trap catch	Composition	Total trap catch
300 μg I + 6 μg II (100:2)	156a	0 μg I + 0 μg II	0a
300 μg I + 12 μg II (100:4)	97ab	100 μg I + 4 μg II	239b
300 μg I + 18 μg II (100:6)	68ab	300 μg I + 12 μg II	383b
300 μg I + 24 μg II (100:8)	89ab	1000 μg I + 40 μg II	357b
300 μg I + 36 μg II (100:12)	34bc	3000 μg I + 120 μg II	360b
0 μg I + 0 μg II	2c		
1000 μg I + 0 μg II (100:0)	0a	0 μg I + 0 μg II	0a
1000 μg I + 20 μg II (100:2)	10ab	100 μg I + 4 μg II	86b
1000 μg I + 40 μg II (100:4)	25b	300 μG I + 12 μg II	131b
1000 μg I + 80 μg II (100:8)	3a	1000 μg I + 40 μg II	137b
1000 μg I + 160 μg II (100:16)	1a	3000 μg I + 120 μg II	163b
0 μg I + 0 μg II	1a		

[a]The two 1992 tests were conducted in the same bog early and late in the season (top and bottom). The two 1993 tests were conducted in two different bogs from June 20 to July 28. There were four replications of all tests. Catches followed by the same letter were not significantly different ($P = 0.05$).

tistically from the 100:2. In the second test, the 100:2 and 100:4 ratios elicited greater trap catch than the other ratios. Taken together these tests indicate that the titer of *E*8,*Z*10–15:Ac relative to *E*9–15:Ac (100:4), estimated from the mass spectral data, is a reasonable ratio for the most effective lure. In 1993, tests were conducted to determine the most effective dosage (Table 5). Between 100 and 3000 μg/septum, there was no difference in trap catch in either test. Even though the 100-μg dose was statistically equivalent to the other doses, its values are lower in both tests and may indicate that significantly lower catches would be obtained if dosage were further decreased. Considering the long half-lives of 1400–2200 days at 20°C expected from the pheromone components (McDonough, 1991; McDonough et al., 1993), doses of 300–1000 μg/gray septum should provide an effective lure for the entire season.

This is the first report of the determination of the sex pheromone of an *Acrobasis* species. Other *Acrobasis* species may also contain 15-carbon-based female sex pheromones. In field screening experiments, 13 male *Acrobasis rufilimbalis* Wileman were captured in traps baited with (*Z*)-9-pentadecen-1-ol acetate (Mayer and McLaughlin, 1991; Ando et al., 1977).

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IDENTIFICATION, SYNTHESIS, AND BIOACTIVITY OF A MALE-PRODUCED AGGREGATION PHEROMONE IN ASSASSIN BUG, *Pristhesancus Plagipennis* (HEMIPTERA: REDUVIIDAE)

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Abstract—*Pristhesancus plagipennis*, a large Australian assassin bug, possesses three pairs of dorsal abdominal glands (DAGs). In the male, the anterior and posterior glands are hypertrophied and secrete an attractant pheromone. Gas chromatography-mass spectrometry (GC-MS) analyses of male DAG extracts and airborne volatiles emitted from calling males showed the pheromone signature to be dominated by a novel component. Subsequent chemical manipulations, GC-MS, and chiral-column analyses established its identity as (*Z*)-3-hexenyl (*R*)-2-hydroxy-3-methylbutyrate. Minor components included 3-methylbutanol, 2-phenylethanol, (*Z*)-3-hexenol, decanal, (*E*)-2-hexenoic acid, and three minor hexenyl esters. Bioactivity studies using laboratory olfactometers and outdoor flight cages demonstrated attraction by female *P. plagipennis* to calling males, heptane extracts of male posterior DAGs and a synthetic formulation of the (*Z*)*R* enantiomer of the major ester, alone or in combination with other components of male anterior and posterior DAGs. Males were also attracted to the major ester. The racemate and *S* enantiomer of the ester were not attractive. Contamination of the (*Z*)*R* enantiomer with 30–60% of the *E* isomer also made the compound nonattractive. This is the

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first report of an aggregation pheromone in the Reduviidae. The prospects for pheromonal manipulation of *P. plagipennis* populations to enhance the value of this predator in horticultural ecosystems, are discussed.

Key Words—*Pristhesancus plagipennis*, assassin bug, Hemiptera, Reduviidae, dorsal abdominal glands, (*Z*)-3-hexenyl (*R*)-2-hydroxy-3-methylbutyrate, aggregation pheromone.

INTRODUCTION

The chemical ecology of assassin bugs (Reduviidae), the largest family of predaceous land bugs (Schaefer, 1988), has been little studied. Aldrich (1988) suggested that the sedentary mode of capturing prey and use of the rostrum for stinging defense may have contributed to reduction of the metathoracic gland and nymphal dorsal abdominal glands (DAGs). Most reduviids possess two pairs of unique glands: Brindley's glands situated laterally under the first abdominal tergite and ventral glands at the junction of the thorax and abdomen (Staddon, 1979). Secretions from Brindley's glands in hematophagous reduviids (Triatominae) consist primarily of isobutyric acid (Blum, 1981). Clear functions for reduviid exocrine glands have not been established.

Pristhesancus plagipennis Walker is a large (2–2.5 cm), common Australian reduviid that is part of the natural enemy complex operating against the spined citrus bug, *Biprorulus bibax* Breddin (Pentatomidae) in Queensland helping to prevent economic damage (Summerville, 1931; Murray, 1987; James, 1992). Dissection of adult *P. plagipennis* revealed the presence of three well-developed paired DAGs, with the anterior and posterior pair being much larger in males than females. In addition, males were observed to adopt a characteristic calling posture accompanied by release of volatiles (Aldrich, 1991). Recently, *B. bibax* has become a serious pest of citrus in southern Australia (James, 1989) where *P. plagipennis* does not occur naturally. Integrated management of *B. bibax* in southern areas is focused on utilizing and strengthening the natural enemy complex, by introducing parasitoids and predators from elsewhere in Australia (James, 1993). Initial introduction of *P. plagipennis* to the Murrumbidgee Irrigation Area in southern New South Wales were made in 1991–1992, but were hampered by difficulties in collecting and rearing sufficient numbers of bugs.

Associated studies on pheromones of *B. bibax* (James and Warren, 1989; James et al., 1990) led us to examine the exocrine gland morphology of *P. plagipennis*. This paper verifies the existence of sexually dimorphic DAGs in *P. plagipennis* and describes the identification, synthesis, and bioactivity of a male-produced aggregation pheromone emanating from these glands.

METHODS AND MATERIALS

Insects and Gland Extracts. Bugs (all stages) were collected at regular intervals during 1990–1993 from an unsprayed citrus grove at the Maroochy Horticultural Research Station, Nambour, and from Cotton trees (*Hibiscus tiliaceus* L.) in the Maroochydore district. The collecting area is approximately 100 km north of Brisbane, Queensland. *P. plagipennis* was maintained in the laboratory on *Tenebrio molitor* L. larvae and pupae supplemented with moths from a light trap.

Adult *P. plagipennis* were killed by storage in a freezer. After thawing, male and female bugs were dissected under tap water. The abdominal cavity of each bug was opened up dorsally exposing the pheromone glands. All fat body surrounding the glands was carefully removed. Pheromone glands were excised, blotted with tissue paper, and placed individually in small conical vials containing approximately 250 μl of CH_2Cl_2. They were then macerated with a melting point capillary tube.

Airborne Trapping of Bug Volatiles. Active trapping of airborne compounds was achieved by placing single insects in an all-glass system comprising a cylinder of ca. 150 ml with charcoal pre- and post-filters. Air was aspirated at ca. 30 ml/min through the series-connected system comprised of an activated carbon prefilter, cylinder, and Swinney Luer-Lok filter holder packed with ca. 30 mg activated carbon. Sampling normally continued for 24 hr.

Trapped compounds were desorbed from the carbon by detaching the filter holder from the apparatus, attaching it to a Luer-Lok syringe and eluting with CH_2Cl_2. Passive adsorption was achieved using proprietary stainless steel adsorption tubes (Perkin Elmer) packed with ca. 200 mg 60–80 mesh Tenax-TA. Subsequent desorption was achieved thermally at 200°C using the automated Perkin Elmer ATD-50 instrument, interfaced to the gas chromatograph–mass spectrometer (GC-MS), with helium carrier gas.

Chemical Analysis. Gland extracts and solvent-desorbed solutions were chromatographed on a nonpolar capillary column (0.25 μm film, 0.25 mm ID, 30 m DB-5, J&W Scientific, Folsom, California) housed in a Hewlett Packard 5890 GC. Injection was split and the oven was programmed from 45°C (2 min delay) to 260°C at 15°C/min. The carrier gas was nitrogen. GC-MS analyses of solutions and thermally desorbed analytes were performed on a capillary column of identical specifications to that described above, housed in a Finigan 1020B instrument. Mass spectra were generated by a 70 eV electron impact. Injection was splitless and the carrier gas helium. Resolution of synthetic racemic ester and determination of the absolute configuration of the natural compound was achieved by chromatography on a CDX-B (J&W) column of identical dimensions to that described above and operated isothermally at 100°C.

Chemical Manipulations and Syntheses. Transesterification of the posterior DAG contents was achieved by adding 1 ml methanolic boron trifluoride (14%) to the gland extract. After standing overnight, 5 ml aqueous sodium bicarbonate and 1 ml hexane were added, the mixture shaken, and the hexane solution removed.

The racemic major ester was synthesized by azeotropic distillation of water from a benzene solution (150 ml) of 5 g (*Z*)-3-hexenol, 5.5 g (*RS*)-2-hydroxy-3-methylbutyric acid and 50 mg *p*-toluenesulfonic acid. After 3 h, the solution was cooled, extracted with aqueous sodium bicarbonate, dried, and reduced under vacuum. Synthesis of the *R* enantiomer employed the acid derived from oxidative deamination (Mori, 1976) of D-valine as follows. (The *S* enantiomer was synthesized in identical fashion from L-valine). A solution of 10 g sodium nitrite in 20 ml water was added dropwise to 10 g D-valine in 40 ml molar sulfuric acid, with the temperature maintained below 5°C. The solution was allowed to warm to room temperature and stirred overnight, its pH adjusted to 3 if necessary, and then concentrated under vacuum. (A lower pH, resulting from the use of more than an exact equivalent of sulfuric acid, was associated with significant isomerization at the double bond during subsequent esterification). The resultant semisolid was extracted into ether, the ether dried and concentrated, and the residue dissolved in chloroform and dried further. Evaporation of the solvent yielded 8.2 g (81%) acid, which was used without further purification. The ester (12 g, 85%) derived from this chiral acid was distilled under reduced pressure (120°C, 5 mm). The level of contaminating *E* isomer was generally less than 5%. The racemic acid, amino acid enantiomers, and alcohol used in the syntheses were Aldrich Chem. Co. reagents, as were the remaining components identified in gland extracts and included in artificial blends.

Bioactivity Studies: Forced Air Olfactometer. A Perspex Y-tube olfactometer was used to determine the response of mated female *P. plagipennis* to heptane extracts of male posterior dorsal abdominal glands (DAGs), live calling males, and various formulations of synthetic pheromone. 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 (30.0 ± 0.5°C, 50 ± 10% relative humidity). 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 6–8 hr and four to

seven females were used per test. Records of movement were taken at 10-min intervals with bugs returned to the holding chamber.

Male DAG extracts were prepared by excising the posterior paired glands from a single bug under a microscope and placing them in a glass vial containing 1 ml of heptane. The glands were lightly crushed with a glass rod, and vials were covered with plastic film with a single pin hole. A vial with heptane only was used in the control chamber.

Live males, separated from females for at least three weeks, were presented in glass specimen tubes with muslin lids. A single male was presented at a time and the experiment commenced when calling behavior was observed.

Five formulations of synthetic pheromone were tested: (1) the racemate; (2) the natural enantiomer (*Z*)*R*, (3) the unnatural enantiomer *S* of the major male posterior DAG ester and two blends of the ester [(*Z*)*R* enantiomer] with minor components identified in airborne trappings and DAG extracts (4) 75% ester, 12.5% 2-phenylethanol, 12.5% 3-methylbutanol and (5) 60% ester, 15% 2-phenylethanol, 15% 3-methylbutanol, 5% (*E*)-2-hexenoic acid, 5% (*Z*)-3-hexenol). All synthetic materials were used at a rate of 100 mg/ml heptane in a glass vial covered with plastic film (punctured). Vials with heptane only served as controls.

Five to 19 replicates were performed for each material (synthetic samples, DAG extracts, live males) over a five-month period. Female *P. plagipennis* were "rested" for three to four days between each test to avoid habituation and were isolated from males during rest periods. To compensate for possible minor asymmetry in the olfactometer or experimental conditions, test and control samples were alternated between arms between each test. After each test, the olfactometer was washed thoroughly in hot water and acetone. Sporadic tests using heptane only in both chambers demonstrated neutrality of the system to female *P. plagipennis*. Data were subjected to chi-squared analysis.

Bioactivity Studies: Still-Air Olfactometer. A cage–funnel trap arrangement was used as a dual-choice still-air olfactometer. Two types of cage were used: (1 steel frame and meshed (60 × 30 × 30 cm) and (2 wooden framed, cloth meshed (60 × 45 × 45 cm). Funnel traps were constructed from Perspex cylinders (27 × 15 cm) with two holes (4 cm diam.) on opposite sides. Each hole was covered by an inwardly projecting screen funnel. Traps were baited with test material, control solutions (heptane only), or left unbaited. Bugs responded to traps by locomotion and/or flight. Tests were conducted at 27 or 30°C (±0.5°C) and ran for periods of 6–96 hr. Where tests ran for 24 hr or more, photophase was 15 hr. Most tests were conducted using 4–33 virgin or mated female *P. plagipennis* and the number of trapped bugs was recorded twice daily (0800 and 1600 hr). One series of tests used 13–17 mated males. Trapped bugs were reliberated in the cage. DAG extracts, calling males, and synthetic samples of the (*Z*)*R* enantiomer of the major male posterior DAG ester and two

blends of the ester with minor components were tested against females [80% blend: 80% ester, 10% (*Z*)-3-hexenol, 5% 2-phenylethanol, 2% decanal, 2% 3-methylbutanol, 1% (*E*)-2-hexenoic acid; 60% blend: 60% ester, 15% 2-phenylethanol, 15% 3-methylbutanol, 5% (*E*)-2-hexenoic acid, 5% (*Z*)-3-hexenol]. In addition, synthetic samples of the *R* enantiomer of the ester that were "contaminated" with 30–60% of the *E* isomer, were also tested. The (*Z*)-*R* enantiomer was also tested against males. DAG extracts were prepared and presented in glass vials as described previously. Single calling males were presented in glass specimen tubes with muslin lids. Synthetic materials were prepared at a rate of 25 mg/ml heptane and presented in uncovered glass vials. Three to seven replicates were conducted for each material. Female *P. plagipennis* were rested for at least three days between each test and were isolated from males. Food (moths, mealworms) was available for the bugs at all times during tests. Data were subjected to analysis of variance.

Bioactivity Studies: Flight Cage. Two outdoor flight cages (A: 3 × 3 × 1.8 m and B: 6.6 × 3 × 1.8 m) were used to test the response of mated female *P. plagipennis* to three synthetic materials: (1) (*Z*)*R* enantiomer of the major male posterior DAG ester, (2) 60% ester blend, and (3) 80% ester blend (blend formulations as described previously). The flight cages were constructed of a steel rod framework covered with green shadecloth. Cages were placed in an open sunlit grassy area.

Experiment 1 was conducted in flight cage A over 43 days (March 3–April 15). Six cylinder/funnel traps (same design as described previously) were hung from the central strut of the cage, spaced evenly from one side of the cage to the other. Two traps were baited with 25 mg of the (*Z*)*R* enantiomer in 1 ml of heptane, two with the 60% blend in 1 ml of heptane and two with heptane alone. All solutions were held in glass vials covered with punctured plastic film. Pheromones were replaced and traps repositioned weekly. Ten female *P. plagipennis* were released into the cage. Food for the bugs was available at all times with insects from a light trap released in the cage on alternate days. Traps were checked daily (1400–1500 hr). Trapped bugs were recorded and released back into the cage. Four females were still alive at the end of the experiment.

Experiment 2 was conducted in flight cage B during November 15–22, 1993. Four cylinder/funnel traps were attached to supporting stakes of potted citrus (Valencia orange) plants, 1.5 m tall. Eight plants were placed in a line from one end of the cage to the other (6.6 m) and traps attached to alternate plants. Two traps were baited with 25 mg of the *R* enantiomer in 1 ml of heptane, and two traps contained vials of heptane only. Traps with pheromones were separated by control traps. Pheromones were replaced at three-day intervals and traps were checked twice daily (0800 and 1400 hr). Twenty-five female *P. plagipennis* were released in the cage and retrieved at the conclusion of the experiment.

RESULTS

Dissection of adult *P. plagipennis* verified the presence of three well-developed paired DAGs (Aldrich, 1991). There was marked sexual dimorphism with the anterior and posterior glands being much larger in males than females (Figure 1). The male posterior DAG was always larger (2–3×) than the anterior DAG and usually antlerlike. The mid-DAGs of both sexes were morphologically similar.

Gland Extracts. Chromatograms of the male posterior gland extracts were dominated (>90%) by a component whose mass spectrum failed to match any NIST data-base entry; *m/z* (%): 113(1), 99(9), 83(23), 82(100), 76(5), 73(43), 67(50), 55(24), 43(5), 41(10), 39(4). However, the presence of several abundant fragments consistent with a hexenyl group, together with the occurrence of (*Z*)-3-hexenol as a minor component in the chromatogram (confirmed by coinjection of authentic material) suggested a (*Z*)-3-hexenyl ester. Transesterification of the crude gland extract with boron trifluoride in methanol yielded increased quantities of the hexenol and a component whose mass spectrum closely matched (NIST data base) that of the methyl ester of 2-hydroxy-3-methylbutyric acid. Subsequent synthesis of the (*Z*)-3-hexenyl ester of this acid yielded a compound whose retention time (nonpolar column) and mass spectrum duplicated the nat-

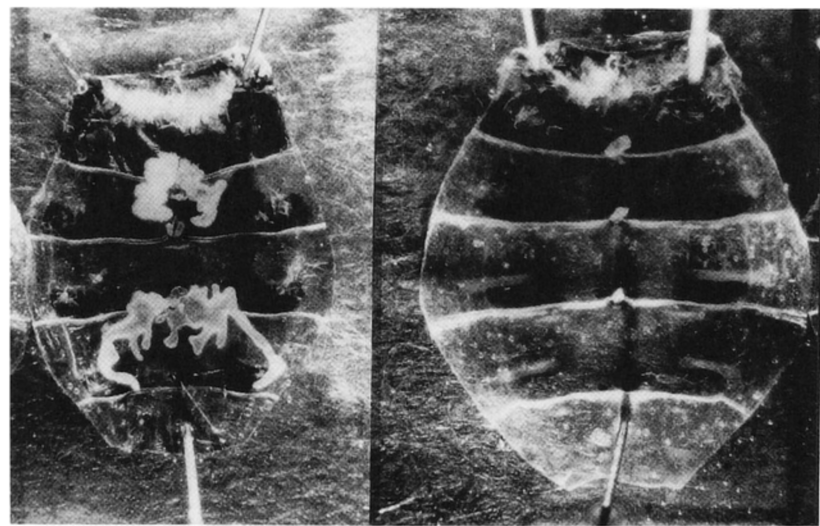

FIG. 1. Male (left) and female (right) *P. plagipennis* dissected to show anterior (upper), mid, and posterior dorsal abdominal glands.

ural compound exactly. Further characterization of the natural compound by gas chromatography on a chiral column established its absolute configuration as *R*, with an ee of >98%. Minor posterior gland components included decanal and three (*Z*)-3-hexenyl esters whose characterization is incomplete (Moore et al., 1993).

The male anterior DAG chromatographic profile was dominated by 3-methylbutanol (>75%), with 2-phenylethanol and (*E*)-2-hexenoic acid, all confirmed by coinjection of authentic standards.

The mid-DAG contents of both males and females were chromatographically identical and include at least 10 components. Chemical characterization is incomplete, but is considered irrelevant in the present context. The small size of the other female glands has so far precluded definitive study.

Airborne Volatiles Analysis. Chromatograms of airborne samples associated with males that appeared to be calling (deflexing abdomen and arching wings) trapped on carbon or Tenax displayed similar profiles. They were dominated by the major posterior DAG component (ca. 60%), but included the minor components present in this gland and the anterior DAG. No mid-DAG components were present. These profiles formed the basis for the artificial blends (Aldrich, 1991; Moore et al., 1993).

Bioactivity Studies: Forced-Air Olfactometer. Female *P. plagipennis* were significantly (<0.001) attracted to male posterior DAG extracts, calling males, the natural enantiomer *R* of the DAG major ester, and two blends containing the (*Z*)*R* enantiomer and associated minor components. There was no response

TABLE 1. ATTRACTION OF MATED FEMALE *P. plagipennis* TO VARIOUS NATURAL AND SYNTHETIC PHEROMONE SOURCES IN FORCED-AIR OLFACTOMETER TESTS

Test material	Replicates	Responses (%) to		χ^2
		Test material	Control	
Male posterior DAG extract	5	117[a] (69.6)	61	17.6
"Calling" Male	19	295[a] (74.7)	100	96.3
Racemate of male posterior DAG ester	12	91 (47.6)	100	0.42
Natural enantiomer (*R*) of male posterior DAG ester	17	273[a] (83.2)	55	144.9
Unnatural enantiomer (*S*) of male posterior DAG ester	17	142 (46.7)	162	1.3

[a]Significantly greater than control response ($P < 0.001$).

to the racemate of the major ester or the unnatural enantiomer *S* (Table 1). The natural enantiomer (*Z*)*R* appeared to be the most attractive material tested with bugs responding in a ratio of almost 5:1.

Bioactivity Studies: Still-Air Olfactometer. Female *P. plagipennis* were significantly attracted ($P < 0.05$) to male posterior DAG extracts, calling males, the (*Z*)*R* enantiomer of the DAG major ester, and the two blends of the (*Z*)*R* enantiomer and minor components (Tables 2–3). Males were also significantly attracted to the (*Z*)*R* enantiomer (Table 4). There was no response to synthetic materials contaminated with the *E* isomer of *R* (Table 5). The response rate of females in these tests was fairly consistent at ca. 22–31%. Females tested three to seven days since last mating did not respond to the (*Z*)*R* enantiomer, but did so when retested at seven to ten days after mating (Table 6).

Bioactivity Studies: Flight cage. Both experiments demonstrated a clear response by female *P. plagipennis* to find and enter synthetic pheromone-baited traps (Table 7). Females appeared to respond equally well to (*Z*)*R* and the (*Z*)*R* blend.

TABLE 2. ATTRACTION OF VIRGIN AND MATED FEMALE *P. plagipennis* TO MALE POSTERIOR DAG EXTRACTS AND CALLING MALES IN STILL-AIR OLFACTOMETER TESTS

				Responses (*N*) to	
Replicate	Females (*N*) and mating status	Duration of test (hr)	Temperature (°C)	Male posterior DAG extract	Heptane (control)
1	4 mated	48	30	1	0
2	9 mated	20	30	2	0
3	8 mated	24	30	1	0
4	11 mated	24	30	1	0
5	11 mated	6	27	2	0
6	7 mated	34	30	1	0
Mean ± SE				1.3 ± 0.2[a]	0
				Calling male	Unbaited (control)
1	4 virgin	39	30	2	0
2	6 virgin	6	27	1	0
3	5 virgin	48	30	1	0
4	17 virgin	48	30	2	0
Mean ± SE				1.5 ± 0.25[a]	0

[a] Significantly more than control ($P < 0.05$).

TABLE 3. ATTRACTION OF VIRGIN AND MATED FEMALE *P. plagipennis* TO (*Z*)*R* ENANTIOMER OF MALE POSTERIOR DAG MAJOR ESTER AND BLENDS CONTAINING (*Z*)*R* AND ASSOCIATED MINOR COMPONENTS IN STILL-AIR OLFACTOMETER TESTS

Replicate	Females (*N*) and mating status	Duration of test (hr)	Temperature (°C)	Responses (*N*) to (*Z*)*R*	Responses (*N*) to Heptane (control)
1	11 virgin	48	27	2	0
2	11 virgin	31	27	2	0
3	20 virgin	32	30	4	0
4	18 virgin	54	30	3	1
5	18 virgin	31	30	6	0
6	18 mated	96	30	4	0
7	14 mated	96	30	4	1
Mean ± SE				3.6 ± 0.5[a]	0.25 ± 0.2
				(*Z*)*R* blend	Heptane (control)
1	12 mated	48	30	3[b]	0
2	21 virgin	48	30	3[c]	0
3	8 virgin	54	30	4[c]	1
4	10 virgin	31	30	6[c]	1
Mean ± SE				4 ± 0.6[a]	0.5 ± 0.25

[a] Significantly more than control ($P < 0.05$).
[b] 60% blend.
[c] 80% blend (see text).

TABLE 4. ATTRACTION OF MATED MALE *P. plagipennis* TO (*Z*)*R* ENANTIOMER OF MALE POSTERIOR DAG MAJOR ESTER

Replicate	Males (*N*)	Duration of test (hr)	Temp (°C)	Responses (*N*) to (*Z*)*R*	Responses (*N*) to Heptane (control)
1	13	96	30	5	1
2	17	96	30	1	0
3	17	96	30	4	1
Mean ± SE				3.3 ± 1.0[a]	0.7 ± 0.3

[a] Significantly more than control ($P < 0.05$).

TABLE 5. RESPONSES OF VIRGIN AND MATED FEMALE *P. plagipennis* TO *E* ISOMER CONTAMINATED (30–60%) (*Z*)*R* ENANTIOMER OF MALE POSTERIOR DAG MAJOR ESTER AND 60% BLEND CONTAINING (*Z*)(*E*)*R* AND ASSOCIATED MINOR COMPONENTS IN STILL-AIR OLFACTOMETER TESTS

Replicate	Females (*N*) and mating status	Duration of test (hr)	Temperature (°C)	Responses (*N*) to (*Z*)(*E*)*R*	Heptane (control)
1	12 virgin	48	30	0	0
2	7 virgin	96	27	0	0
3	7 virgin	96	27	1	0
4	10 virgin	96	30	0	1
5	13 virgin	48	27	0	0
Mean ± SE				0.2 ± 0.2[a]	0.2 ± 0.2
				60% (*Z*)(*E*)*R* blend	Heptane (control)
1	12 virgin	96	30	0	0
2	21 virgin	96	30	0	1
3	33 virgin	96	30	1	0
4	15 virgin	96	30	0	1
5	11 virgin	96	30	1	0
6	14 virgin	96	30	1	1
Mean ± SE				0.5 ± 0.2[a]	0.5 ± 0.2

[a] No significant difference from control ($P > 0.05$).

DISCUSSION

This study presents the first evidence of an aggregation pheromone produced by a reduviid. Male *P. plagipennis* possess three pairs of DAGs, of which the anterior and posterior pair are considerably larger than the corresponding glands in females (Aldrich, 1991). Calling males release a pleasant-smelling odor from these glands comprised largely (ca. 60%) of a novel ester, (*Z*)-3-hexenyl (*R*)-2-hydroxy-3-methylbutyrate, with 3-methylbutanol, 2-phenylethanol, (*Z*)-3-hexenol, decanal, (*E*)-2-hexenoic acid, and three minor hexenyl esters, as minor components. The major ester alone, or in combination with minor components, was attractive to female *P. plagipennis* in olfactometer and flight-cage tests. The major ester was also attractive to males, indicating that the compound functions as an aggregation pheromone. It is possible that addition of one or more of the minor hexenyl esters might increase potency of the pheromone and this should be studied further.

TABLE 6. EFFECT OF TIMING OF LAST MATING ON RESPONSIVENESS OF FEMALE *P. plagipennis* TO (*Z*)*R* ENANTIOMER OF MALE POSTERIOR DAG MAJOR ESTER IN STILL-AIR OLFACTOMETER TESTS (SAME FEMALES USED IN BOTH SERIES)

				Responses (*N*) to	
Replicate	Females (*N*)	Duration of test (hr)	Temperature (°C)	(*Z*)*R*	Heptane (control)
3–7 days since mating					
1	16	48	30	0	1
2	10	48	27	0	0
3	10	48	27	0	0
Mean ± SE				0	0.3 ± 0.3
7–10 days since mating					
1	16	48	30	2	0
2	10	48	27	3	0
3	10	48	27	3	0
Mean ± SE				2.7 ± 0.3[a]	0

[a] Significantly greater than control ($P < 0.05$).

TABLE 7. NUMBER OF FEMALE *P. plagipennis* TRAPPED IN PHEROMONE-BAITED AND UNBAITED TRAPS IN TWO FLIGHT-CAGE EXPERIMENTS

	(*Z*)*R*	60% (*Z*)*R* blend	Unbaited
Experiment 1, March 3–April 15, 1993 (10 females)	5	5	0
Experiment 2, November 15–22, 1993 (25 females)	5		0

Only the natural *R* enantiomer of the major ester was attractive. Nonattractivity of the racemate indicates the *S* enantiomer inhibits the action of the antipode. Inhibition of the biological activity of one enantiomer by its antipode has been noted before (Tumlinson et al., 1977), but a situation where a synthetic racemate successfully mimics a chiral pheromone because of the biological inactivity of the antipode is more common (Mori, 1989).

The presence of 30–60% of the *E* isomer in samples of the major ester rendered the compound unattractive to *P. plagipennis*. Progressive environmental isomerization of the pheromone of the pea moth (*Cydia nigricana* F.) was reported to inhibit attraction of this insect (Witzgall et al., 1993). Preliminary tests indicate the pheromone of *P. plagipennis* does not undergo rapid isomer-

ization when exposed to field or laboratory conditions. However, it is clear that excessive isomeric contamination should be avoided during synthesis of the compound. It is possible that the minor ($<5\%$) E isomer contamination that was present in the bioactive samples of $(Z)R$ in this study may have limited the responses shown by *P. plagipennis*. A totally pure formulation, although difficult to achieve, should also be tested. Mating of *P. plagipennis* appeared to be followed by a period of about seven days when females did not respond to pheromone. It is also quite likely that responses to the pheromone vary with physiological age. Most tests in this study were conducted using young, virgin females, which are likely to show greatest response to an aggregation pheromone.

No attempt was made in this study to determine the effect of pheromone dosage on attraction of female *P. plagipennis*. Doses used were high (100 and 25 mg) and may have been excessive. Responses by females to male posterior DAG extracts were comparable to those for synthetic baits, despite, presumably, considerably lower doses of released pheromone. Excessive pheromone doses can cause adaptation, habituation, or confusion in target insects (Bartell, 1982). The effect of lower doses of pheromone on attraction of *P. plagipennis* should be evaluated.

An effective, synthetic aggregation pheromone for *P. plagipennis* would be of considerable value to more effective and widespread utilization of this predator in managing populations of the citrus stink bug pest *B. bibax*. Pheromone could be used to concentrate *P. plagipennis* populations in citrus orchards to enhance biological control. Pheromone could also be used to remove *P. plagipennis* from orchards prior to application of insecticides. Pheromone-baited traps may provide the ability to mass-trap *P. plagipennis* for release at sites or in regions in which it currently does not occur. The value of *P. plagipennis* in citrus orchards is not confined to regulating *B. bibax*. As a general predator, it also preys on other citrus pests such as caterpillars, mealybugs, weevils, etc. (James, 1994). Other perennial horticultural ecosystems in Queensland and northern New South Wales also benefit from the presence of *P. plagipennis*. In addition to being a tool for harnessing the biological control potential of *P. plagipennis* in horticultural crops, synthetic pheromone would also serve as a powerful research tool, enabling field evaluation of the role of this predator in biological control of specific pests. Assassin bugs as biological control agents have received even less attention than other general predators (Schaefer, 1988), largely due to the difficulties involved in evaluation (Luck et al., 1988).

Pheromonal manipulation of predatory heteropterans is the basis of HOPE (husbandry of pest enemies), a concept presented by Aldrich (1991). The first commercially available product for HOPE is the Spined Soldier Bug Attractor, based on the male-produced aggregation pheromone of this important North American predatory pentatomid (*Podisus maculiventris* Say) (Aldrich et al.,

1984). Aimed at the home garden market, the pheromone attracts *P. maculiventris* to aid in garden pest control. A similar market exists in Australia for a *P. plagipennis* attractant.

Much research remains before the use of pheromones in husbandry of *P. plagipennis* can be practiced. Rigorous field evaluations of dose and blends are required to ensure an optimal formulation is produced. More information is also needed on the role of aggregation pheromone in *P. plagipennis* biology to ensure appropriate use of and expectations from synthetic material.

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VOLATILE SECRETIONS OF OLD WORLD ARMY ANT *Aenictus rotundatus* AND CHEMOTAXONOMIC IMPLICATIONS OF ARMY ANT DUFOUR GLAND CHEMISTRY

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Abstract—The Dufour glands of *Aenictus rotundatus* contain a complex mixture of terpenoids with geranylgeraniol comprising over 50% of the secretion. Some novel compounds have been tentatively identified as higher homologs of 1,3,3-trimethyl-2,7-dioxabicyclo[2,2,1]heptane based on GC-MS data. The Dufour gland secretion of *A. rotundatus* is more similar in composition to the secretions of members of the subfamily Ecitoninae than to its closer relatives from the tribe Dorylini, a result that further complicates studies on the phylogeny of army ants. The mandibular glands of *A. rotundatus* contain a mixture of 4-methyl-3-heptanone and limonene in trace amounts, and the well-developed postpygidial glands contain methyl anthranilate only.

Key Words—Hymenoptera, Formicidae, army ants, Dorylinae, *Aenictus*, *Dorylus*, *Eciton*, Ecitoninae, Dufour gland, mandibular gland, postpygidial gland, ant secretions, chemotaxonomy.

INTRODUCTION

Army ants are characterized by their nomadic existence and group predation behavior (Wilson, 1958). They are divided into two subfamilies, each with a distinct zoogeographical distribution: the Ecitoninae in the New World and the Dorylinae in the Old World tropics (Hölldobler and Wilson, 1990). The Dory-

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linae are further divided into two tribes: the Dorylini (single genus *Dorylus*) and the Aenictini [single genus *Aenictus* (Gotwald, 1982)]. Due to the considerable differences in external morphology and based on a geological interpretation of their geographical distribution, it was suggested that the two tribes developed separately (Gotwald, 1979). Furthermore, the common presence, in *Aenictus* and *Eciton*, of a conspicuous epithelium associated with the inner part of the seventh abdominal sternite has led to the suggestion that there is a possible relationship between the Aenictini and the Ecitoninae, in spite of their very different geographical distribution (Jessen, 1987). However, it has since been demonstrated that the Dufour glands of members of the Aenictini and Dorylini possess a unique crenellate lining, which distinguishes them from other ants and suggests both tribes should be classified within a single subfamily, the Dorylinae (Billen and Gotwald, 1988).

Previous work has been carried out in our laboratories on the volatile secretions of *Dorylus molestus* (Dorylini) (Bagnères et al., 1991), *Eciton burchelli*, *Labidus praedator*, and *L. coecus* (Ecitoninae) (Keegans et al., 1993) as part of a chemotaxonomic survey of army ant secretions. Here we report a study of the Dufour gland, mandibular gland, and postpygidial gland secretions of *Aenictus rotundatus* (Aenictini) and draw attention to the phylogenetic implications of the results.

METHODS AND MATERIALS

Live worker ants of *Aenictus rotundatus* were collected, from their raiding column, in Nairobi, Kenya, and immediately flown to Leuven. The ants were immobilized by cooling over liquid nitrogen and dissection was carried out under a binocular microscope (as described by Morgan, 1990). The dissected glands were dried, sealed in glass capillaries, and stored in a refrigerator until ready for analysis by gas chromatography–mass spectrometry (GC-MS).

GC-MS was performed (as described by Bagnères et al., 1991) on a 5% phenyl–95% dimethylsiloxane phase of 0.25 μm film thickness in a fused silica capillary column (12 m $\times$ 0.2 mm). Helium was used as the carrier gas at 1 ml/min. The oven temperature was programmed from 30°C to 270°C at 7°/min.

Commercially available samples of 6-methyl-5-hepten-2-one (Koch Light), geranylacetone (Aldrich), limonene (Aldrich), and methyl anthranilate (Aldrich) were used to confirm identifications based on mass spectra. Samples of β-springene (P. Baekström) and geranyllinalool and geranylgeraniol (R. Lucas, Quest International) were received as gifts.

1,3,3-Trimethyl-2,7-dioxabicyclo[2,2,1]heptane was synthesized from 6-methyl-5-hepten-2-one via the γ,δ-epoxide (by a modification of the method

of Gaoni, 1968). To a solution of the ketone (0.5 g, 3.95 mmol) in THF–H_2O (3:4, 50 ml) a solution of magnesium monoperoxyphthalate (1.2 g, 2.43 mmol) in THF–H_2O (3:4, 30 ml) was added with stirring. The reaction mixture was left at room temperature for 2 hr before the addition of saturated sodium hydrogen carbonate solution (50 ml). The neutral solution was extracted with ether (50 ml) and the extract washed with water (20 ml), dried (magnesium sulfate), and concentrated by rotary evaporator to yield a colorless oil (0.51 g). GC-MS analysis of the product showed essentially a single peak corresponding to the γ,δ-epoxide of 6-methyl-5-hepten-2-one [M^+ 142(2), 127(3), 84(30), 83(11), 82(8), 72(16), 59(11), 43(100)). NMR data (δ1.23(s, 2 × CH_3), δ1.69(m, CH_2CH_2CO), δ2.13(s,CH_3CO) and δ2.50(m, epoxidic **H** and CH_2CO)] and IR data [ν1718(carbonyl), 1377, 1358 and 1162 cm^{-1} (epoxide)] were found to be identical to those previously reported (Gaoni, 1968).

Cyclization was achieved by heating the epoxyketone over a few grains of silica gel for 20 min to yield 1,3,3-trimethyl-2,7-dioxabicyclo[2,2,1]heptane (95%). GC-MS analysis revealed that the bicyclic compound had a similar mass spectrum to the epoxyketone but it eluted 4 min earlier [M^+ 142(3), 127(3), 84(20), 83(10), 82(16), 72(31), 59(5), 42(100)]. NMR data [δ1.13(s, CH_3), δ1.19(s,CH_3), δ1.35–2.30(m, 2 × CH_3), δ1.51(s, CH_3), δ4.16(d,**CH**)] was consistent with that previously reported (Gaoni, 1968).

A mixture of farnesene isomers was prepared by the dehydration of nerolidol (Parry, 1978). GC-MS analysis of the products showed a 70% conversion to the six farnesene isomers.

A mixture of geranylneral and geranylgeranial was prepared by PCC oxidation (Corey and Suggs, 1975) of geranylgeraniol. GC-MS analysis showed a 90% conversion to an equilibrium mixture of geranylneral [M^+ 288(2), 245(2), 136(8), 107(15), 84(20), 81(50), 69(97), 41(100)] and geranylgeranial [M^+ 288(2), 245(2), 136(9), 107(12), 84(31), 81(42), 69(99), 41(100)].

4-Methyl-3-heptanone was prepared in quantitative yield by hypochlorite oxidation of 4-methyl-3-heptanol.

RESULTS

The Dufour gland secretion of *A. rotundatus* was found to be dominated by terpenoids (Figures 1 and 2, Table 1). Geranylgeraniol **(17)** was the major component, comprising 50.8% of the secretion. The other principal components included an equilibrium mixture of geranylneral **(16)**, which is masked by **17** in Figure 1, and geranylgeranial **(18)**, and β-springene **(12)**. The bicyclic acetal 1,3,3-trimethyl-2,7-dioxabicyclo[2,2.1]heptane **(1)**, 6-methyl-5-hepten-2-one **(2)**, geranylacetone **(4)**, and geranyllinalool **(15)**, present in the secretion, have been found in the Dufour glands of New World army ants (Keegans et al., 1993). We have also found two pairs of isomeric compounds in the gland of

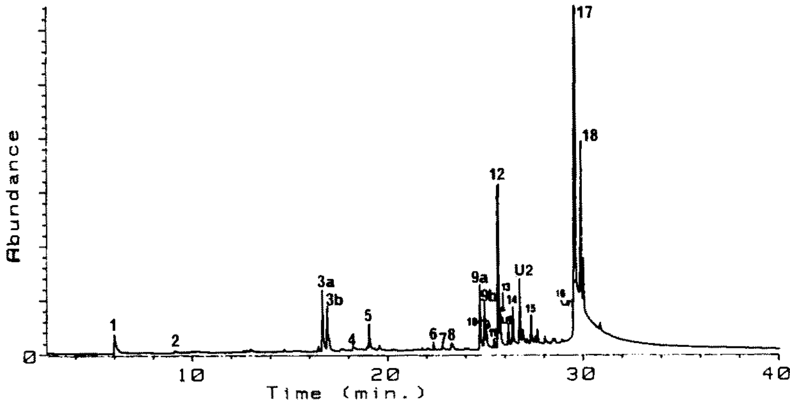

FIG. 1. Total ion chromatogram of the volatile compounds in the Dufour gland of *Aenictus rotundatus* (the numbering of the peaks corresponds to that in Figure 2 and Table 1).

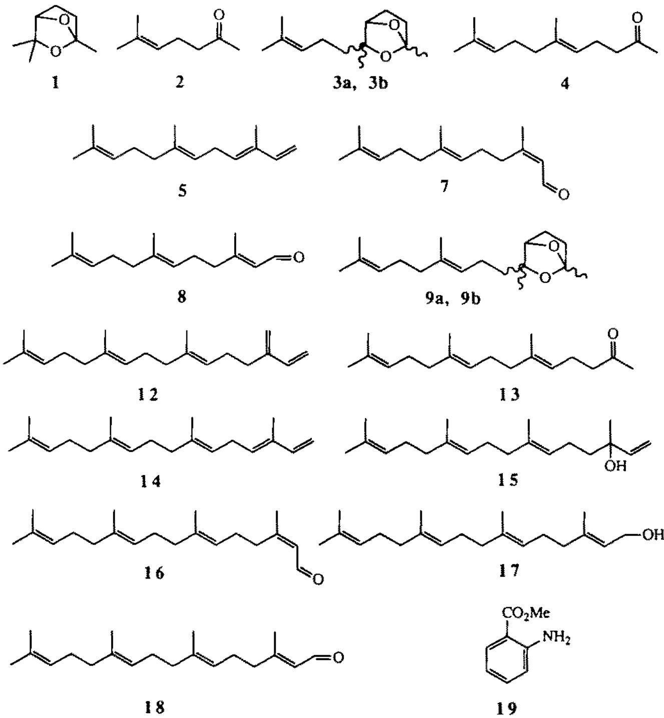

FIG. 2. The chemical structures of the volatile compounds from *Aenictus rotundatus*. The structures of **3a, 3b** and **9a, 9b** are tentative.

TABLE 1. PERCENTAGE COMPOSITION OF VOLATILES FROM DUFOUR GLAND SECRETION OF *A. rotundatus*

Compound	Mean % (N = 5)	SD
1. Bicyclic acetal	1.2	1.1
2. 6-Methyl-5-hepten-2-one	t[a]	
3a. Bicyclic acetal	2.9	1.9
3b. Bicyclic acetal	2.5	1.7
4. Geranylacetone	0.6	0.2
5. (*E,E*)-α-Farnesene	0.8	1.0
6. Heptadecane	0.6	0.1
7. (*Z,E*)-Farnesal	t	
8. (*E,E*)-Farnesal	0.6	0.3
9a. Bicyclic acetal	2.9	1.4
9b. Bicyclic acetal	2.1	0.9
10. Nonadecene	1.4	0.3
11. Nonadecane	0.6	0.1
12. β-Springene	7.6	1.9
13. Farnesylacetone	1.5	0.9
U1. Unknown 1	1.1	0.1
14. α-Springene	2.2	0.6
U2. Unknown 2	2.8	1.4
15. Geranyllinalool	1.7	0.5
16. Geranylneral	4.6	1.0
17. Geranylgeraniol	50.8	12.6
18. Geranylgeranial	12.1	0.5
Total/μg	53.9	24.4

[a] t = trace component (<0.5%).

A. rotundatus, for which we propose the structures **3a, 3b** and **9a, 9b** (Figure 2), by analogy to **1.** These compounds were also found by Keegans et al. (1993) but remained unidentified at that time.

In addition to the terpenoids present in the Dufour gland, small amounts of heptadecane **(6)**, nonadecene **(10)**, and nonadecane **(11)** were also detected. The Dufour gland of *A. rotundatus* was the major source of volatiles of workers with each gland containing a mean of 54 (±24) μg.

Due to the small amount of volatile material in the heads of *A. rotundatus*, 10 heads were used for each injection (mean amount = 19 ± 9 ng, $N = 3 \times 10$). The major component was identified as 4-methyl-3-heptanone (mean 78 ± 13%) and the minor component as the monoterpene limonene (mean 22 ± 13%). The amount reported may not be truly representative of normal conditions, for many species of ant tend to discharge their mandibular gland secretion when seriously disturbed.

The postpygidial glands of *A. rotundatus* are well developed and were found to contain methyl anthranilate **(19)** (mean 104 ± 100 ng, $N = 10$) as the only detectable component.

No volatiles were detected in the poison gland or sternal gland of *A. rotundatus*, although both elicited a trail-following response in the ants.

DISCUSSION

The Dufour gland secretion of *A. rotundatus* contains a complex mixture of structurally related terpenoids. Geranylgeraniol, the major component, has been previously identified as a component of the Dufour gland secretion of *Formica fusca*, *F. nigricans*, and *F. polyctena* (Bergström and Löfqvist, 1973). We have tentatively identified higher homologues of the bicyclic acetal **(1)**. The mass spectra of **3a** and **3b**, shown in Figure 3, have the same general appearance as that of **1**. The molecular ions at *m/z* 210 would correspond to the molecular formulae of **3a** and **3b** ($C_{13}H_{22}O_2$) and the spectra show fragment ions at *m/z* 127, 84, and 43, which are important ions in the mass spectrum of compound **1**. The relatively low retention times of **3a** and **3b** provided further evidence for the bicyclic structure, since they eluted before geranylacetone **4** just as **1** eluted before 6-methyl-5-hepten-2-one **(2)**. The two forms of **3** are probably due to *cis-trans* isomerism of the side chains about the rings.

Compounds **9a** and **9b** gave mass spectra of the same general appearance as **1**, **3a** and **3b** and had molecular ions at *m/z* 278. We suggest therefore that they have the structure shown in Figure 2.

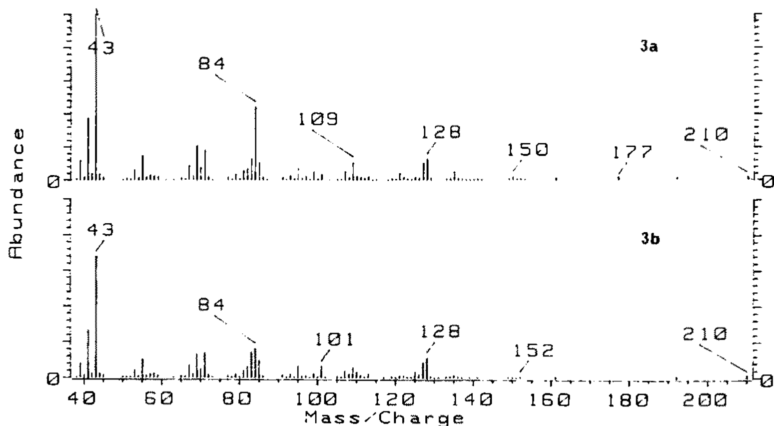

FIG. 3. The mass spectra of compounds **3a** and **3b**, tentatively identified as bicyclic acetals.

2. R= CH_3

4. R = (isoprenyl chain) CH_2

13. R = (isoprenyl chain) CH_2

Epoxidation

Cyclisation

1. R= CH_3

3. R = (isoprenyl chain) CH_2

9. R = (isoprenyl chain) CH_2

FIG. 4. A possible biosynthetic relationship between the isoprenylacetones and the bicyclic acetals.

The presence of 6-methyl-5-hepten-2-one **(2)**, geranylacetone **(4)**, and farnesylacetone **(13)** was yet further evidence for the bicyclic structure of **3** and **9**, since the isoprenylacetones can be thought of as their biosynthetic precursors, via the γ,δ-epoxides (Figure 4). It is known that γ,δ-epoxides are relatively unstable and readily decompose to the bicyclic acetals on distillation and exposure to acid (Gaoni, 1968). We therefore coinjected a synthetic sample of the epoxide of 6-methyl-5-hepten-2-one into the GC-MS with an *A. rotundatus* Dufour gland. However, the epoxide did not cyclize, demonstrating that the bicyclic acetals were real components in the secretion and not decomposition products brought about by heating the samples in the injection port.

Several attempts to synthesize the γ,δ-epoxide of geranylacetone, and thus the bicyclic acetal, were made. Treatment of geranylacetone with one equivalent of epoxidizing agent gave the undesired 9,10-epoxide, while attempted selective reduction of the corresponding bis-epoxide with sodium cyclopenadienyldicarbonyliron gave no product. We were thus unable to confirm the structures of compounds **3** and **9.**

Comparison of the Dufour gland secretion of *A. rotundatus* with those of the Dorylini [*Dorylus molestus* and *D. nigricans*, (Bagnères et al., 1991)] and Ecitoninae [*Eciton burchelli*, *Labidus praedator*, and *L. coecus* (Keegans et al., 1993)] is very interesting, as, on the limited information available, the chemistry of *A. rotundatus* appears to be closer to the Ecitoninae than the Dorylini. The Dufour glands of members of the Dorylini examined to date contain essentially linear hydrocarbons with (Z)-9-tricosene as the major component, while the Ecitoninae contain a mixture of terpenoids and linear hydrocarbons. Compounds **1, 2, 3, 4, 6, 10, 11,** and **15** from the Dufour gland of *A. rotundatus* are also present in the Dufour gland of *E. burchelli*. Thus, the chemical results appear to contradict the morphological study, which was used as evidence against the

triphyletic origin of army ants (Billen and Gotwald, 1988) and support that which linked the Aenictini and the Ecitoninae (Jessen, 1987). We hope to obtain more army ant species in the future to examine this curious result further.

The major volatile from the heads of *A. rotundatus*, like *E. burchelli* and *L. coecus*, is 4-methyl-3-heptanone, which is a common component of the mandibular gland secretions of myrmicine ants, where it often functions as an alarm pheromone (Attygalle and Morgan, 1984). However, its role in army ant communication is unknown, and the use of such a common compound as a guide in ant chemotaxonomy is limited.

Postpygidial glands are well developed in the Aenictini. The only component detected in the postpygidial glands of *A. rotundatus* was methyl anthranilate, which has been found previously in the mandibular glands of males of *Camponotus* species (Brand et al., 1973) where it is suspected to act as a sex pheromone. The function of methyl anthranilate in *A. rotundatus* is unknown, but in an unidentified *Aenictus* species (close to *A. laeviceps*) we have demonstrated that, together with methyl nicotinate, it acts as the trail pheromone (Oldham et al., 1994).

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ETHANOL AND AMBROSIA BEETLES IN DOUGLAS FIR LOGS WITH AND WITHOUT BRANCHES

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Abstract—November-felled Douglas fir (*Pseudotsuga menziesii* (Mirb.) Franco) logs with and without branches were left lying on the forest floor through August. In May, as the logs were being colonized by ambrosia beetles, *Trypodendron lineatum* (Oliv.) and *Gnathotrichus retusus* (LeConte), the ethanol, acetaldehyde, and water concentrations in the delimbed logs were significantly higher than in the branched logs. Since both log types received the same rainfall, lower water contents in branched logs was probably the result of absorbed water being transported through the branches via capillary movement and evaporation. Lower tissue water levels could have prevented the establishment and maintenance of anaerobic conditions, thus limiting the synthesis of acetaldehyde and ethanol in the branched logs. By late August, the beetle densities in delimbed logs were 9–16 times greater than in the branched logs. Log ethanol concentrations could be a key chemical factor affecting the ambrosia beetle attack densities. Acetaldehyde concentrations in the logs also may have affected the attack densities.

Key Words—*Pseudotsuga menziesii*, *Trypodendron lineatum*, *Gnathotrichus retusus*, Coleoptera, Scolytidae, anaerobic respiration, volatile attractants, host selection.

INTRODUCTION

In Pacific Northwest forests, conifer logs lying on the forest floor during spring may be attacked by ambrosia beetles *Trypodendron lineatum* (Oliver), *Gnathotrichus sulcatus* (LeConte) and *G. retusus* (LeConte). These insects function as initiators of decomposition by boring through the bark and excavating egg galleries in the sapwood, which they inoculate with microorganisms to grow and utilize as food (Dowding, 1984; Carpenter et al., 1988; Schowalter et al.,

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1992). *Trypodendron lineatum* prefer to attack aged logs felled during the previous summer, fall, or winter, while fresh spring-felled logs receive little or no attack (Prebble and Graham, 1957; Chapman, 1961; Johnson, 1964; Dyer and Chapman, 1965; Johnson and Zingg, 1969). *Gnathotrichus sulcatus* and *G. retusus* also attack aged logs, but they are less discriminatory and will attack logs aged for only short periods (Prebble and Graham, 1957; Johnson, 1964; Johnson and Zingg, 1969; Cade, 1970; Cade et al., 1970; McLean and Borden, 1977).

During host selection, ambrosia beetles will discriminate among conifer species. Logs of Douglas fir, *Pseudotsuga menziesii* (Mirb.) Franco, and western hemlock, *Tsuga heterophylla* (Raef.) Sarg., are preferred over Pacific silver fir, *Abies amabilis* Dougl. ex Forbes, and western red cedar, *Thuja plicata* Donn ex D. Don, by both *T. lineatum* and *Gnathotrichus* spp. (Johnson, 1958; Chapman, 1961; Zhong and Schowalter, 1989). Even when logs of a single species are felled on the same date there can be considerable variability in ambrosia beetle densities (Dyer and Chapman, 1965).

Aging log tissues produce ethanol (Cade, 1970; Cade et al., 1970; Moeck, 1970; Kelsey, 1994), which functions as a primary attractant for the initial pioneering beetles searching for suitable host logs (Moeck, 1970; Nijholt and Shönherr, 1976; Klimetzek et al., 1986; Liu and McLean, 1989; Schroeder and Lindelöw, 1989). The ethanol may also function as an arrestant and attack stimulant as the beetles approach or make contact with the log surface (McLean and Borden, 1977; Salom and McLean, 1990; Kelsey, 1994). When beetles begin boring into the bark, aggregation pheromones are released, providing a signal for other species members to respond. Beetle attraction to the pheromones may be synergistically enhanced by the simultaneous release of ethanol and α-pinene from the log tissues (McLean and Borden, 1977; Vité and Bakke, 1979; Borden et al., 1980; Shore and McLean, 1983).

Ethanol may be found in the phloem and sapwood of trees (Crawford and Baines, 1977; Crawford and Finegan, 1989; Kimmerer and Stringer, 1988; MacDonald and Kimmerer, 1991). It can be synthesized in all living tree tissues by anaerobic respiration when the supply of O_2 for aerobic respiration is inadequate (Kimmerer and MacDonald, 1987; Kimmerer and Stringer, 1988; Harry and Kimmerer, 1991; MacDonald and Kimmerer, 1991). Placing fresh conifer tissues in a N_2 atmosphere (Moeck, 1970) or submerging tissues in water (Graham, 1968; Cade, 1970; Cade et al., 1970) will quickly stimulate anaerobic respiration and ethanol synthesis.

Other than demonstrating that ethanol is present in aging or anaerobic log tissues (Cade, 1970; Cade et al., 1970; Moeck, 1970; MacDonald and Kimmerer, 1991; Lindelöw et al., 1992), there have been no studies to validate a relationship between ethanol concentrations in host logs and ambrosia beetle attack behavior. It was recently reported (Kelsey, 1994) that November-felled

Douglas fir logs left lying in the forest produced substantial quantities of ethanol, whereas logs felled in January did not. The November-felled logs were the first to be attacked by ambrosia beetles in the spring. Moreover, a correlation was observed between log ethanol concentrations measured in March and ambrosia beetle densities counted in August. As the spring ambrosia beetle attack began, it was noted that the residual crown portion of each November-felled log, with branches still attached, were only lightly attacked relative to the delimbed logs cut from their base. Similar responses have been reported previously (Johnson, 1961, 1964; Johnson and Zingg, 1969).

If the quantity of ethanol produced in conifer logs is a key factor regulating ambrosia beetle attack behavior, then a measurable difference in ethanol quantities would be expected for these two log types experiencing dissimilar levels of attack. The delimbed logs and their residual branched crowns were sampled in May to verify that the lower ambrosia beetle attack densities were associated with logs containing lower quantities of ethanol.

METHODS AND MATERIALS

Study Site. This study was conducted in two stands of Douglas fir near Corvallis, Oregon, in the Oregon State University, Paul M. Dunn Research Forest. Stand 1 was 11.2 ha, 140–207 m elevation, average slope 12°, with a southeast aspect. Prior to harvest there were 272 trees/ha with a 20.3-cm or greater diameter at breast height (DBH). Average DBH for these trees was 38.4 cm, and average height of the dominant trees was 25.9 m. Stand 2, about 320 m northeast of stand 1, was similar in composition and the site used in the previous experiment (Kelsey, 1994). Both stands were harvested in August 1991 by removing all trees in patches of about 0.6–0.9 ha.

Study Trees and Logs. Six Douglas fir trees were selected from a residual portion of stand 1 and their crowns felled at 15–18 m above ground on November 12, 1991, leaving the upright stems to serve as snags for wildlife. In stand 2, two trees were selected in each of three blocks of residual forest separated by the harvest strips. Their crowns were felled on November 17, 1991. In both stands, the day after felling, a 2.44-m bolt was cut from the base of each crown and delimbed. The remaining portion of the crown was left in one long piece with branches intact. The number and location of the attached branches, and their degree of damage from felling, varied considerably among the crowns. Some crown logs had intact branches along their entire length, whereas others had intact branches only at the upper end of the log beyond the portion sampled for analysis. Two of the residual crowns were broken during felling, creating logs without attached branches. They were not used in the analysis, thus reducing the number of trees from 12 to 10.

Tissue Sampling. On May 11, 1992, the top and side positions of each delimbed log were sampled with an increment borer at about 0.61, 1.22, and 1.83 m from the base. One composite tissue sample from each position was obtained by combining the three sapwood cores (1.0 cm depth) and three phloem cores (with attached cambium) in separate sealed vials (15 × 45 mm). Vials were transported on ice to the laboratory where they were stored in a −36°C freezer. All of these logs had been previously sampled (Kelsey, 1994) and the holes plugged with corks. The May samples were bored 8 cm toward the crown from the nearest cork.

On May 12, tissues from the corresponding branched crown logs were collected by compositing increment cores at 0.61, 1.83, and 3.05 m for one sample and cores at 4.27, 5.49, and 6.71 m for a second sample. These logs had not been sampled previously.

Sample Preparation. Samples were prepared for analysis in the coldroom (2–3°C) by dicing the phloem and slicing the sapwood cores into disks. Phloem (0.12–0.13 g fresh weight) or sapwood (0.21–0.22 g fresh weight) were weighed into autosampler vials (22 × 75 mm OD, 22.4 ml) and sealed with a septum. After the samples were heated at 102°C for 20 min to deactivate their enzymes, they were cooled to room temperature and placed in the autosampler or held in the coldroom (less than 12 hr) until the analysis of previous samples was completed.

Headspace Analysis. Concentrations of ethanol, acetaldehyde, and α-pinene were analyzed with a Perkin Elmer HS40 headspace autosampler connected to a Hewlett Packard 5890 gas chromatograph (GC). A Supelcowax 10 capillary column (0.32 ID × 30 m, 0.25 μm film thickness) was directly connected to the autosampler by passing it through the GC injector port and heated transfer line. The GC temperatures were 250°, 50°, and 50°C isothermal, for the detector, injector, and column oven, respectively. The HS40 temperatures were 100°, 100°, and 60°C for the sample, needle, and transfer line, respectively; the timing was programmed at 20 min for sample thermostating; 1 min for vial pressurization; 0.04 min for injection; and 0.2 min for needle withdrawal. Helium was the carrier gas. Samples were analyzed in the multiple headspace extraction mode with two injections per vial and venting between injections (Kolb, 1982; Kolb et al., 1984). The instrument was calibrated with vials containing 5 μl of standard solution. After analysis, the samples were oven dried at 102°C for 16 hr, cooled in a desiccator 30 min, and weighed. Concentrations of the volatiles were calculated as described by Kolb et al. (1984) and reported as micrograms per gram dry weight (μg/gdw) of tissue.

Ambrosia Beetle Measurements. From August 26 to September 1, the ambrosia beetle entrance holes were counted in 20 × 20-cm quadrats of exposed sapwood. On delimbed logs, the quadrats were placed over each sampling hole along the top and sides, nine per log. On branched logs, a line of four quadrats was placed along each position, between the three bore holes used in preparing

the composite samples. One quadrat was located on both sides of the center hole at half the distance to each outside hole. Another quadrat was located near each outside hole. This provided eight quadrats from the top and each side, or 24 per log. Beetles excised from galleries were verified by the Oregon State University Entomology Extension Service. *Gnathotrichus retusus* was the only species of *Gnathotrichus* captured from galleries in August. Its entrance holes were separated from those of *T. lineatum* with a No. 53 wire gauge drill bit (Kinghorn, 1957).

Statistical Analysis. The data were analyzed as a strip plot design with individual trees as blocks and log segments and positions on the log as strips. The SAS (SAS Institute Inc., 1988) General Linear Model (GLM) procedure with type III sums of squares was used for analysis of variance (ANOVA) for each of the response variables (ethanol, acetaldehyde, water, α-pinene, *T. lineatum* densities, and *G. retusus* densities). Fisher's protected LSD test ($\alpha = 0.05$) was used for preplanned comparisons of means. Compound concentrations in the phloem and sapwood at each log position were averaged for analysis (see additional comments below). Shading and random positioning of logs on the ground made comparisons of the two side positions meaningless, so these measurements were combined as a single variable and weighted for analysis. All measurements were log transformed to ensure homogeneity of variance and normality. Geometric means with back-transformed standard errors are presented.

This experiment was not designed to evaluate how the chemical components in the phloem and sapwood would separately affect beetle attack behavior and subsequent colonization. However, it was apparent that the concentrations of some compounds were not the same in both tissues. Therefore, before averaging the tissue concentrations for analysis, it was necessary to determine whether interactions existed between the tissues and log types. This was accomplished by analyzing the data as a strip–strip plot design with separate chemical concentrations for each tissue. This analysis indicated no interactions between the log types and tissues for any of the constituents (ethanol, acetaldehyde, water, and α-pinene); thus, the two tissue concentrations were averaged for the simpler strip plot design described above.

To further evaluate the relationships between beetle densities and the various log parameters, the categorical variables (log type and position on log) and the continuous variables (ethanol, acetaldehyde, water, and α-pinene) were analyzed together (Neter et al., 1989) with a multiple regression model using the SAS GLM procedure with type II sums of squares. Continuous numeric variables were transformed as for the ANOVA above. The initial model included all possible second- and third-order interactions between the categorical and continuous variables. The data were then analyzed using the initial model to generate the sums of squares, F, and P values. Nonsignificant ($P \geq 0.05$) third-order terms were removed and the data analyzed again using the reduced model.

TABLE 1. SOURCE OF VARIATION IN AMBROSIA BEETLE DENSITIES AS DETERMINED BY MULTIPLE REGRESSION

Source of variation	*df*	Mean square	*P*
Trypodendron lineatum			
Tree (block)	9	6.1515	0.0001
Ethanol × log type	2	84.1948	0.0001
Error	28	0.8767	
Gnathotrichus retusus			
Tree (block)	9	10.4594	0.0310
Acetaldehyde × position	2	93.2810	0.0001
Error	28	4.1946	

Removal of nonsignificant terms at each iteration was at the highest order interaction in the model. This process was repeated until only significant variables and interactions remained as shown in Table 1.

Since acetaldehyde is the metabolic precursor to ethanol during anaerobic respiration, their concentrations in the tissues may be related. To check for a relationship, the concentrations of these two compounds in the phloem and sapwood of each log type were subjected to regression analysis.

RESULTS

Ethanol, acetaldehyde, and water concentrations in the delimbed logs were significantly higher than in the branched logs (Figure 1). α-Pinene concentrations were higher in the delimbed logs than in the branched logs, but the difference was not significant ($P = 0.34$) (Figure 1). Ethanol was the only compound whose concentrations differed among positions ($P = 0.02$), with the log tops (34.0 μg/gdw) containing more ethanol than the sides (23.4 μg/gdw). When tissue concentrations were separated for analysis, the water content of the phloem (98.1%) was significantly greater ($P = 0.009$) than the sapwood (75.4%), whereas the sapwood ethanol concentration (31.4 μg/gdw) was about double the amount in the phloem (16.9 μg/gdw; P = 0;063). Acetaldehyde and α-pinene concentrations were not different between tissues.

In stand 2, some delimbed logs had been attacked by ambrosia beetles on March 8 and all delimbed logs had been attacked by March 23 (Kelsey, 1994). Attacks on branched logs were not observed until later in the spring. Densities of *T. lineatum* and *G. retusus* recorded in late August were much greater (P = 0.0001 and 0.0007, respectively) in the delimbed logs than in the branched logs (Figure 2). Log sides were attacked more heavily ($P = 0.002$) by *G. retusus* than the log tops (Figure 3). *Trypodendron lineatum* showed no preference for log position (Figure 3).

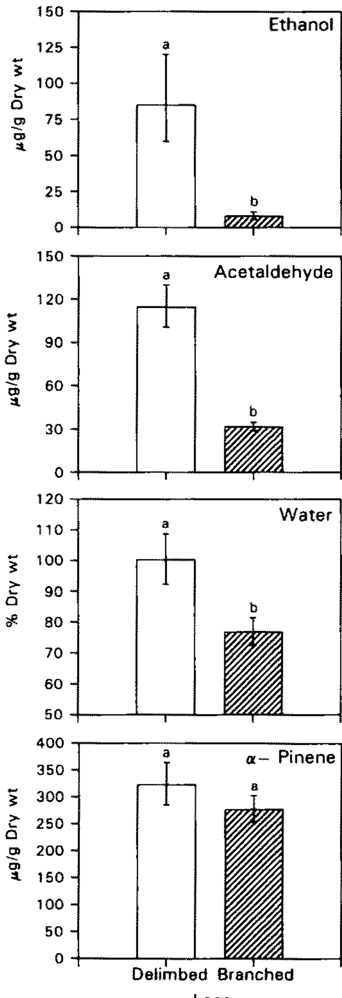

FIG. 1. Mean concentrations ($\pm$SE, N = 10) of ethanol, acetaldehyde, water, and α-pinene in delimbed and branched logs when sampled in May.

Of all the variables entered into the initial multiple regression model, the interaction between ethanol concentrations and log type (Table 1) best explained the variation in *T. lineatum* densities, and the interaction between acetaldehyde concentrations and position on the log best explained the variation in *G. retusus* densities. Acetaldehyde concentrations were correlated with ethanol concentrations in the phloem (R^2 = 0.47, P < 0.0001) and sapwood (R^2 = 0.43, P <

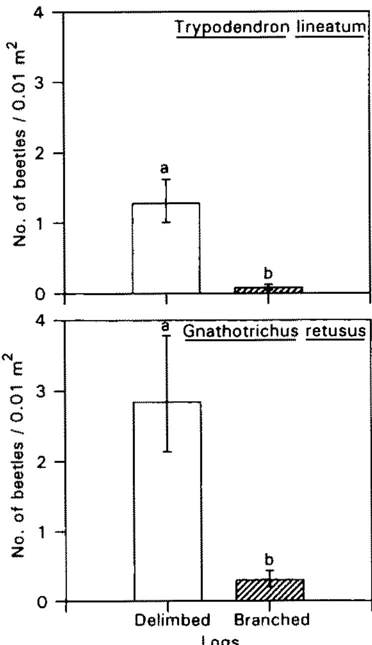

FIG. 2. Mean ambrosia beetle density (±SE, N = 10), counted in August, in delimbed and branched logs.

0.0001) of delimbed logs and sapwood (R^2 = 0.41, $P < 0.0001$) of branched logs. Concentrations of these compounds were not correlated (R^2 = 0.16, P = 0.0018) in the phloem of the branched logs.

Delimbed logs had a mean diameter of 53.7 cm at their base compared to 49.0 cm for the crown logs at their base. The difference was not significant.

DISCUSSION

Logs with branches were more lightly attacked by *T. lineatum* and *G. retusus* in comparison to shorter, delimbed logs cut from the same tree. Johnson (1961, 1964) observed a similar behavior. In the present experiment, the lightly attacked branched logs contained significantly less water, ethanol, and acetaldehyde than logs without branches. The low tissue water content in branched logs could be the cause for their low ethanol and acetaldehyde concentrations.

The delimbed logs from stand 2 in this study were felled and sampled in November, then resampled in January and March as part of another experiment

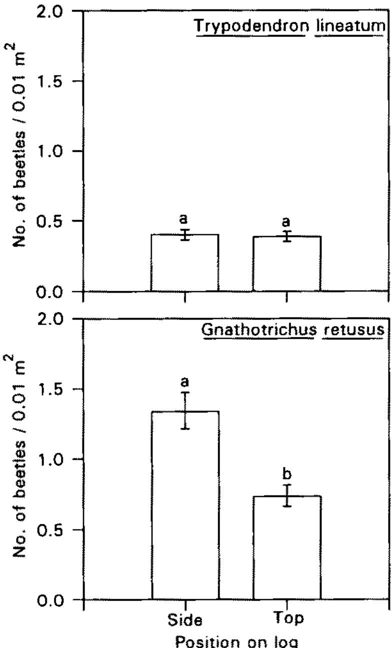

FIG. 3. Mean ambrosia beetle densities ($\pm$SE, $N = 20$), counted in August, in the sides and tops of all logs.

to measure ethanol production over time in logs felled during different seasons (Kelsey, 1994). Additional logs were felled in January and March. By March, ethanol concentrations in the phloem and sapwood of the November-felled logs were significantly higher than in the same tissues of January-felled logs. This was attributed to environmental differences between the logs because at both dates tissues from freshly felled logs produced equal quantities of ethanol when placed under anaerobic conditions in the laboratory. The greater quantity and more even distribution of rainfall received by the November-felled logs was considered the key factor for greater ethanol production compared to January-felled logs.

It was suggested (Kelsey, 1994) that rainwater entering the November-felled logs interfered with the rate of gas exchange between the respiring cells and the external atmosphere, similar to the induction of ethanol synthesis in fresh conifer tissues submerged in water (Graham, 1968; Cade, 1970; Cade et al., 1970). Once a tissue receives sufficient rainwater to interfere with gas exchange, anaerobic conditions could be established by aerobic respiration depleting the cellular O_2 and generating CO_2. As the O_2 levels drop, the tricar-

boxylic acid cycle slows down and organic acids accumulate, causing the pH to drop. The lower pH activates pyruvate decarboxylase (Davies, 1980; Roberts et al., 1984; Harry and Kimmerer, 1991), the enzyme that converts pyruvate to acetaldehyde and releases CO_2. The acetaldehyde is then converted to ethanol by alcohol dehydrogenase with NADH as a cofactor. Consequently, rainwater entering the logs would not directly create or control the establishment of anaerobic conditions, but it may play an important role in initiating the process.

Evaporation from twigs and needles on the branches could have facilitated the movement of absorbed water out of tissues by capillary action, similar to transpirational water movement in live trees. Sapwood in branched Douglas fir and western hemlock logs dried faster and to a lower final water content than sapwood in delimbed logs sampled at monthly intervals over an entire year (Johnson, 1964; Johnson and Zingg, 1969). Shading and canopy cover reduced the rate and extent of drying.

Less tissue water in branched logs could prevent or delay the establishment of anaerobic conditions or greatly shorten the time that anaerobic conditions were maintained. This would limit the synthesis and accumulation of ethanol, which in turn would reduce the number of ambrosia beetles entering the log, in contrast to logs without branches and a higher tissue water content. The effectiveness of branches at reducing the tissue water content of logs in this study may have been enhanced by the limited winter rainfall. Monthly precipitation (measured near Corvallis at the Hyslop Crop Science Field Research Laboratory, a short distance from the study site) for November through May was 300 mm below the 30-year average (1961 to 1990).

Trypodendron lineatum showed no preference for log positions, whereas *G. retusus* attacked the log sides more heavily than the tops, even though there was more ethanol in the tops. This is the same result observed in the seasonal log study (Kelsey, 1994). In other studies, *T. lineatum* avoided log tops (Prebble and Graham, 1957; Lindgren et al., 1982). Dyer (1963) observed this preference by *T. lineatum* but only when logs were in direct sunlight; no preference was observed for shaded logs. In the present study, variable log shading probably minimized any preference for log positions by *T. lineatum*. On Vancouver Island, British Columbia, *G. retusus* attacked all positions equally, whereas on the same logs *G. sulcatus* avoided the tops (Lindgren et al., 1982).

Multiple regression analysis indicated that the variation in *T. lineatum* densities was best explained by the interaction of log type, delimbed or branched, and the ethanol concentration of the tissues (Table 1). The variation in *G. retusus* densities, however, was best explained by the interaction of acetaldehyde concentrations and position on the log. Mean acetaldehyde concentrations were greater but less variable than the mean ethanol concentrations in the logs (Figure 1). Concentrations of these two compounds were correlated in the sapwood of both log types and the phloem of the delimbed logs. Ambrosia beetle response

to acetaldehyde has received only minor attention relative to the many studies with ethanol described earlier. In olfactometer bioassays, *T. lineatum* did not respond to acetaldehyde released alone, but in the field, some traps releasing a combination of acetaldehyde and methanol, with or without ethanol, caught more beetles than traps releasing ethanol alone (Moeck, 1970). In olfactometer bioassays, 13% of the *G. sulcatus* males were attracted to a 1% solution of acetaldehyde in water (Cade, 1970). It was not tested at lower concentrations or in combination with ethanol. The response of ambrosia beetles to acetaldehyde released alone or in combination with ethanol and α-pinene warrants further study.

Differences in chemical concentrations between log types was not the result of an inherent gradient up the bole. This was clearly demonstrated by the similarity in concentrations of the chemical constituents between the two composite samples from the branched logs separated by 4 m. This was about the same distance separating samples collected from the two log types. Secondly, any confounding effect on beetle densities due to differences in bole diameters was eliminated since bole diameters at the base of the delimbed and branched logs were not significantly different.

In conclusion, this experiment demonstrates that the difference in log chemistry measured during the initial attack and early colonization of delimbed and branched logs is a good indicator of the subsequent level of ambrosia beetle attack. Of the compounds analyzed, ethanol probably had the most influence on attack behavior, although acetaldehyde also could have had some effect, particularly on *G. retusus*. A high tissue water content in logs producing high concentrations of acetaldehyde and ethanol is consistent with the suggestion (Kelsey, 1994) that rain absorbed by logs might interfere with gas exchange between respiring tissues and the atmosphere. Depletion of O_2 with replacement by CO_2 could lead to anaerobic respiration and the synthesis of ethanol. A lower tissue water content in branched logs, probably resulting from capillary movement and evaporation of absorbed rainwater out through the branches, could have interfered with the establishment and maintenance of anaerobic tissues, thus restricting the synthesis of acetaldehyde and ethanol in the branched logs.

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BEHAVIORAL RESPONSES OF *Littoraria irrorata* (SAY) TO WATER-BORNE ODORS

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Abstract—Behavioral responses of the gastropod mollusc *Littoraria* (=*Littorina*) *irrorata* indicate that it can discriminate among environmental odors. Snails were assayed for responses to 11 odors from plants and animals potentially representing food, shelter, location in the environment, and predators. Crushed conspecifics were included as an alarm odor. Except for odor of crushed conspecifics, all odor sources were water-borne from living intact organisms. Behavioral responses were categorized as no response, positive response, or negative response. For some analyses, negative responses were subdivided into withdrawing and turning responses. Snails responded positively to several plant odors. They did not respond to odors of intact conspecifics, fiddler crabs, or grass shrimp. They responded negatively to odors of a plant found at the upper limit of their minimal habitat, predatory blue crabs, crushed conspecifics, predatory gastropods, and ribbed mussels. Odors of blue crabs on different diets affect the type of negative response the snails display.

Key Words—Gastropods, chemoreception, periwinkle, *Littoraria irrorata*, odor, snail, behavior, environmental odors, alarm responses

INTRODUCTION

Studies of chemically mediated behavior in gastropods address feeding (Carr, 1967a,b), prey detection (Carriker, 1955; Blake, 1960; Rittschof and Gruber, 1988), alarm responses to crushed conspecifics, mucus trail following (Snyder, 1967; Crisp, 1969; Trott and Dimock, 1978), alarm responses to mucus trails (Gore, 1966; Dix and Hamilton, 1993), alarm responses to predator odor and contact (Bullock, 1953; Feder, 1963; Gore 1966; Hoffman et al., 1978; Hoffman

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and Weldon, 1978), and alarm in response to body juices released upon damage of related gastropods (Atema and Burd, 1975; Atema and Stenzler, 1977). Snyder (1967) reports over 25 genera of gastropods show alarm responses to juices from crushed conspecifics.

Littoraria irrorata (Say) was not included in Snyder's (1967) survey of alarm responses by gastropods to crushed conspecifics. Dix and Hamilton (1993) tested the responses of *L. irrorata* to contact chemical stimulants from predatory and nonpredatory mollusks. This species exhibits an escape response when touched with the mucus of a predaceous gastropod (Dix and Hamilton, 1993), responding most strongly to the mucus of crown conch (*Melongena corona*). This response was characterized as an increase in crawling speed and in the frequency of cephalic tentacle taps. Another species of *Littoraria*, *L. scutulata*, responds to the odor of the predatory ochre star, *Pisaster ochraceus*, by moving rapidly away from the source of the odor (Feder, 1963).

We report responses to water-borne chemical cues by salt marsh periwinkles *Littoraria* (=*Littorina*) *irrorata* (Say). *L. irrorata* are herbivorous gastropods usually associated with the marsh cordgrass *Spartina alterniflora* (Baxter, 1983; Warren, 1985; Hamilton, 1976, 1977). Periwinkles migrate down the stems of the cordgrass to feed on the sediment at low tide and return to the stems of grass ahead of the incoming tide. This migration has been termed an avoidance behavior (Warren, 1985; Dix and Hamilton, 1993; Hamilton, 1976, 1977). By migrating up grass stems, the snails avoid aquatic predators such as blue crabs *Callinectes sapidus* (Rathbun), tulip snails *Fasciolaria* spp., and crown conchs *Melongena corona* (Gmelin) that move into and feed in the cordgrass with the tide (Warren, 1985; Hamilton, 1976). Common organisms in *L. irrorata* habitat include fiddler crabs *Uca* spp., grass shrimp *Palaemonetes* spp., and ribbed mussels *Geukensia demissa* (Dillwyn). Common plants near *S. alterniflora* habitat include *Salicornia virginica*, *Distichlis spicata* immediately above *Spartina*, and *Borrichia frutescens*. *S. virginica* is usually found in a band in the high intertidal. *B. frutescens* is found above the *S. virginica* zone.

We have examined the responses of *Littoraria irrorata* to odors of crushed conspecifics, marsh grass, and predatory and nonpredatory mollusks and crustaceans. Finally, because responses of freshwater snails to predator odors are affected by predator diet (Crowl, 1990; Crowl and Covich, 1990), we tested responses of snails to odors of fasted blue crabs and blue crabs fed periwinkle and shrimp diets. We demonstrate that water-borne chemical cues could play an important role in the daily life of *L. irrorata*.

METHODS AND MATERIALS

Animals. All test organisms were collected from habitats in the vicinity of the Duke University Marine Laboratory in Beaufort, North Carolina. *Littoraria*

irrorata (Say) were gathered from the west side of the Pivers Island Bridge and in the salt marsh north of Route 70 and west of the Beaufort bridge, Beaufort, North Carolina. Snails were kept in 2.4-liter plastic boxes containing 5 ml of seawater to keep snails moist and stimulate their movement.

Animals were tested with two different protocols. In the first protocol, a fresh group of snails was tested for each odor. These animals were collected 2–3 hr prior to testing and maintained as described above. Once testing was complete, animals were released. In the second protocol, snails were collected and tested with four odors in the following order: 100-kDa filtered seawater (FSW), *Spartina alterniflora*, predatory blue crabs *Callinectes sapidus* (Rathbun), and crushed conspecifics. Trials were approximately 12 hr apart. Water was changed two to three times daily for these groups. In this protocol, snails were maintained up to four days in the laboratory. In no case was an individual tested more than once with any odor.

Odors. All odors tested (Table 1) were prepared by soaking target species in FSW for a period of 1–3 hr and decanting the water. Each odor preparation was used for only one group of 36 animals. FSW was used as a control. Odors tested are displayed in Table 1, along with the weight of the animal or substance and the volume of FSW used.

Blue crabs fasted on diets of *L. irrorata* or grass shrimp were maintained as follows. Crabs were housed in individual 10-gallon aquaria. Those that were fed only snails or only grass shrimp were monitored to determine consumption. Once the prey was consumed, crabs were moved to individual buckets with fresh FSW and the odor was prepared as described above.

TABLE 1. WEIGHT OF ODOR SOURCE AND VOLUME OF WATER USED IN PREPARATION OF ODORS

Odor Source	Wet weight (g)	Seawater
Seawater	0	100 ml
Live conspecifics (in shells)	30	100 ml
Grass shrimp (15 males)	18	200 ml
Sand fiddler crabs (males and females)	50.7	375 ml
Crushed conspecifics (20)	39.2	20 ml
Ribbed mussels	108	350 ml
Horse conch	484.3	3 liter
Tulip snails (2)	603.5	3.5 liter
Blue crabs (7)	400–500	1–1.5 liter
Borrichia scandins	80	1 liter
Distichlis splicata	80	1 liter
Salicornia virginica	80	1 liter
Spartina alterniflora	80	1 liter

Bioassays. Snails were tested as follows: A Pasteur pipet was used to make a 1-cm-wide ring of odor water 1 cm from the edge of an 8-cm-diameter finger bowl. An active snail was selected from the holding container, was placed in the center of the ring, and a blind erected because movement of the observer startled the animals. Once the snail touched the odor ring, its response was determined. The animal's first response was recorded as either withdraw, turn, follow, or no response. Withdraw was characterized by retraction of tentacles and foot. Turn was recorded when a snail turned away from the ring after contact. Follow was used if the snail followed the ring after contact. No response was recorded if the animal crawled directly through the odor ring. The proportion of each of these response types to an odor allowed us to categorize the group response to the odor as positive (follow), negative (turn and withdraw), or neutral (no response).

Statistics. Responses to odors were compiled and tested for dependence of response upon odor using the G test of independence (Sokal and Rohlf, 1981). The Z test of proportions (Walpole, 1974) was used to analyze responses when responses of the same snails were compared (Sokal and Rohlf, 1981). At the first level of analysis, turn and withdraw responses were combined and considered as a negative response. For preparations showing high proportions of negative responses, negative responses were split into turn and withdraw responses and reanalyzed.

RESULTS

In an initial series of experiments, *Littoraria irrorata* were tested for responses to five environmental odors. Snails in this series exhibited two obvious categories of response: neutral and negative (Figure 1). Odors of crushed conspecifics and blue crab evoked a negative response in more than 50% of the animals tested, while odors of marsh grass, live conspecifics, and filtered seawater elicited no response from more than 50% of the test animals. These groups of responses were dramatically different (G = 162.27, df = 4, $P \ll 0.001$). Examination of the data suggested a trend toward positive responses when snails are exposed to odors of marsh grass and little response to seawater and live conspecifics.

The results of experiment 1 suggested that marsh grass might be attractive to *L. irrorata*. A second experiment (Figure 2) focused on differences in responses of snails exposed to seawater and to odor of *S. alterniflora*. Seventy-two snails were tested first with filtered seawater, then with the odor of marsh grass. Approximately 25% of the animals tested responded positively to marsh grass, while 8% responded positively to seawater. Snails displayed a significant positive response to the odor of marsh grass (Figure 2, Z = 2.68, $P < 0.01$).

The finding that snails responded positively to the odor of *S. alterniflora*

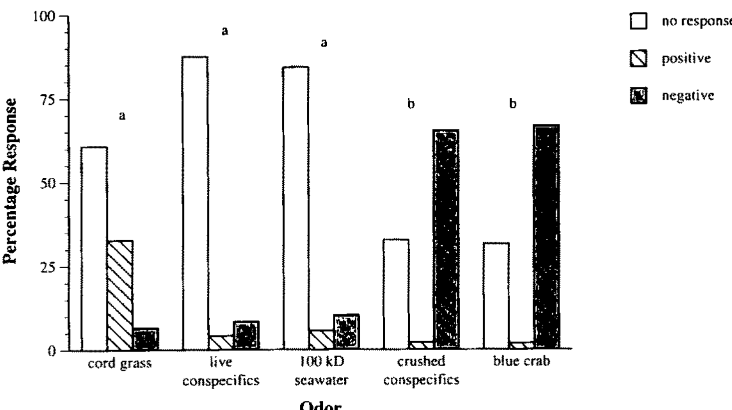


FIG. 1. Response of *Littorina irrorata* to five environmental odors. Odors can be broken into two general categories according to the most common response observed: neutral and repellent in this experiment. Marsh cordgrass (N = 107), live conspecifics (N = 72), and filtered seawater (N = 108) are neutral odors. They evoke low percentages of positive and negative responses. Predatory crab (N = 108) and crushed conspecific odors (N = 144) elicit high percentages of negative (turn or withdraw) responses. Snails respond to cues that may signal food, refuge, and conspecifics. Letters above bars represent nonsignificant subsets (G = 162.27, df = 4, $P < 0.001$). We observed a trend for snails to be attracted to marsh cordgrass.

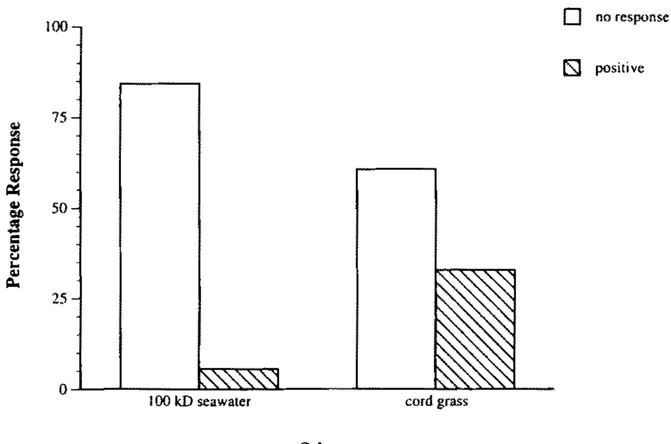


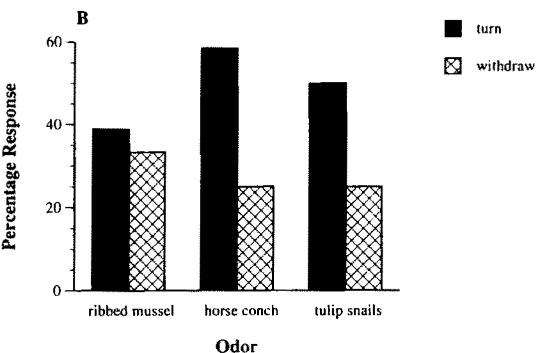
FIG. 2. Responses of 72 snails tested first with filtered seawater and then with the marsh cordgrass *Spartina alterniflora*. A significant proportion of snails responded positively to marsh cordgrass (Z = 2.68, $P < 0.01$).

induced us to ask if snails responded similarly to any plant odor. We tested common plants from four different elevations in the marsh: *S. alterniflora* (snail habitat), inundated at each high tide; *Distichlis spicata*, immediately above snail habitat and inundated at most high tides; *Salicornia virginica*, inundated on spring tides, and *Borrichia frutescens*, plants which are rarely inundated. Analysis of all bioassay responses (N = 107 for cordgrass; N = 36 for the other treatments) of the four plant odors by frequency analysis showed snails responded differently to plant odors ($G = 34.3$, $df = 3$, $P < 0.01$). The difference in overall response was due to differences in the response to *Distichlis* and *Salicornia* and *Distichlis* and *Spartina*. When only positive and negative snail responses were analyzed, there were clear differences between *Distichlis* and the other three plants. Snails responded negatively most frequently to *Distichlis* and positively most frequently to the other three plant odors.

Figure 3 displays the results of assays testing odors of mollusks other than *L. irrorata*. The odors of live conspecifics, ribbed mussel, horse conch, and tulip snail were test odors. Responses to conspecific odors were different than those to the other three test odors, which were negative compared to odor of undamaged conspecifics ($G = 70.99$, $df = 3$, $P < 0.001$; Figure 3A). The percentage negative response was broken into its turn and withdraw components to test for differences in negative response type (Figure 3B). Negative responses were not statistically different for all three test odors ($G = 1.69$, $df = 2$, $P > 0.05$).

Because *L. irrorata* had not previously been tested for alarm response (cf. Snyder, 1967), snails were tested for responses to crushed conspecifics. Responses of snails to the odor of crushed conspecifics were significantly negative compared to a seawater control (Figure 4A; $G = 72.4$, $df = 1$, $P < 0.001$). Figure 4B displays the negative response broken into turn and withdraw components. Of the 94 animals that displayed a negative response, 84 exhibited a withdraw response, while only 10 turned away from the odor after contact.

Snails were assayed for their ability to discriminate among odors of predatory and nonpredatory crustaceans (Figure 5). Over 50% of the snails tested responded negatively to the odor of an intact blue crab, but not to odors of intact fiddler crabs and grass shrimp ($G = 50.37$, $df = 2$, $P < 0.001$). A more detailed study of snail responses to blue crab odors and crab diet was conducted. It was postulated that perhaps diet played a role in predator odor. To test this, two blue crabs were maintained on different diets. One was fed only crushed *L. irrorata*, while the second was fed only grass shrimp. Figure 6A displays the total negative response, which is identical for both cases ($G = 0.354$, $df = 1$, $P > 0.05$). However, significantly more snails withdrew than turned when exposed to odor of crabs that ate snails (Figure 6B; $G = 7.66$, $df = 1$, $P < 0.01$). In a second experiment, snails were tested with odors of fasted blue crabs and with odors of crabs fed snails. Snails responded to fasted and fed crab odors



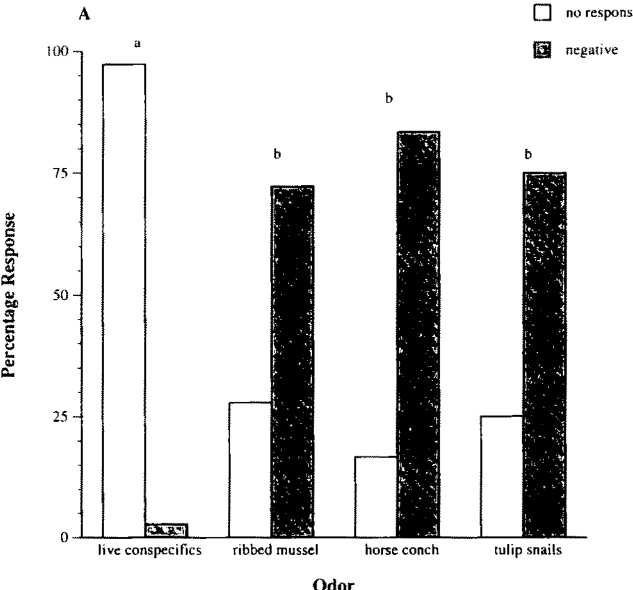
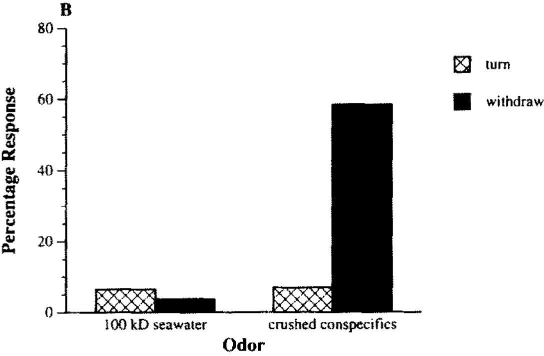


FIG. 3. Responses of 72 snails (seawater) and 36 snails (all other treatments) were determined. (A) Odors of two predatory gastropods and one bivalve all evoked significantly negative responses when compared to odor of live conspecifics ($G = 70.99$, $df = 3$, $P < 0.001$). Letters over bars group nonsignificant subsets. (B) Negative responses were divided into turn and withdraw categories. Odors of ribbed mussel, horse conch, and tulip snail did not evoke significantly different types of negative responses ($G = 1.69$, $df = 2$, $P > 0.05$).



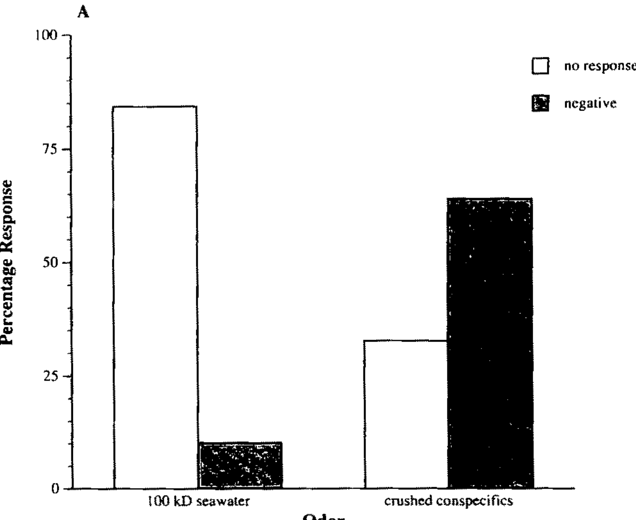


FIG. 4. (A) Snails exhibit a significant negative response to the odor of crushed conspecifics ($G = 72.4$, $df = 1$, $P \ll 0.001$). (B) Negative responses broken into turn and withdraw categories. Over 60% of the animals responding negatively withdrew when they contacted the odor of crushed conspecifics.

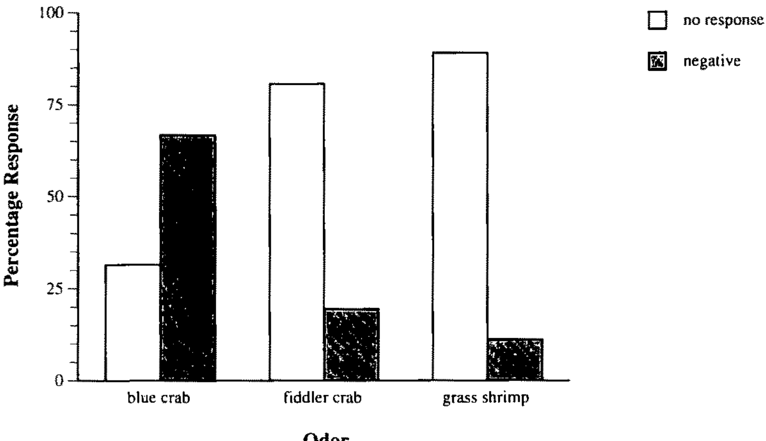


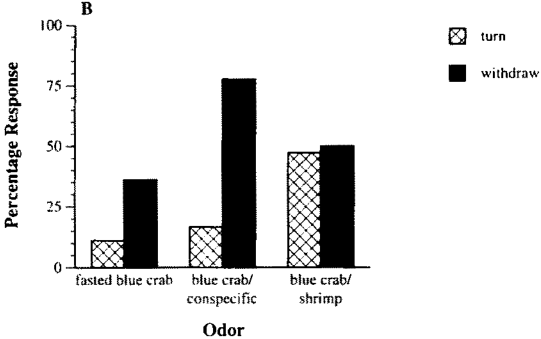
FIG. 5. Snails respond specifically to odors of different crustaceans. Snail predators, blue crabs evoke a significantly higher percentage of negative responses than nonpredatory crustaceans ($G = 50.37$, $df = 2$, $P < 0.001$).

similarly. Seventy-six percent of the negative responses to odor of crab fed snail withdrew. Eighty-eight percent of the snails exposed to odor of fasted blue crab withdrew (Ho crab fed snail = crab fasted $G = 1.32$, $df = 1$, $P > 0.05$).

DISCUSSION

In the laboratory, marsh periwinkles *Littoraria irrorata* responded to many odors common to their environment. *L. irrorata* responded in four ways upon contact with aqueous odors: (1) Snails ignored the odors by crawling directly through them. Snails crawled directly through filtered, aged seawater and seawater with the odor of living intact conspecifics, fiddler crabs, and grass shrimp. (2) Snails showed attraction by turning into the odor. Attraction was seen in approximately 30% of snails in response to the odor of marsh grass, *Spartina alterniflora*, *Salicornia virginica*, and *Borrichia scandins*. The final two kinds of snail responses are both kinds of repulsion. We consider them as separate responses because snails show clear differences in responses to different sources of repulsive odors. (3) Snails turned and crawled away from the odor. Snails turned away from the odor of other mollusks and blue crabs fed a diet of shrimp. (4) Snails withdrew into their shells upon contact with odor. Snails withdrew in response to odors of crushed conspecifics, odors of field-collected blue crabs, odors of fasted blue crabs, and blue crabs fed a diet of snails.

Of the responses we categorized above, repulsion (alarm) is the best studied



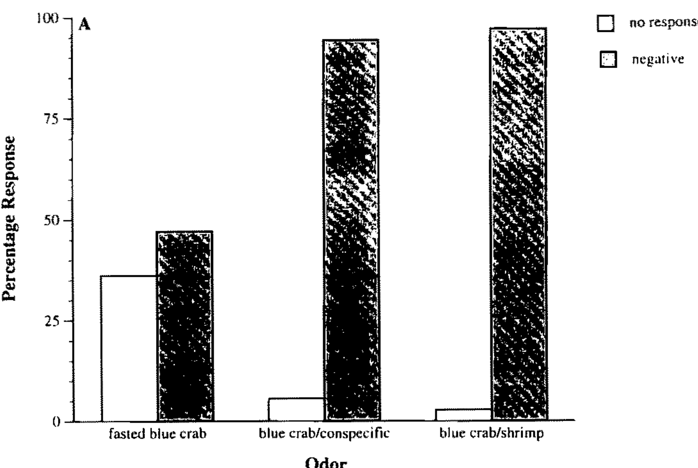


FIG. 6. (A) Responses of 36 snails to odors of blue crabs fasted or maintained on different diets were uniformly negative ($G = 0.354$, $df = 1$, $P > 0.05$). As in Figure 3, negative responses were divided into turn and withdraw categories. (B) Snail responses to odors produced by blue crabs are affected by prior diet of the crab. A shrimp diet results in higher proportions of snails that turn. Snail diets and fasting result in higher proportions of snails that withdraw.

in gastropods (Snyder, 1967; Atem and Burd, 1975; Atema and Stenzler, 1977). However, there is no documented study of *L. irrorata* response to conspecific juices. This species was not included in Snyder's 1967 survey of gastropod reactions to intraspecific extracts. The withdrawal response to conspecific juices that we observed is similar to that of the species tested by Snyder (1967). *L. irrorata* also shows clear alarm in response to slime trails of predatory snails (Dix and Hamilton, 1993). Snails increased both speed of movement and number of tentacle taps after direct contact with the crown conch *Melongena corona*, a natural predator. This type of response has been found in other gastropod species as well (Feder, 1963; Hoffman and Weldon, 1978; Hoffman et al., 1978).

Past studies have focused on direction of movement (Dix and Hamilson, 1993) and self-burial (Snyder, 1967) as a means of determining alarm response. By using crawling behavior to measure snail reactions, we can discriminate avoidance, following, and neutral responses to odors. For instance, after a snail contacts an odor ring, withdrawal and movement directed away from the ring are considered negative responses. Sixty-five percent of snails exposed to crushed conspecifics displayed these behaviors. Directed crawling in the path of the ring is defined as a positive response. Even in statistically significant cases, positive responses were in low percentage. Less than one third of the snails tested responded by following any odor. Movement through the ring of odor, as is done by 84% of snails responding to seawater, is classified as a neutral response.

Sets of odors that have a large number of negative responses can be statistically tested to determine if negative responses are different. From this analysis, the responses of snails to crushed conspecifics and to odors of blue crab fed *L. irrorata* or fasted blue crabs are of one type and responses to intact mollusks (nonself) are of another. Nonself mollusk odors all evoked significant negative responses, regardless of the source animal's status as a predator or nonpredator. However, in contrast to responses to crushed conspecifics, the majority of the negative responses recorded were turn rather than withdraw. We suggest that this response to nonself mollusk odors is because snails have a reasonable chance of escaping by running from slow-moving sources. Perhaps it is more efficient to simply run away from all other mollusks rather than to discriminate potential predatory from the nonpredatory mollusks. Mollusk odors may be repulsive because they signal the presence of predators, such as mud crabs, that live among the mollusks.

L. irrorata may respond positively to the odor of *Spartina alterniflora* because it provides food (Warren, 1985) or because it provides refuge (Hamilton, 1976, 1977). Because responses to *S. alterniflora* are similar to those of *S. virginica* and *B. scandins* and less than 50% of the snails respond, we hypothesize that they are feeding responses.

Grasses could also be a refuge from slow-moving predators such as large predatory snails (Dix and Hamilton, 1993; Warren, 1985). However, the study

by Dix and Hamilton (1993) found that *L. irrorata* discriminate between the mucus of predatory and nonpredatory mollusks and even respond differently to the mucus of different predators. The difference between our study and that of Dix and Hamilton is a result of the methods used and the questions asked. We allowed animals to contact only the bathwater of other mollusks, while Dix and Hamilton directly applied mucus of other mollusks to the foot region of *L. irrorata*. Thus, our study focused on fundamentally different odors than did Dix and Hamilton. It is possible that our method of presenting chemosensory information did not provide a significantly strong signal to enable snails to differentiate between predator and nonpredator mollusk odors. These data suggest that snails cannot discriminate predatory from nonpredatory mollusks by waterborne odors.

In addition to negative responses to mollusks, negative responses to *D. spicata* may occur because this odor cues the snails that they are too high in the intertidal. Typically, *L. irrorata* residue high on the stalks of the marsh grass *Spartina alterniflora* during high tide and migrate to the bottoms of the stalks or surrounding sediments during low tide (Baxter, 1983; Dix and Hamilton, 1993). The grasses serve as a refuge from tidally limited predators such as blue crabs (Hamilton, 1976, 1977; Warren, 1985; Baxter, 1983; Dix and Hamilton, 1993).

Snail responses to crustacean odors are quantitatively and qualitatively different than to the mollusk odors. Snails discriminate between odors of predatory and nonpredatory crustaceans. They ignore odors of grass shrimp and fiddler crabs. Responses to predatory odors (blue crabs) are always negative, but change depending on the dietary history of the crab. Our data suggest that the odor of shrimp-fed blue crabs is very different than that of either snail-fed or fasted blue crabs. A diet-dependent response to predator odor has been documented in freshwater snails as well (Crowl and Covich, 1990; Crowl, 1990). Members of the stream-dwelling species *Physella virgata virgata* exhibit a change in reproductive strategy only when exposed to the odor of crayfish on a diet of *P. v. virgata* (Crowl, 1990).

Previous studies of blue crab predation on periwinkles (Hamilton, 1976; Baxter, 1983) have reported that blue crabs are able to reach up to 7 cm above the water line to remove *L. irrorata* from the stalks of marsh grasses. Most of the blue crabs observed by Hamilton (1976) had a distinct and efficient predation style. In some cases, shells of *L. irrorata* were crushed at the apex (Hamilton, 1976). Most were manipulated to the mouth region using both chelipeds and several other appendages, where they were chipped and crushed by the major cheliped (Hamilton, 1976; Warren, 1985). Large snails survived attacks by large crabs (Hamilton, 1976; Baxter 1983). Crowl and Covich (1990) reported preferential predation of small size classes of freshwater snails by crayfish.

Thus, crustaceans in general are fast-moving and efficient predators. It is

unlikely that a periwinkle on the substratum could outrun a predatory blue crab. A periwinkle is less likely to be injured when hidden in its shell than when crawling away with soft body parts exposed. This could explain why snails withdraw when exposed to blue crab odor, yet ignore the odor of fiddler crabs and grass shrimp that are not snail predators.

It is clear that environmental odors elicit at least four kinds of behavioral responses in *L. irrorata*. The source of the odor determines which behavior the snail will display. *L. irrorata* has potential for using olfaction in sensing key environmental features including food, refuge, and predators.

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LONG-CHAIN FREE FATTY ACIDS: SEMIOCHEMICALS FOR HOST LOCATION BY WESTERN CORN ROOTWORM LARVAE

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Abstract—A bioassay-driven sequential fractionation scheme was used to isolate fractions of a crude dichloromethane maize seedling extract behaviorally active to larvae of the western corn rootworm, *Diabrotica virgifera virgifera* LeConte. (Z,Z)-9,12-Octadecadienoic (linoleic) acid, (Z)-9-octadecenoic (oleic) acid, and octadecanoic (stearic) acid were identified from a purified fraction of maize extract that was attractive to western corn rootworm larvae in choice tests with equal levels of carbon dioxide on both sides of the choice. When synthetic linoleic, oleic, and stearic acids were tested together in the amounts and proportions found in the attractive fraction (1000, 800, and 300 ng of linoleic, oleic, and stearic acids, respectively), significantly more western corn rootworm larvae were found on the side with synthetic free fatty acids plus carbon dioxide than on the side with carbon dioxide alone. Results of the choice-test bioassays were not significantly different when the synthetic blend of free fatty acids was substituted for the purified maize fraction. Neither the purified extract nor the synthetic blend was behaviorally active in preliminary single-choice experiments without carbon dioxide. Linoleic, oleic, and stearic acids were also tested individually in the choice test bioassay with carbon dioxide on both sides of the choice to determine a dose–response curve. Linoleic and oleic acid each had one dose that was significantly attractive in conjunction with carbon dioxide on both sides of the choice, but stearic acid was not active in the doses tested.

Key Words—*Diabrotica virgifera virgifera*, fatty acids, linoleic acid, oleic acid, stearic acid, semiochemical, attractants, western corn rootworm, host location, Coleoptera, Chrysomelidae, *Zea mays*, kairomone.

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INTRODUCTION

The western corn rootworm, *Diabrotica virgifera virgifera* LeConte, is a serious pest of maize (*Zea mays* L.) throughout much of the maize-growing regions of North America. Larval damage to maize roots results in reduced plant height, reduced yield, and lodging of maize, which interferes with harvesting (Branson et al., 1980). An estimate of crop losses and treatment costs for corn rootworms is in the range of $1 billion annually (Metcalf, 1986).

Because of their economic importance, the chemical ecology of western corn rootworm adults has been extensively studied. Specific attractants and feeding stimulants as well as sex pheromones have been identified for adults (Guss et al., 1982; Metcalf and Metcalf, 1992; and references therein), but it is the larval stage of the western corn rootworm that causes most of the economic damage (Krysan and Miller, 1986). Research on the chemical ecology of corn rootworm larvae began with Branson's (1982) demonstration that western corn rootworm larvae were attracted to the roots of maize and prefer maize roots over the roots of broadleaf plants in choice tests. Carbon dioxide is a general plant metabolite that is released into the soil by the roots of maize and other plant species (Massimino et al., 1980). Although attractive to many soil organisms (Jones and Coaker, 1977; Doane et al., 1975; Pline and Dusenbery, 1987; and references therein) including western corn rootworm larvae (Strnad et al., 1986; Strnad and Bergman, 1987; Hibbard and Bjostad, 1988), carbon dioxide alone does not appear to provide a basis for western corn rootworm larvae to discern the roots of maize from the roots of broad-leaved plants. Hibbard and Bjostad (1988) demonstrated that volatiles in addition to carbon dioxide are involved in orientation to germinating maize kernels. Host cues appear to dominate over general cues. Strnad and Dunn (1990) demonstrated that, immediately after contact with maize, western corn rootworm larvae were not attracted to carbon dioxide.

Hibbard and Bjostad (1990) used a bioassay-driven sequential fractionation scheme modified from Silverstein et al. (1967) to isolate several behaviorally active compounds from an attractive crude maize extract. Two fractions were found that were significantly attractive in choice tests. One of the attractive compounds was 6-methoxy-2-benzoxazolinone (MBOA), which functions as a volatile semiochemical in conjunction with carbon dioxide in host location by western corn rootworm larvae (Bjostad and Hibbard, 1992). We now report the chemical identification of the second attractive fraction.

METHODS AND MATERIALS

Larvae and Maize Source. A nondiapausing strain of the western corn rootworm was obtained in June 1986 from the USDA-ARS laboratory in Brook-

ings, South Dakota. The colony was maintained with the methods of Jackson (1986) as modified by Hibbard and Bjostad (1988). Second-instar larvae (5–7 days old and 5–8 mm long) were chosen for bioassays because initial bioassays indicated that they behaved similarly to first-instar larvae (the instar that must locate a host) and second-instar larvae were more robust for the large numbers of bioassays required. To prevent possible effects from previous testing, each larva was used only once in the bioassays. Larvae were removed from containers with maize seedlings growing in soil less than 1 hr before testing. Dried whole kernels of maize (cv. 3090, Pioneer Hi-Bred International, Inc., Johnston, Iowa), not treated with insecticide or fungicide, were washed, soaked, and placed on moist germination paper (Steel Blue, Anchor Paper Company, St. Paul, MN). Maize seedlings [germination stage 6, (Ritchie et al., 1992)] were recovered after three to five days to obtain maize semiochemicals.

Extraction and Isolation of Maize Semiochemicals. As reported in Hibbard and Bjostad (1990), approximately 80 g of moist maize seedlings were extracted by placing them in a glass seed-holding tube (30 cm × 30 mm, tapering to 12 mm) for 3–6 hr and subsequently dripping dichloromethane through until 4 ml of solution had been collected. The extracts were then concentrated to less than 10 μl with a nitrogen stream, and the solution was separated into fractions with a 1.83 m × 2 mm ID packed gas chromatography column (3% OV-101 on Gaschrom Q, mesh 100/120, Alltech Associates Inc., Deerfield, Illinois) in a Hewlett-Packard 5890 gas chromatograph temperature programmed from 60°C (held for 1 min) to 260°C (held for 9 min) at a rate of 10°C. With the detector and noncolumn gases turned off, fractions were collected every 3 min during the 30-min run in 22.9-cm transfer pipets (Baxter Scientific Products, McGaw Park, Illinois) that were snugly connected to the end of the column with a specially fitted Teflon connector. The collection efficiency of this system ranged from as low as 50% to as high as 85% with known compounds with similar retention times. The transfer pipets were rinsed with 200 μl of dichloromethane, and the solutions were set aside for later bioassays. Approximately 40 g equiv of seedling extract was tested in the bioassays based on the collection efficiency of the system. In a second set of GC collections, the most active fraction from the first set of separations (fraction 8, 21–24 min) was reinjected and fractions were collected every minute. Transfer pipets used for collecting fractions were rinsed with 200 μl of dichloromethane, and the solutions were set aside for later bioassays.

Choice-Test Bioassays with Purified Maize Volatiles. Because carbon dioxide is highly attractive to western corn rootworm larvae (Strnad et al., 1986), we designed a bioassay to test for any additional influence of other corn volatiles (Hibbard and Bjostad, 1988). The bioassay apparatus consisted of three plastic Petri dishes (5 cm diam.) connected in series by Teflon tubing (10 mm diam.). Holes (12 mm) were melted into the bottom of end dishes to allow attachment

of 35-cm glass tubes. The dichloromethane solutions of fractions were placed on 0.1 g of glass wool, and an equal amount of dichloromethane was placed on another piece of glass wool. The solvent was allowed to dry, and the glass wool pieces were added to either end of the choice-test bioassay. The 35-cm glass tubes were prepared by flushing with carbon dioxide from a gas cylinder for 10 sec at a flow rate of 8 liters/min to give 4 mmol carbon dioxide per mol air at bioassay onset on both sides of the choice. A 5-min equilibration time was allowed before adding larvae. Ten larvae were placed in the center dish of the choice test, and the number of larvae that had reached either of the end dishes was recorded after 1 hr. Bioassays were conducted in the dark at room temperature and were replicated a minimum of 10 times.

Chemical Analysis. Electron-impact (EI, 70 eV) mass spectra of the purified maize fraction were obtained on a HP 5890 Series II gas chromatograph coupled with a HP 5970 mass selective detector and a HP Series 9000 data system (Hewlett-Packard, North Hollywood, California). A HP-1 methyl silicone column (12 m × 0.2 mm × 0.25 μm film, Hewlett-Packard, North Hollywood, California) was temperature programmed from 60°C (held for 2 min) to 260°C (held for 5 min) at a rate of 10°C/min. The mass spectra obtained were analyzed visually and with a probability-based computer mass spectral library search of the NIST/EPA/NIH 75K spectral library (Hewlett-Packard, Palo Alto, California). Because the computerized mass spectral library search indicated good matches, synthetic samples of (*Z*,*Z*)-9,12-octadecadienoic (linoleic) acid, (*Z*)-9-octadecenoic (oleic) acid, and octadecanoic (stearic) acid were obtained (Sigma Chemical Company, St. Louis, Missouri) and analyzed as described above. BSTFA (*N*,*O*-bis(trimethylsilyl)trifluoroacetamide, Pierce Chemical Company, Rockford, Illinois 61105) was purchased and diazomethane was prepared [from Diazald (Aldrich Chemical Company, P.O. Box 355, Milwaukee, Wisconsin 53201) as described in Fieser and Fieser (1967)], and used to derivatize the purified maize fraction and synthetic linoleic, oleic, and stearic acids. Both reactions are done at room temperature with virtually 100% conversion within a few seconds for diazomethane and a few hours for BSTFA. The retention time and mass spectra of the derivatized maize volatiles and synthetic free fatty acid derivatives were compared on the HP-1 column using conditions described above. In addition, they were analyzed by coinjection using two different capillary gas chromatograph columns, both programmed from 60°C (held for 1 min) to a final temperature of 240°C (held for 11 min) at a rate of 10°C, in a Hewlett Packard 5890 gas chromatograph. The columns used were an Econocap methyl silicone (30 m × 0.25 mm ID × 0.25 μm film, Alltech Associates, Inc., Deerfield, Illinois) and an HP-5 cross-linked 5% phenyl methyl silicone (30 m × 0.25 mm ID × 0.25 μm film, Hewlett-Packard, North Hollywood, California). The injection port was set at 240°C, the detector was set at 250°C, and the column head pressure was set at 240 kPa.

Behavioral Bioassays with Synthetic Free Fatty Acids. The purified maize volatile fraction (isolated as described above) was found to contain linoleic acid, oleic acid, and stearic acid in the ratio of 10:8:3 (see Results below). Aliquots from this maize fraction tested in behavioral bioassays had 1000 ng linoleic, 800 ng oleic, and 300 ng stearic acid. Synthetic linoleic, oleic, and stearic acid dilutions were prepared and tested in these quantities using the choice-test bioassays described above. In addition, synthetic linoleic, oleic, and stearic acid were tested individually for behavioral significance in doses of 10 ng, 100 ng, 1 μg, and 10 μg each in the same choice-test bioassay. All treatments were replicated a minimum of 10 times.

Statistical Analysis. The statistical package SAS (SAS Institute Inc., 1990) was used in all data analysis. Analysis of variance (ANOVA) were used followed by Duncan's (1955) multiple range test when significant F values were found in the ANOVA.

RESULTS

Bioassays with Purified Maize Volatile Fraction. The purified maize fraction was significantly attractive ($P < 0.01$) in choice tests with equal levels of carbon dioxide on both sides of the choice (Figure 1A).

Identification. The purified behaviorally active maize fraction contained three major peaks comprising over 98% of the area in the total ion current chromatogram. The EI mass spectrum of the peak with a retention time of 18.41 min on a methyl silicone column included peaks at m/z 280(19.3), 195(0.8), 182(1.5), 164(2.4), 163(2.3), 151(3.5), 150(7.3), 149(5.3), 138(4.2), 137(5.7), 136(8.4), 135(6.3), 124(9.3), 123(11.8), 122(8.3), 121(6.9), 110(22.3), 109(26.9), 96(40.6), 95(60.5), 81(87), 79(34.7), 68(55.3), 67(100), 55(52.5), and 54(40.4); the EI mass spectrum of the peak with a retention time of 18.46 min included peaks at m/z 282(3.9), 264(17.4), 235(2.9), 221(4.7), 220(5.4), 180(5.1), 166(4), 165(4.8), 139(6.5), 138(9), 127(9.1), 123(14), 111(26.6), 98(33), 97(53), 96(35), 84(41), 83(65), 82(34), 81(39), 69(81), 55(100), and 41(55); and the EI mass spectrum of the peak with a retention time of 18.61 min included peaks at m/z 284(47), 241(26), 227(6), 129(46), 115(14), 101(8), 98(15), 97(23), 83(28), 73(100), 60(75), 57(60), 55(53), 43(61), and 41(41). When the mass spectra of these peaks were analyzed with a probability-based computer mass spectral library search, the best matches were (*Z*,*Z*)-9,12-octadecadienoic (linoleic) acid, (*Z*)-9-octadecenoic (oleic) acid, and octadecanoic (stearic) acid, respectively, for the three chromatographic peaks. When synthetic linoleic, oleic, and stearic acid were injected onto the same GC-MSD under identical operating conditions, the retention times and EI mass spectra were in full agreement with the three peaks in the purified fraction.

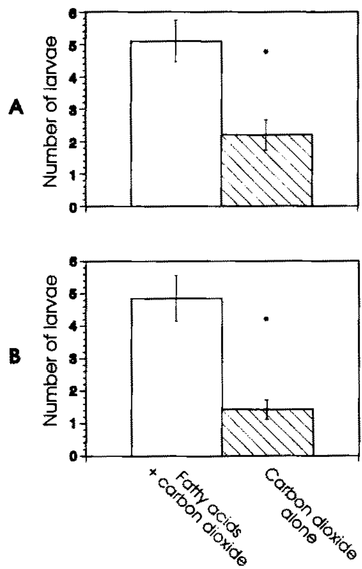

FIG. 1. Choice test bioassays of purified maize volatile fraction (A) and synthetic linoleic (1 μg), oleic acid (800 ng), and stearic acid (300 ng) combined (B) plus carbon dioxide versus carbon dioxide alone. Significant differences within each graph are indicated by an asterisk (*).

The retention times of the three peaks in the purified maize fraction and synthetic linoleic, oleic, and stearic acid were also identical on the Econocap methyl silicone column and on an HP-5 phenyl methyl silicone column (verified identical by coinjection). The retention times of the BSTFA and diazomethane products of the three peaks in the purified maize fraction and the corresponding BSTFA and diazomethane products of linoleic, oleic, and stearic acid were also identical on both the Econocap methyl silicone and the HP-5 phenyl methyl silicone columns (verified identical by coinjection).

On the basis of the EI mass spectra and gas chromatographic retention times of the natural and synthetic compounds and their derivatives, the structure of the unknown attractants were determined to be (*Z*,*Z*)-9,12-octadecadienoic (linoleic) acid, (*Z*)-9-octadecenoic (oleic) acid, and octadecanoic (stearic) acid.

Bioassays with Synthetic Free Fatty Acids. When the free fatty acid blend was tested together in the same proportion and concentration as was isolated from maize, significantly more western corn rootworm larvae chose the side with 1000 ng linoleic, 800 ng oleic acid, and 300 ng stearic acid than chose the side with carbon dioxide alone (Figure 1B). When synthetic linoleic, oleic, and

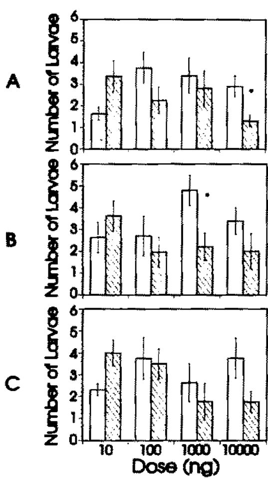

FIG. 2. Choice test bioassays of synthetic linoleic acid (A), oleic acid (B), and stearic acid (C) plus carbon dioxide (clear bars) versus carbon dioxide alone (hatched bars). Significant differences within each graph are indicated by an asterisk (*).

stearic acids were tested separately in choice tests for a dose–response curve, linoleic and oleic acid each had at least one does that was significantly attractive, but no dose of stearic acid was significantly attractive (Figure 2). Neither the purified extract nor the synthetic blend was behaviorally active in preliminary single-choice experiments without carbon dioxide.

DISCUSSION

We have used a bioassay-guided sequential fractionation scheme to isolate behaviorally active portions of a crude dichloromethane maize seedling extract and have identified (*Z*,*Z*)-9,12-octadecadienoic (linoleic) acid, (*Z*)-9-octadecenoic (oleic) acid, and octadecanoic (stearic) acid from one of the most behaviorally active fractions of an attractive crude maize extract. Long-chain fatty acids are the second set of compounds identified from maize roots as behaviorally active to western corn rootworm larvae, one of the most important insect pests in North America. The only compound previously isolated and identified from maize as an attractant (again, in choice tests with carbon dioxide on both sides of the choice) for western corn rootworm larvae is MBOA, 6-methoxy-2-benzoxazolinone (Bjostad and Hibbard, 1992). MBOA is found almost exclusively in the Gramineae (Niemeyer, 1988) and is toxic to a number of insect species

(Klun and Brindley, 1966; Campos et al., 1988; Nicollier et al., 1982) as well as bacteria (Nicollier et al., 1982; Beck and Stauffer, 1957) and fungi (Beck and Stauffer, 1957; Wahlroos and Virtanen, 1958; Beck and Smissman, 1961). Unlike MBOA, linoleic, oleic, and stearic acids are not limited to members of the grass family. In fact, in a preliminary survey of host and nonhost plants for western corn rootworm, linoleic, oleic, and/or stearic acids were found in surface extracts of all 10 species examined to date (BEH, unpublished data). Carbon dioxide is also released into the soil by most plant species (Massimono et al., 1980). Of the five semiochemicals known for western corn rootworm larvae, four appear to be prevalent for many plant species, and only one is apparently specific to grasses. Despite their almost ubiquitous presence in nature, a limited number of studies have found long-chain fatty acids to be of importance as behavioral cues to arthropods. Phelan et al. (1991) found oleic and linoleic acids to be components of ovipositional host-finding cues for the navel orangeworm, *Amyelois transitella* (Walker). Other studies that found fatty acids to be semiochemicals include those of Rickli et al. (1992), who demonstrated that the ectoparasitic mite, *Varroa jacobsoni* Oud responded significantly to palmitic acid, and Allan et al. (1988), who demonstrated that saturated free fatty acids (C_{14}–C_{22}) found in vaginal washes of *Dermacentor variabilis* and *D. andersoni* serve as components of the genital sex pheromone.

We have used a nondiapausing colony of western corn rootworm larvae in laboratory behavioral bioassays extensively since 1986, periodically comparing small numbers of a diapausing field strain in the same bioassays. Most tests have indicated no significant differences in the responsiveness of the two strains to corn semiochemicals. However, Hibbard et al. (1994) demonstrated that a diapausing strain of larvae responded more than a nondiapausing larvae in response to a crude dichloromethane extract of germinating corn seedlings or MBOA granules (in conjunction with an insecticide). A physiological difference may exist between the two strains in their responsiveness to semiochemicals, but it appears that if a difference does exist, the field strain responds more to semiochemicals.

Current insecticide application practices of maize growers for *Diabrotica* spp. larval control appear to be compatible with semiochemical techniques for enhancing the efficiency of insecticide use. Most insecticides in current use for control of corn rootworm larvae are granular systemic insecticide formulations (Mayo, 1986), and semiochemicals such as MBOA and long-chain free fatty acids can easily be formulated with granular insecticides currently used to control corn rootworm larvae. Hibbard and Bjostad (1989) demonstrated in laboratory experiments that a cryogenic collection of maize seedling volatiles could be used to attract western corn rootworm larvae to insecticide to increase insecticide efficacy. MBOA has been also demonstrated to increase insecticide efficacy in the laboratory, but field experiments with MBOA formulated onto clay granules with the experimental insecticide chlorethoxyphos (Fortress, E.I. Du Pont, Wil-

mington, Delaware) did not always provide a significant difference in root damage compared to treatments with insecticide alone (Hibbard et al., 1994). Addition of the long-chain free fatty acid blend to insecticide granules along with MBOA may increase efficacy more than MBOA alone.

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ALLELOPATHIC POTENTIAL OF MENTHOFURAN MONOTERPENES FROM *Calamintha Ashei*

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Abstract—A reversed-phase HPLC analysis was used to separate and quantify five menthofuran monoterpenes in *Calamintha ashei* leaf soaks and washes. (+)-Evodone and desacetylcalaminthone were the major constituents of both soaks and washes. Concentrations of (+)-evodone and desacetylcalaminthone were as high as 0.66 and 0.74 mM, respectively, in leaf soaks. The highest concentration of monoterpenes in leaf washes obtained by misting was 0.021 mM. Aqueous solubilities of the menthofurans were determined to exceed concentrations required for growth inhibition. Bioassays of individual *Calamintha* monoterpenes demonstrated effects on germination as low as 0.05 mM for (+)-evodone. An equimolar mixture of desacetylcalaminthone and (+)-evodone reduced *Rudbeckia hirta* germination by 17% at a combined concentration of 0.025 mM for *Leptochloa dubia*. Confirmation of allelopathic effects by *Calamintha ashei* will require long-term bioassays of *Calamintha* menthofurans on the growth of native sandhill species under conditions comparable to the harsh environment of the Florida scrub.

Key Words—Allelopathy, *Calamintha ashei*, Florida Scrub, menthofurans, monoterpenes.

INTRODUCTION

Calamintha ashei (Weatherby) Shinners is one of several perennial shrubs of the Florida scrub community for which evidence of allelopathic effects on pines

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and grasses of the sandhills is accumulating (Richardson and Williamson, 1988; Fischer et al., 1988; Weidenhamer and Romeo, 1989; Williamson et al., 1989). Several menthofuran monoterpenes (Figure 1) have been isolated from *Calamintha*, including (−)-calaminthone **(3)**, (+)-desacetylcalaminthone **(2)**, (+)-evodone **(1)**, and menthofuran **(5)** (Tanrisever et al., 1988; Macias et al., 1989), as well as six highly unstable compounds, which include 4α,5β-diacetoxymenthofuran **(4)** (Menelaou, 1990).

Proving allelopathic interference with the growth of one plant by another requires not only the identification of potential phytotoxins from the source plant, but the demonstration that they reach the target plant in sufficient concentration to cause the observed inhibition of growth (Fuerst and Putnam, 1983). The lack of such quantitative data has prompted some criticisms of research in allelopathy. As Radosevich and Holt (1984) assert, "Research in this area must be designed specifically to prove that a toxic substance is produced and that it accumulates or persists long enough at concentrations in the environment sufficient to inhibit development of other plants. . . . It is the failure to isolate, identify, and confirm these elusive chemicals as having allelopathic properties that allows skepticism."

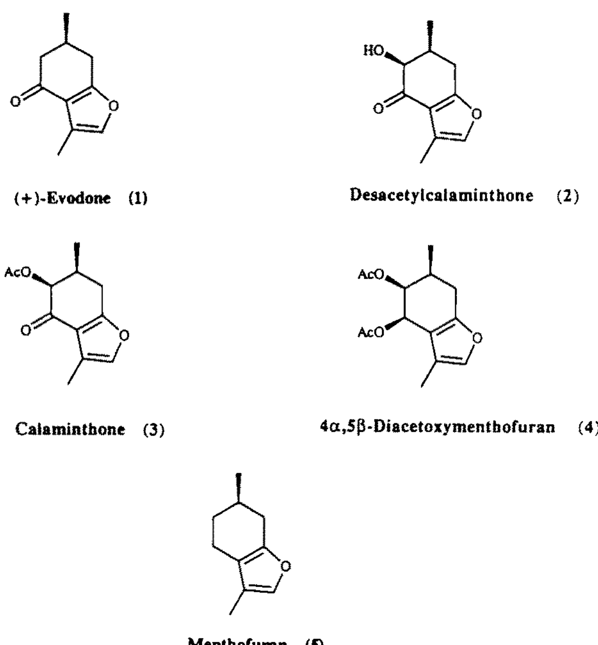


FIG. 1. Monoterpenes from *Calamintha ashei*.

The proposed mechanism for the release of allelochemicals from *Calamintha* and other scrub perennials is the washing of the compounds 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: (1) evaluated the phytotoxicity of the menthofuran monoterpenes in bioassays; (2) determined the aqueous solubilities of these compounds; and (3) quantified the release of the menthofuran monoterpenes in leaf soaks and leaf washes that were collected monthly for one year.

METHODS AND MATERIALS

Source and Purity of Menthofuran Monoterpenes

Standards of the *Calamintha* monoterpenes were previously isolated and purified in our laboratories (Tanrisever et al., 1988; Macias et al., 1989; Menelaou, 1990). Menthofuran was purchased from Eastman Kodak Co., Rochester, New York. Purities of calaminthone (**3**), desacetylcalaminthone (**2**), (+)-evodone (**1**), and menthofuran (**5**) were determined by gas chromatography to be 85%, 100%, 100%, and 95%, respectively. The 85% calaminthone contained 15% desacetylcalaminthone. A voucher specimen of *Calamintha ashei* is deposited at the University of South Florida herbarium in Tampa, Florida (sheet 137245).

Bioassays of Menthofuran Monoterpenes

Assays were conducted in 480 ml glass jars lined with one sheet of Whatman No. 1 filter paper. Lids were lined with foil and tightly sealed to prevent loss of the monoterpenes through volatilization. Each jar contained 25 seeds of one of four test species with 5 ml of test solution. The monocot species tested were the native sandhill grasses, little bluestem [*Schizachyrium scoparium* (Mich x.) Nash cv. Cimarron] and green sprangletop [*Leptochloa dubia* (H.B.K.) Nees]. Dicot species tested were blackeyed-susan (*Rudbeckia hirta* L.), also a sandhill species, and lettuce (*Lactuca sativa* L. cv. Great Lakes 118). Treatments were replicated six times for little bluestem and three times for the other test species. Assays were carried out in the dark at room temperature (23–25°C) and terminated by freezing after three days for lettuce and five days for the other species. Seeds were considered to have germinated if the root protruded at least 1 mm.

Aqueous stock solutions of calaminthone, desacetylcalaminthone, (+)-evodone, menthofuran, and a decomposing solution of the unstable $4\alpha,5\beta$-diacetoxymenthofuran were prepared by sonication for 1 hr. The concentrations of the stock solutions were 1.0 mM except for calaminthone (0.80 mM) and

menthofuran. In the case of the latter, not all of the monoterpene dissolved. The undissolved material adsorbed to filter paper and was separated from the solution by filtration, and the actual concentration of the menthofuran stock solution was determined by UV absorbance measurements to be 0.13 mM. Stock solutions were diluted to 1/2, 1/4, 1/10, and 1/20 strength for bioassays. An equimolar mixture (0.5 mM total) of desacetylcalaminthone and (+)-evodone, the two major monoterpenes of *C. ashei*, was also prepared and diluted. Distilled water controls were run concurrently with double the replication of test solutions.

Data were analyzed by comparing the treatment means to the corresponding control using the least-squares means test of the general linear models procedure of the Statistical Analysis System (SAS) programs (SAS Institute, 1985). Radicle length data were subjected to logarithmic transformations prior to analysis. A significance level of $P = 0.05$ was used.

Solubility Determinations

Stock solutions (10,000 mg/liter) of calaminthone, desacetylcalaminthone, (+)-evodone, and menthofuran were prepared in ethanol and serially diluted to 1000, 100 and 10 mg/liter. Standard solutions were stored under refrigeration in crimp-top vials with Teflon seals.

Duplicate saturated solutions of individual monoterpenes were prepared, equilibrated, and analyzed by gas chromatography as described previously (Weidenhamer et al., 1993). Solutions of the solid monoterpenes [calaminthone, desacetylcalaminthone and (+)-evodone] were filtered through a 0.2-μm nylon membrane prior to analysis. The injection technique for the liquid menthofuran was as follows: A 10-μl syringe containing 1 μl ethanol behind 1 μl of air was inserted from below into a vial, which was carefully maintained in a stable, inverted position. Two microliters of solution were drawn from the lower part of the vial into the syringe, which was then carefully removed from the vial. One microliter of solution was expelled and the needle wiped down thoroughly. The 1-μl sample was then drawn into the syringe barrel and injected. This technique has been found to give reproducible results for solubility determinations (Weidenhamer et al., 1993). Contamination by droplets of liquid monoterpenes, which occasionally collect at the surface of air bubbles that remain at the septum, produces anomalously high and variable data and was not a problem with menthofuran.

Quantification of Monoterpene Release

Collection and Preparation of Leaf Soaks and Leaf Washes. The plants sampled belong to a stand of approximately 30 individuals in a sand pine scrub community near Sun Ray, Florida. Samples were collected monthly for one year. On each sampling date, one to three (usually two) samples of leaf wash

and fresh foliage were collected from different plants. These samples were shipped in a cooler (10–20°C) during transport from the field to the laboratory (24 hr).

Leaf wash samples were obtained by misting water on a portion of a plant in the field and collecting the runoff in a 15-cm-diameter glass funnel. The misting was continued for approximately 1 min after droplets began to form on the leaves, usually requiring a volume of approximately 40 ml. For each sample, four or five such mistings over different portions of an individual shrub were combined.

Leaf soaks were prepared in the laboratory. For each sample, 25 g foliage (leaves and stems) was soaked in 100 ml water for 24 hr at 4°C. Leaf soak and mist samples were then filtered and 10-ml aliquots of each sample frozen at −20°C until analysis. Samples were stored in tightly capped glass vials wrapped with plastic film. No degradation of the monoterpenes was observed in these samples.

HPLC Analytical Method. HPLC analyses of leaf soaks and washes were performed on a Hewlett Packard 1090 liquid chromatograph equipped with a diode array detector and autoinjector (250-μl syringe). Detection channels were set at 220 nm (16-nm bandwidth) and 275 nm (8-nm bandwidth). Chromatograms were recorded and analyzed on a Hewlett Packard HPLC Chemstation (Series 300 Computer). The column (250 × 4.6 mm) had a 10-μm particle size, octylsilyl stationary phase (Phenomenex, Torrance, California). Analyses were performed at ambient temperature with a flow rate of 1.0 ml/min.

Two solvents were used: (A) HPLC grade methanol and (B) distilled, deionized water. Compounds were eluted with a linear gradient elution profile of 50–100% methanol over 0–18 min, with a 10-min equilibration period between runs. Injection volume was 100 μl. Detector response was linear in the range of 1–10 μg (Table 1). Approximate detection limits were 0.001 mM. The precision of peak area measurements with repeated injections of the same sample was ±0.9%.

RESULTS

Bioassays

Bioassays of (+)-evodone revealed strong inhibitory activity, with germination (Figure 2) being inhibited more than radicle growth (Figure 3). 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. Radicle elongation was inhibited in three of the four target species, but generally to a lesser degree than germination. Activity

TABLE 1. SUMMARY OF HPLC RETENTION TIMES, REGRESSION ANALYSES ON PEAK AREA, AND RESPONSE FACTORS (RF) FOR FIVE *Calamintha* MONOTERPENES

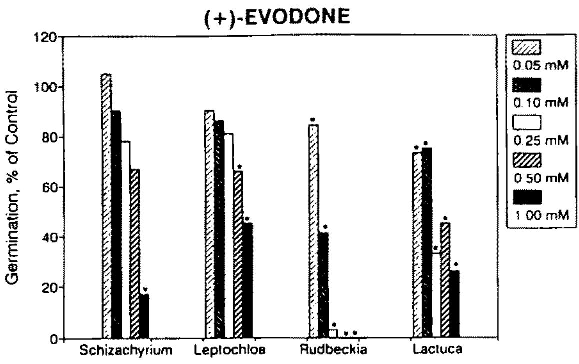
Compound	t_R (min)	Wavelength	R^2	*RF*
Desacetylcalaminthone	7.37-7.47	220	1.00	1.018 ± 0.010
		275	1.00	0.977 ± 0.014
Calaminthone	9.55-9.61	220	1.00	0.769 ± 0.032
		275	1.00	0.715 ± 0.032
(+)-Evodone	10.44-10.52	220	1.00	1.000
		275	1.00	1.000
4α,5β-diacetoxymenthofuran	12.09-12.11	220	1.00	0.993 ± 0.018
Menthofuran	16.23-16.29	220	1.00	1.405 ± 0.007

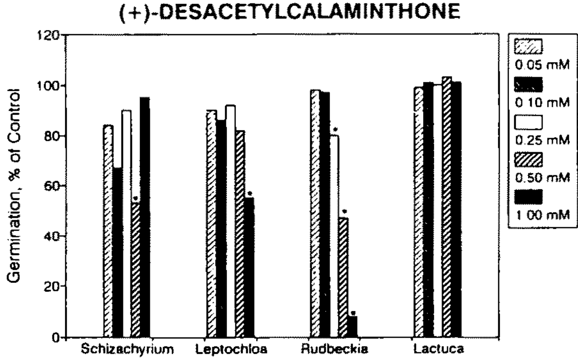
of desacetylcalaminthone, the second major constituent of *Calamintha*, was limited to inhibition of germination and radicle elongation of *Leptochloa* at 1.0 mM and germination of *Rudbeckia* as low as 0.25 mM (Figures 2 and 3).

The equimolar mixtures of (+)-evodone and desacetylcalaminthone showed inhibitory effects generally intermediate between the effects of the single compounds, indicating that their combined effects are additive rather than synergistic or antagonistic. For example, *Schizachyrium* germination was 53%, 67%, and 59% for 0.5 mM solutions of desacetylcalaminthone, (+)-evodone, and the equimolar mixture, respectively (Figure 2). *Lactuca* germination was 100%, 33%, and 66% for 0.25 mM solutions of desacetylcalaminthone, (+)-evodone, and the equimolar mixture, respectively (Figure 2). In a few cases, effects of the mixture appear to be more than additive. *Rudbeckia* germination was only slightly reduced by 0.25 mM desascetylcalaminthone (80% germination), but was greatly reduced by both 0.25 mM (+)-evodone (3% germination) and the 0.25 mM equimolar mixture (6% germination).

Effects of the other three monoterpenes were much less, so these data are not shown here. Calaminthone exhibited strong inhibition of *Rudbeckia* at 0.8 mM but showed few effects at lower concentrations, and no consistent effects were noted on the other three species. The degrading solution of 4α,5β-diacetoxymenthofuran exhibited modest inhibition of radicle elongation of the two grasses at 1.0 mM, but no inhibition at lower concentrations. The stock solution of menthofuran was only 0.13 mM, so its serial dilutions (1/2, 1/4, 1/10, 1/20 of stock) were much less concentrated than solutions of the other monoterpenes. Inhibition occurred in only one case—germination of *Rudbeckia* in the 0.13 mM solution was 71% of the control germination. Furthermore, menthofuran stimulated 30–100% increases in radicle elongation of *Leptochloa* at all five concentrations.

Effects on Germination





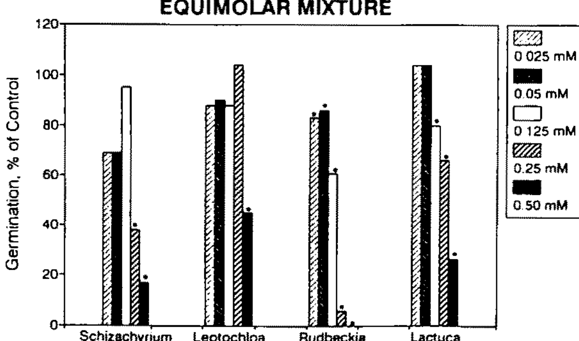


FIG. 2. Effect of (+)-evodone, (+)-desacetylcalaminthone, and an equimolar mixture of these two compounds on the germination of test species. Means significantly different from the control ($P = 0.05$) are designated by an asterisk.

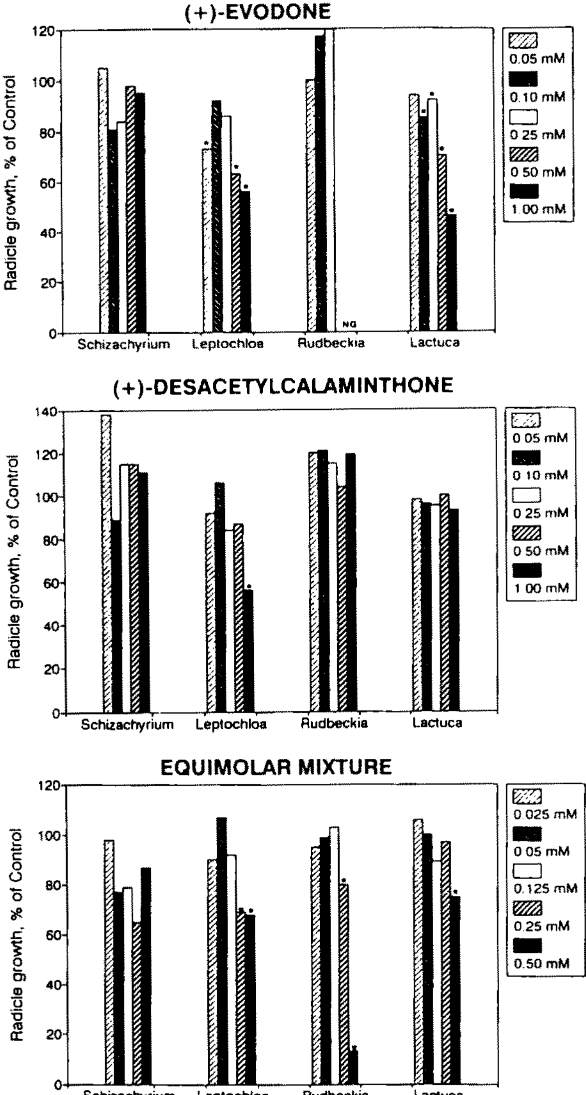


FIG. 3. Effects of (+)-evodone, (+)-desacetylcalaminthone, and an equimolar mixture of these two compounds on the radicle growth of test species. Means significantly different from the control ($P = 0.05$) are designated by an asterisk.

Solubility Determinations

Aqueous solubilities of the compounds, determined by gas chromatography, were 2.49 mM (409 ppm) for (+)-evodone, 5.58 mM (1005 ppm) for desacetylcalaminthone, and 4.36 mM (972 ppm) for calaminthone. Menthofuran showed evidence of degradation over the 72-hr equilibration period, such that its final measured solubility of 1.69 mM (254 ppm) represented only 40% of the soluble products in solution. These degradation products may have increased the solubility of menthofuran. The inability to prepare a 1.0 mM stock solution of menthofuran for bioassay is likely due to its lower solubility in undegraded form. No solubility determination was attempted for the highly unstable $4\alpha,5\beta$-diacetoxymenthofuran.

Quantification of Monoterpene Release

HPLC Method. The relative elution of the *Calamintha* monoterpenes correlates well with the lipophilicity of the molecules. Desacetylcalaminthone, the most polar compound, eluted first at 7.40 min, and menthofuran, the most lipophilic, eluted last at 16.23 min (Table 1, Figure 4A). Greater than optimal resolution was maintained for the analysis due to the potential production of other compounds at certain times of the year as well as breakdown products of the monoterpenes.

HPLC Analyses of Leaf Soaks. (+)-Evodone and desacetylcalaminthone were the major monoterpene constituents of *Calamintha* leaf soaks (Table 2, Figure 4B). In leaf soaks, the concentration of (+)-evodone ranged from trace levels to 0.66 mM, and concentrations of desacetylcalaminthone ranged from trace levels to 0.74 mM. Differences between samples taken on the same date indicate considerable variation in concentration and relative proportions of these two compounds between individual plants at the location sampled (Table 2). With the exception of desacetylcalaminthone in the February 3 sample, concentrations of (+)-evodone and desacetylcalaminthone were higher during the growing season of April–November than during the winter months of December–March (Table 2).

HPLC Analyses of Leaf Washes. (+)-Evodone and desacetylcalaminthone were also the major monoterpene constituents of *Calamintha* leaf washes (Table 2, Figure 4C). In leaf washes, total concentrations of monoterpenes in no case exceeded 0.021 mM. As with the leaf soaks, there appears to be a seasonal component to the monoterpene concentrations in leaf washes. With the exception of one sample in October, which did not contain detectable amounts of (+)-evodone (<0.001 mM), both (+)-evodone and desacetylcalaminthone were found to occur in trace (0.001–0.005 mM) or quantifiable amounts in all samples for the period May–December, while being below detectable levels in most samples for the period January–April (Table 2). It is obvious from the HPLC

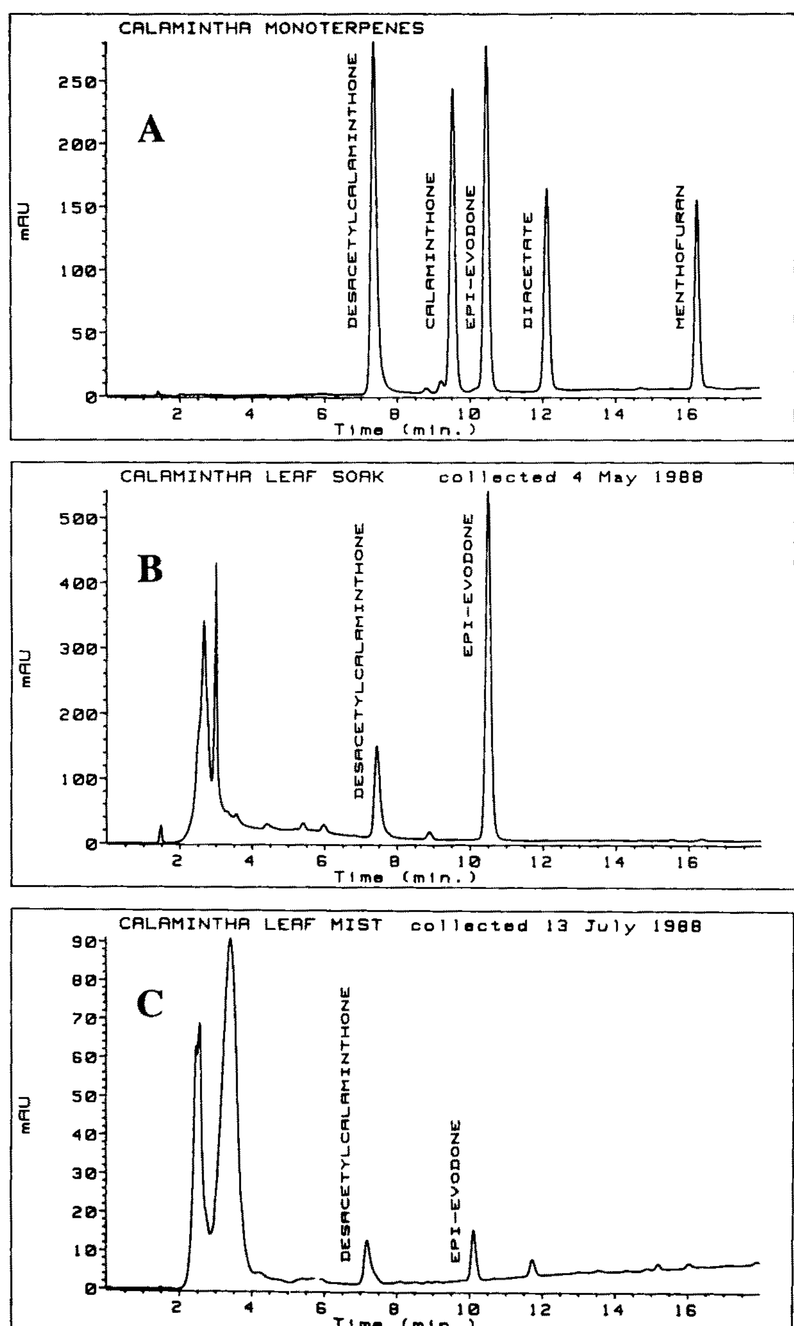


FIG. 4. Representative HPLC chromatograms of: (A) standard mixture of *Calamintha* monoterpenes, (B) *Calamintha* leaf soak, and (C) *Calamintha* leaf wash.

TABLE 2. SUMMARY OF HPLC DATA FOR LEAF SOAK AND LEAF WASH SAMPLES COLLECTED MONTHLY THROUGHOUT 1988[a]

Date	Leaf soak concentration (mM)					Leaf mist concentration (mM)				
	EV	DES	CAL	DMF	MF	EV	DES	CAL	DMF	MF
Jan. 6	0.017	0.118	—	—	Tr	—	—	—	—	Tr
Feb. 3	0.090	0.737	—	—	Tr	—	Tr	—	—	Tr
						—	Tr	—	—	Tr
Mar. 2	0.055	0.048	—	—	Tr	—	—	—	—	Tr
	—	Tr	—	—	Tr	—	—	—	—	Tr
Apr. 13	0.663	0.087	—	—	Tr	Tr	—	—	—	Tr
	0.582	0.080	—	—	Tr	Tr	—	—	—	Tr
	0.389	0.172	—	—	Tr					
May 4	0.210	0.054	—	—	Tr	0.012	0.008	—	—	Tr
	0.033	0.067	—	—	Tr	0.012	0.009	—	—	Tr
June 1	0.110	0.218	—	—	Tr	0.008	Tr	—	—	Tr
	0.580	0.195	Tr	—	Tr	Tr	Tr	—	—	Tr
July 13	0.401	0.193	—	—	Tr	Tr	Tr	—	—	Tr
	0.041	0.082	—	—	Tr	Tr	Tr	—	—	Tr
Aug. 10	0.135	0.049	—	Tr	Tr	Tr	Tr	—	—	—
	0.535	0.370	—	—	Tr	Tr	Tr	—	—	—
Sept. 14	0.365	0.052	Tr	Tr	Tr	Tr	Tr	—	—	—
	0.416	0.352	Tr	Tr	Tr	Tr	Tr	—	Tr	Tr
Oct. 12	0.290	0.643	—	Tr	Tr	—	0.006	—	Tr	Tr
	0.265	0.010	—	—	Tr	Tr	0.006	—	—	Tr
Nov. 9						0.008	0.006	—	—	Tr
						0.008	0.006	—	—	Tr
Dec. 6	0.202	0.210	Tr	Tr	Tr	Tr	Tr	—	—	Tr
	0.043	0.033	—	—	Tr	Tr	Tr	—	—	—

[a]Tr = <0.005 mM, — not detected. Approximate detection limits 0.001 mM. EV = (+)-evodone, DES desacetylcalaminthone, CAL = calaminthone, DMF = 4α,5β-diacetoxymenthofuran, and MF = menthofuran.

chromatograms (Figure 4C) that the leaf washes contain significant amounts of more polar constituents, but these are as yet unidentified.

DISCUSSION

The aqueous solubilities of desacetylcalaminthone and (+)-evodone, the two major menthofuran constituents of *Calamintha ashei*, exceed concentrations necessary for inhibition of germination and radicle growth of the sandhill species tested. Calaminthone and menthofuran, which had low activity in the bioassays, also had relatively high solubilities (> 1 mM). These data, as well as solubility data for 28 other monoterpenes (Weidenhamer et al., 1993), clearly demonstrate that oxygenated monoterpenes have solubilities in excess of 100 mg/liter, a concentration that is sufficient for many of these compounds to be potent inhibitors.

We previously 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, which occurs in copious quantities in *Calamintha* foliage (Fischer et al., 1988; Tanrisever et al., 1988). While prior work unambiguously established the formation of micelles in leaf soaks of these plants (Tanrisever et al., 1988), the ecological significance of this observation must be questioned for two reasons: first, oxygenated monoterpenes are sufficiently soluble to exert biological activity, and second, ursolic acid does not increase monoterpene solubility (Weidenhamer et al., 1993). Preliminary data using sonication to solubilize the monoterpenes showed no effect of ursolic acid on solubilization rate, but these studies need to be conducted in ways that more precisely simulate the natural situation.

Concentrations of desacetylcalaminthone and (+)-evodone in the leaf soaks are generally high enough to cause the inhibition of germination and radicle elongation observed in bioassays. The apparent seasonal variation in menthofuran concentrations may also explain the results of initial bioassays that showed a pronounced seasonal component in the inhibition of grass seed germination, with the greatest inhibition coming during the early summer (Richardson and Williamson, 1988).

On the other hand, concentrations of desacetylcalaminthone and (+)-evodone in the leaf washes, which were collected so as to simulate the action of rainfall, appear to be too low to cause inhibitory effects. The maximum total concentration of menthofurans in leaf washes was 0.021 mM in a May 4 sample (Table 2). An equimolar mixture of desacetylcalaminthone and (+)-evodone reduced *Rudbeckia* germination by 17% at a combined concentration of 0.025 mM, but the germination of sandhill grasses was not affected below concentrations of 0.125 mM for *Schizachyrium* and 0.25 mM for *Leptochloa* (Figure 2).

Implications for Hypothesis of Allelopathy. The proposed mechanism for the release of *Calamintha* allelochemicals is via the washing of the compounds from leaves and decaying litter by frequent rains during the growing season, which begins with the onset of heavy rains in June and extends through September. Leaf soaks may be indicative of the total release of menthofurans to be expected from leaf litter, whereas the misting of fresh leaves may simulate release during rainfall. However, our experimental technique does not mimic harsh rainstorms with multiple impacts of large water droplets on the leaf surface. It is possible that large raindrop impacts may cause more trichomes to rupture and thereby release larger quantities of monoterpenes from the leaves. Furthermore, it is not known whether monoterpenes washed from the leaves are replaced by additional biosynthesis. A determination of whether washing of leaves or of litter is more important will require more detailed analytical studies of release in the field.

A further consideration, which is of concern in all studies of allelopathy, is the question of realism in bioassays (Hollis et al., 1982). Single applications of a higher concentration of an allelochemical are less realistic than periodic doses of a lower concentration. Germination bioassays are generally carried out under the former conditions, while longer-term growth studies usually involve regular applications of an inhibitor. 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 deleterious effects on growth (Williamson and Weidenhamer, 1990). In this study, (+)-evodone was found in 20 of 21 leaf soaks and 17 of 23 leaf washes, and desacetylcalaminthone was found in 21 of 21 leaf soaks and 18 of 23 leaf washes. While data are accumulating in the allelopathy literature on the concentrations of allelochemicals in soil at a single point in time, the more crucial question is how much of an allelochemical is available over a period of time. Answering this question in the case of *Calamintha* will require measurements of the total amounts of desacetylcalaminthone and (+)-evodone 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 a prolonged period of time and then removed for analysis.

Also complicating the interpretation of the results of the present study is the fact that environmental parameters such as temperature, nutrient limitation, and plant density can significantly intensify plant response to an allelochemical (Einhellig and Eckrich, 1984; Einhellig, 1987; Williamson et al., 1992; Weidenhamer et al., 1989). Macronutrient levels in the Florida scrub are typically very low (Kalisz and Stone, 1984; Richardson, 1985). On sunny days, surface temperatures often exceed 50°C on the well-drained sands of the Florida scrub (Richardson, 1985), and significant moisture stress can occur during the dry

season and during pauses in summer rains. Plant densities in the Florida scrub are low, and this may also contribute to enhanced phytotoxicity of scrub allelochemicals (Weidenhamer et al., 1987, 1989).

The effect of hydrocinnamic acid, a breakdown product of ceratiolin from the scrub endemic (Ceratiola ericoides), is intensified by reduced levels of nitrogen and potassium (Williamson et al., 1992). It is not known whether the effects of *Calamintha*'s menthofuran monoterpenes will also be intensified in the harsh scrub environment.

It is perhaps significant that the major menthofuran constituents of *Calamintha*, desacetylcalaminthone, and (+)-evodone are also the most active in the bioassays conducted (Figures 2 and 3). The question of whether other compounds might be involved in the allelopathic effects of *Calamintha* must be considered. *Calamintha* contains a rich mixture of flavonoids in addition to the menthofuran monoterpenes (Hernandez, 1988; Menelaou, 1990), but these do not chromatograph well by reversed-phase HPLC and must be separated by normal-phase HPLC (data not shown). As a result of the low biological activity of these compounds (Menelaou, 1990), their concentrations in the leaf soaks and mists were not quantified. The biological activity of the unidentified polar constituents in the leaf washes is not known. The residence time of the menthofurans in the soil, as well as the activity of their decomposition products, is also unknown.

In summary, many significant questions remain to be answered before the original hypothesis of allelopathic effects by *Calamintha ashei* can be confirmed or refuted. Crucial to this assessment will be long-term bioassays that address the effects of *Calamintha* menthofurans on the growth of native sandhill species under conditions comparable to the harsh environment of the Florida scrub and application regimes that mimic the periodic release of these compounds during the rainy season.

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CHEMICAL DEFENSE OF COMMON ANTARCTIC SHALLOW-WATER NUDIBRANCH *Tritoniella belli* ELIOT (MOLLUSCA: TRITONIDAE) AND ITS PREY, *Clavularia frankliniana* ROUEL (CNIDARIA: OCTOCORALLIA)

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Abstract—Extracts of the dorid nudibranch *Tritoniella belli* and stoloniferan coral *Clavularia frankliniana* were chromatographed and analyzed by ^{1}H NMR and thin-layer chromatography. Three glycerol ethers were detected in *T. belli*, primarily 1-*O*-hexadecyl glycerol (chimyl alcohol). Chimyl alcohol was also detected after gradient flash chromatography and reverse-phase HPLC purification in the tissues of *C. frankliniana*. The common omnivorous predatory Antarctic sea star *Odontaster validus*, a likely predator of benthic invertebrates, showed feeding deterrence to small cubes of *T. belli* mantle tissue placed on the tube feet along the ambulacral feeding groove, while always extruding the cardiac stomach when presented with cubes of shrimp tissue of similar size. Filter-paper disks soaked in an aqueous shrimp solution and then dried were found to elicit a broad range of feeding behaviors in *O. validus*, including movement of the shrimp disk to the mouth, extrusion of the cardiac stomach, and the assumption of a humped feeding posture. Chimyl alcohol-treated shrimp disks caused significant feeding deterrence in sea stars when compared with control disks (solvent plus shrimp treated disks alone). *T. belli* and *C. frankliniana* appear to employ a defensive compound that has been

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found in a variety of temperate and tropical mollusks, where it has been demonstrated to deter fish predators. We provide evidence for further deterrent capabilities of chimyl alcohol and of its trophic relationship in the polar ecosystem of McMurdo Sound, Antarctica.

Key Words—Antarctica, marine benthos, *Tritoniella belli*, Mollusca, nudibranch, *Clavularia frankliniana*, Cnidaria, chemical defense, predator, prey, chimyl alcohol.

INTRODUCTION

The chemical ecology of the Antarctic benthos has only recently become the focus of a number of empirical studies. The marine benthos appears to be environmentally stable and structured primarily by biological factors such as competition and predation (Dayton et al., 1974), although the impact of anchor ice and ice scour at depths less than 30 m can be considerable (Dayton et al., 1969, 1970). The importance of competition and predation combined with the extreme geological age of this environmentally stable benthos increases the likelihood of chemical defense mechanisms in Antarctic marine invertebrates. Although there is a conspicuous absence of browsing fish predators in Antarctica (Eastman, 1993), sea stars are common predators of Antarctic benthic macroinvertebrates (Dayton et al., 1974; Dearborn, 1977; McClintock, 1994). Recent studies have documented chemically mediated feeding deterrent properties in a suite of Antarctic marine invertebrates (Heine et al., 1991; McClintock et al., 1990, 1992a, 1994a,b,c; Slattery et al., 1990), including several species of gastropods (McClintock and Janssen, 1990; McClintock et al., 1992b). Compared with temperate and tropical marine systems (Bakus et al., 1986; Faulkner, 1991), very little information is currently known of the natural products chemistry of Antarctic marine invertebrates (Eggersdorfer et al., 1982; Seldes et al., 1986; Molinski and Faulkner, 1987, 1988; Davies-Coleman and Faulkner, 1991; Blunt et al., 1990; Kong et al., 1992; Perry et al., 1994; Trimurtulu et al., 1994a; Baker et al., 1994, 1995).

The Antarctic nudibranch *Tritoniella belli* is a conspicuous member of the shallow (> 30 m depth) hard-bottom communities along the coast of Ross Island, McMurdo Sound, Antarctica (Dayton et al., 1974). It appears to feed primarily on the common stoloniferan soft coral *Clavularia frankliniana* (Dayton et al., 1974; Slattery, 1994), while hydroids and anemones are occasionally eaten (Slattery, personal observation). No observations of predation on *T. belli* have been made throughout extensive long-term surveys of the benthos (Dayton et al., 1974; Slattery and Heine, personal observation). The purpose of the present study is to provide information on the presence of a bioactive compound in the tissues of *T. belli* and *C. frankliniana*. Moreover, we demonstrate that this bioactive compound is an effective feeding deterrent against one of the most common omnivorous Antarctic sea star predators.

METHODS AND MATERIALS

Chemical Analysis. Fourteen adult individuals of *Tritoniella belli* were collected by hand using SCUBA from Hut Point and Danger Slopes, Ross Island, Antarctica, during the months of October, November, and December 1989 (Figure 1). Individuals were placed in acetone and shipped to Scripps Institution of Oceanography, La Jolla, California, for chemical analysis. Following extraction of whole animals in acetone, the concentrate was partitioned between ethyl acetate and water. The ethyl acetate-soluble portion was subject to gradient flash chromatography yielding eight fractions. From the 1 : 1 ethyl acetate–hexane fraction, two of the glycerol ethers (**1** and **3**, Figure 2) were isolated by normal-phase HPLC (1 : 1 ethyl acetate–hexane) and from the 2 : 1 ethyl acetate–hexane fraction was isolated glycerol ether **2** (Figure 2), which was purified by normal-phase HPLC (7 : 3 ethyl acetate–hexane). Spectroscopic data were used to elucidate the identity of the isolates.

The soft coral *Clavularia frankliniana*, was similarly collected along the coast of Ross Island from Hut Point to Cinder Cones. The freeze-dried animals were extracted successively with hexane, chloroform, methanol, and 70 : 30 methanol–water. The chloroform extract was fractionated by gradient flash chromatography (Still et al., 1978) with hexane and increasing amounts of ethyl

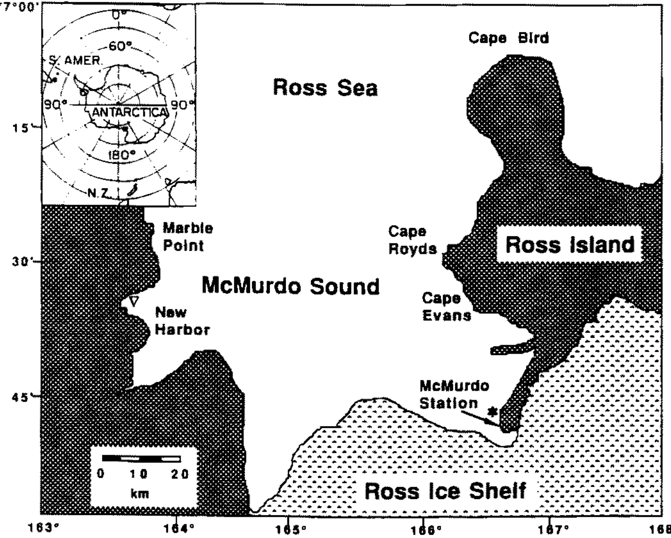

FIG. 1. Map of McMurdo Sound, Antarctica, showing the location (asterisk) of collecting sites of the nudibranch *Tritoniella belli* and the sea star *Odontaster validus*.

Chimyl Alcohol (1)

FIG. 2. Structures of compounds 1–3.

acetate. The 40% ethyl acetate–hexane fraction was then subjected to normal-phase HPLC purification (10 mm × 25 cm Alltech RSIL 10-μ silica gel column, 30% ethyl acetate–hexane, 1.5 ml/min, broad peak with tailing at 45 min) followed by reversed-phase HPLC (10 mm × 30 cm Waters μBondapak RCM 10-μm octadecylsilyl column, 5% water in methanol, 2.0 ml/min, sharp peak at 15 min) to yield, among other fatty acid derivatives, chimyl alcohol (**1**). The isolated chimyl alcohol was characterized by comparison with commercially available material (Sigma), which displayed identical chromatographic behavior, as well as by ^{1}H NMR spectroscopy.

Feeding Deterrent Analysis. The common Antarctic sea star *Odontaster validus* was selected as a potential predator of *Tritoniella belli* and *Clavularia frankliniana*. Although two species of Antarctic fish show deterrence to mantle tissues of *T. belli* (McClintock et al., 1992b), their diets are mostly restricted to amphipods (Eastman, 1993). In contrast, *O. validus* is omnivorous and includes an extremely wide variety of mobile and sessile macroinvertebrate prey in its diet (Peckham, 1964; Pearse, 1965, 1969; Dayton et al., 1970; McClintock et al., 1988). *O. validus* can move rapidly enough to capture *T. belli* (McClintock, laboratory observations), but has never been observed to feed on nudibranchs in the laboratory or field. Individuals of *O. validus* (mean ± 1 SD radius = 44.5 ± 5.3 mm; N = 30) and *T. belli* (mean ± 1 SD length = 82.3 ± 3 mm; N = 3) were collected in September and October 1993, respectively, from near Hut Point between 20 and 30 m depth using SCUBA. Sea stars were held in a large sea water tank (ca. 2 m diameter) with ambient water pumped directly from the sea (−1.5°C). Sea stars tended to climb the side of the tank and remain at the air–water interface with the oral sides of one or two of their arms extended parallel to the water surface. In one bioassay, cubes of fresh mantle tissue of *T. belli* (3 × 3 × 3 mm) were placed on the outstretched tube feet of an arm equidistant between the arm tip and the mouth. Control tissues consisted of cubes of dried shrimp of similar size. When individuals dropped the potential food item, it was considered a rejection response. The time required

for the tube feet to move the tissue to the mouth was recorded, as was the time until rejection or acceptance, with the latter defined as a stereotypic sea star feeding behavior (humped posture with the cardiac stomach extruded against the prey item).

In a second bioassay, individuals were similarly presented circular paper filter disks (5 mm diameter BBC Blank Paper Disks), which had been soaked 24 hr in a 1:6 dry weight–volume ratio of ground dried shrimp mixed with seawater. Preliminary trials indicated that such shrimp-treated disks initiated a predictable stereotypic feeding response in *O. validus*. Experimental shrimp disks were dosed with one of three concentrations of chimyl alcohol (1-*O*-hexadecyl-sn-glycerol, Sigma) in MeOH (20 μl per disk to yield 4, 0.4, and 0.04 mg chimyl alcohol/disk). These concentrations were chosen to bracket the natural concentration found in *T. belli* tissues (see below). Treated and control (shrimp disks alone) disks were allowed to dry before conducting assays. In all trials, 10 replicates were conducted for each treatment, with each lasting 60 min. Tissues and disks were presented in a random sequence and individual sea stars were subject to only one feeding trial per day. Statistical analysis was conducted using a 5×2 contingency table analysis (Sokal and Rohlf, 1981). A lower acceptable probability level was calculated (Bonferroni adjusted probability) when making multiple comparisons to avoid a type II error.

RESULTS

Chemical Analysis. The masses of isolated 1-*O*-hexadecyl glycerol (chimyl alcohol, **1**), 1-*O*-eicosenyl glycerol **(2)**, and 1-*O*-(2-methoxyhexadecyl glycerol, **3**) from 14 *Tritoniella belli* individuals were 26, 13, and 6 mg, respectively (109.5 g animal tissue, after extraction). Therefore, an average adult would contain approximately 1.8 mg of chimyl alcohol. The three isolates displayed ^{1}H NMR signals (Table 1) characteristic of fatty acid glycerols (δ 3.4–3.8, protons on carbon-bearing oxygen; δ 1.22, methylene envelope). While the ^{13}C NMR spectrum failed to resolve all methylene carbons, evaluation of the mass spectrum of **1–3** secured their molecular formula and thus their structure. Subsequent comparison of ^{1}H and ^{13}C NMR data to that previously reported for chimyl alcohol confirmed the structural assignment of **1**.

Successive extraction of 632 g of freeze-dried *Clavularia frankliniana* yielded 4.64 g of chloroform extract composed primarily of fatty acids and their derivatives. HPLC separation of the extract yielded 1 mg of chimyl alcohol **(1)**.

Feeding Deterrent Analysis. Odontaster validus presented cubes of the fresh mantle tissues of *Tritoniella belli* displayed a significant rejection response when compared to acceptance rates for shrimp tissue, with 90% of the individuals rejecting mantle tissues (chi square = 16.363; $P = 0.00005$). Forty percent

TABLE 1. ^1H NMR SPECTRAL DATA [δ, INTEGRATION, MULTIPLICITY, (J IN Hz)] FOR COMPOUNDS 1-3 (200 MHz, $CDCl_3$)

Position	1	2	3
H-1a	3.69, 1H, dd (4, 12)	3.48–3.85, 7H, m	3.69, 1H, dd (4, 12)
H-1b	3.60, 1H, dd (6, 12)		3.60, 1H, dd (6, 12)
H-2	3.82, 1H, m		3.82, 1H, m
H_2-3	3.43, 2H, t (6)		3.43, 2H, t (6)
H_2-1′	3.49, 2H, m		3.49, 2H, m
H_2-2′	1.53, 2H, m	3.35, 1H, m	1.52, 2H, m
H_2-3′	[a]	2.24, 2H, m	[a]
Allylic position	[b]	[b]	1.98, 4H, m
Olefins	[b]	[b]	5.32, 2H, t (5)
Methylene envelope	1.22, 28H, m	1.23, 26H, m	1.23, 26H, m
Terminal methyl	(H_3-16′) 0.85, 3H, t (7)	(H_3-16′) 0.85, 3H, t (7)	(H_3-20′) 0.85, 3H, t (7)
Methyl ether	[b]	3.37, 3H, s	[b]

[a] H_2-3′ in **1** and **3** is under methylene envelope.
[b] Functional group not present.

of the individuals presented *T. belli* mantle tissues carried the tissue with the tube feet to the mouth (mean ± 1 SD time for movement to mouth = 22.8 ± 4.0 min, N = 4). Three of these individuals subsequently rejected the mantle tissue after holding it near the mouth for a mean of 17.2 min. Only one individual offered mantle tissue showed stereotypic feeding behavior (humped posture and extrusion of the cardiac stomach). In contrast, *O. validus* presented similar sized cubes of shrimp tissue always moved the food item from the middle to the arm to the mouth (mean ± 1 SD time for movement to mouth = 2.9 ± 0.9 min, N = 10). Rates of movement prey to the mouth were eightfold more rapid for shrimp than for mantle tissues. When presented shrimp tissues, all individuals showed stereotypic feeding behavior, with feeding commencing after 14.9 ± 4.3 min (X ± 1 SD; N = 10).

All *Odontaster validus* presented shrimp disks used their tube feet to transfer the disks to the mouth (mean ± 1 SD time for movement to the mouth = 4.2 ± 1.5 min, N = 10). Once the disks were carried to the mouth, stereotypic feeding behavior ensued (feeding commenced after 12.7 ± 6.3 min; X ± 1 SD; N = 10). Similar results were obtained for sea stars presented shrimp disks treated with methanol alone, with only one disk rejected. Shrimp disks treated with different concentrations of chimyl alcohol were rejected significantly more often than controls (chi square = 17.881; P = 0.0013; Figure 3). Levels of rejection were significant when comparing any of the three concentrations of

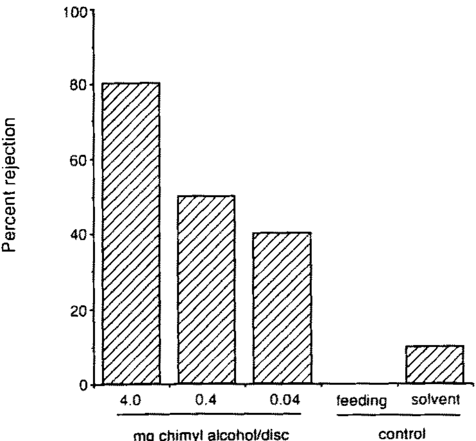

FIG. 3. Histogram showing the dose-responses (percent of disks rejected) of *Odontaster validus* presented shrimp disks treated with different concentrations of chimyl alcohol, $N = 10$ disks per concentration). Also shown is the percentage rejection for feeding (shrimp disks alone) and solvent controls (shrimp disks treated with the same amount of methanol used to load chimyl disks) ($N = 10$ per control treatment).

chimyl alcohol tested against the controls (0.04 mg vs. controls, chi square = 5.88, $P = 0.0153$; 0.4 mg vs. controls, chi square = 7.0726, $P = 0.00783$; 4 mg vs. controls, chi square = 9.8989, $P = 0.00165$). Eighty percent of individuals offered the highest dose of chimyl alcohol rejected the disks. Fifty percent of individuals rejected the medium concentration disks (0.4 mg chimyl alcohol/disks), while an additional 20% of individuals failed to reject the disks, yet never initiated a feeding response. The remaining 30% of the sea stars tested showed feeding behaviors in response to medium concentration disks (0.4 mg chimyl alcohol). At the lowest concentration (0.04 mg chimyl alcohol/disk), 40% of the disks were rejected, with the remaining 60% fed upon. Although the frequency of feeding decreased with decreasing concentration of chimyl alcohol (Figure 3), there were no statistically significant differences between the numbers of disks ingested at the three concentrations tested (chi square = 3.5294, $P = 0.17123$).

DISCUSSION

The results of this study suggest that the Antarctic dorid nudibranch *Tritoniella belli* employs chimyl alcohol to chemically defend itself from the omnivorous Antarctic sea star *Odontaster validus*, a likely predator. Concentrations

of chimyl alcohol up to 45 times lower than levels recovered from an adult individual caused significant feeding deterrence in *O. validus*. The presence of chimyl alcohol in the soft coral *Clavularia frankliniana* may protect this species as well, although comparatively low levels suggest additional bioactive compounds may be present. Slattery (1994) found that *O. validus* showed significant defensive tube-foot retractions to organic extracts of *C. frankliniana*. This study adds another species to a growing list of chemically defended Antarctic molluscs. McClintock and Janssen (1990) found that whole individuals or tissue homogenates of the Antarctic pteropod *Clione antarctica* were unpalatable to the Antarctic fish *Pagothenia borchgrevinki*. In a unique symbiotic relationship, the hyperid amphipod *Hyperiella dilatata* was found to abduct and carry live individuals of *C. antarctica* as a means of providing its own chemical defense (McClintock and Janssen, 1990). The defensive compound in the tissues of *C. antarctica* has recently been identified as a hydroxyketone (Bryan et al., 1994). Mantle tissues of the Antarctic lamellarian gastropod *Marseniopsis mollis* and the dorid nudibranch *Austrodoris mcmurdensis* are rejected by two species of Antarctic fish (*P. borchgrevinki* and *Trematomus bernacchii*) (McClintock et al., 1992b). Moreover, aqueous homogenates of the mantle tissues of both species are cytotoxic to the gametes of the Antarctic regular sea urchin *Sterechinus neumayeri* and cause significant sensory tube-foot retraction in the Antarctic sea stars *Acodontaster conspicuus*, *Diplasterias brucei*, *Perknaster fuscus*, *Odontaster validus*, and *O. meridionalis*. *Marseniopsis mollis* may employ homarine as a feeding deterrent (McClintock et al., 1994c). Davies-Coleman and Faulkner (1991) isolated a series of novel glyceride diterpenes from the whole-body tissues of the Antarctic dorid nudibranch *Austrodoris mcmurdensis*, which are likely to be responsible for bioactivity. Although little information is currently known about the natural products of Antarctic marine invertebrates, a number of similarities have been found between the chemistry of Antarctic marine sponges and their temperate and tropical counterparts (Baker et al., 1994). Chimyl alcohol is apparently another natural product that is not unique to the Antarctic and occurs across wide latitudinal gradients.

Similar to the species described, mantle tissues and aqueous mantle tissue homogenates of *Tritoniella belli* are rejected by two species of Antarctic fish and cause sea urchin gamete mortality and sea star tube foot retractions (McClintock et al., 1992b). In the present study, cubes of fresh mantle tissue of *T. belli* caused significant feeding deterrence in the Antarctic sea star *Odontaster validus*. This common omnivorous sea star feeds on a wide variety of prey including sponges, gastropods, ostracods, shrimp, sea urchins, sea stars, hydroids, ectoprocts, isopods, amphipods, detritus, seal feces, diatoms, and algae (Peckham, 1964; Pearse, 1965, 1969; Dayton et al., 1970, 1974; Dearborn, 1977; McClintock, 1994). As we found chimyl alcohol to be the primary natural product present in *T. belli*, it is likely that this compound is responsible

for the bioactivity (mantle tissue rejection) observed. However, the possibility that other secondary metabolites present in only trace amounts contribute to deterrence can not be discounted. Chimyl alcohol-treated shrimp disks were rejected by *O. validus*, while little or no rejection occurred for shrimp disks treated with solvent carrier or shrimp alone. The concentration of chimyl alcohol actually encountered by a potential predator of *T. belli* is unknown.

Nudibranchs may concentrate and sequester defensive metabolites in their dorsal mantle tissues or in specialized mantle glands or release them in mucus (Cimino et al., 1982; Thompson et al., 1982; Karuso, 1987; Pawlik et al., 1988; Avila et al., 1990; Paul et al., 1990). *Tritoniella belli* produces a mucus that coats the mantle tissues (McClintock, personal observation). As sea stars use chemotactic information from the sensory tube-feet to evaluate prey (Sloan, 1980), it would be most effective to sequester chimyl alcohol in the epithelium of the mantle, where predator contact is most likely. In an attempt to examine in which body tissues chimyl alcohol was located, we recently (November 1993) re-collected a sample of *T. belli* only to find nondetectable levels of chimyl alcohol in any of the body tissues; however, the glycerol ethers were evident in the NMR spectrum. It is unknown whether these other glycerol ethers have feeding-deterrent properties.

Chimyl alcohol has been extracted from other marine molluscs including the chiton *Katherina tunicata*, the gastropod *Thais lamellosa*, the bivalve *Protothaca stamina*, and the octopus *Octopus defleini* (Thompson and Lee, 1965). Moreover, Gustafson and Andersen (1985) have isolated chimyl alcohol from the temperate dorid nudibranchs *Archidoris montereyensis* and *Aldisa sanguinea cooperi*. The bioactive nature of chimyl alcohol was demonstrated by these investigators, with antibacterial activity demonstrated against two common bacterial strains (*Bacillus subtillus* and *Staphylococcus aureus*). Moreover, chimyl alcohol was shown to be an effective fish antifeedant (Gustafson and Andersen, 1985).

Dietary derivation of secondary metabolite chemistry is common in nudibranchs (Karuso, 1987; Faulkner, 1988), with the exception of a select group of nudibranchs that produce defensive compounds de novo (e.g., Anderson and Sum, 1980). Most nudibranchs derive their chemistry from bioactive sponges and instances of those employing soft coral chemistry are rare (Karuso, 1987). The glaucid nudibranch *Phyllodesmium longicirra* feeds exclusively on the alcyonian soft coral *Sarcophyton trocheliophorum*, sequestering dietarily derived cembranes in the cerata (Rudman, 1981). Recently, Baker and Scheuer (1994) demonstrated that the tropical dorid nudibranch *Tritonia welessi* derived defensive punaglandins from a tropical soft coral (*Telesto riisei*). Our study provides one of only a few examples of a nudibranch that likely derives defensive chemistry from a soft coral and the first such example for species living in polar waters.

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BIOLOGICAL ACTIVITY OF (3*R*,5*S*,6*R*)- AND (3*S*,5*R*,6*S*)-3,5-DIMETHYL-6-(METHYLETHYL)-3,4,5,6-TETRAHYDROPYRAN-2-ONE, A PHEROMONE OF *Macrocentrus grandii* (GOIDANICH) (HYMENOPTERA: BRACONIDAE)

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Abstract—In a previous study we reported identification of (3*R**,5*S**,6*R**)-3,5-dimethyl-6-(methylethyl)-3,4,5,6-tetrahydropyran-2-one as a component of the pheromone of *Macrocentrus grandii* Goidanich. The lactone was present in male and female wasps, and laboratory and field bioassays demonstrated that both sources of the lactone elicit flight initiation, upwind anemotaxis, and casting in male wasps. In the present study, the synthetic (3*R*,5*S*,6*R*)- and (3*S*,5*R*,6*S*)-lactone enantiomers (*RSR* and *SRS*, respectively) were bioassayed for biological activity. In wind tunnel studies the *SRS* enantiomer elicited flight initiation, upwind anemotaxis, and casting by male wasps comparable to lactone derived from male and female wasps. Flight response to the *RSR* enantiomer averaged 14 percent of the *SRS* enantiomer. No specific ratio of the stereoisomers was found more attractive than the *SRS* enantiomer alone. Field studies demonstrated the *SRS* enantiomer was active alone in attracting male wasps. When paired with (*Z*)-4-tridecenal (a previously identified female-derived sex pheromone), the *SRS* enantiomer yielded a synergistic response comparable to (*Z*)-4-tridecenal plus female-derived lactone.

Key Words—Pheromone, 3,5-dimethyl-6-(methylethyl)-3,4,5,6-tetrahydropyran-2-one, enantiomer, parasitoid, *Macrocentrus grandii*, Hymenoptera, Braconidae.

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INTRODUCTION

The presence of chiral pheromones has been demonstrated in a variety of insect groups. In such cases, insects generally show discriminatory ability in the detection of optical isomers and their responses are usually limited to the naturally produced enantiomer. In some cases the optimal response is to a specific ratio of enantiomers; in other situations the presence of one isomer is inhibitory (Silverstein, 1988).

No study of chirality and biological activity with parasitoid pheromones has been reported. However, in a related study with semiochemicals, Lewis et al. (1988) evaluated the response of *Microplitis croceipes* (Cresson) and *Microplitis demolitor* Wilkinson to the *R* and *S* enantiomers of 13-methylhentriacontane (a kairomone from the frass of corn earworm larvae). No measurable difference in response was found to the two enantiomers by either parasitoid.

We recently reported the identification of a male- and female-derived pheromone component of *Macrocentrus grandii* Goidanich (a polyembryonic larval parasitoid of *Ostrinia nubilalis* Hübner, the European corn borer) as (3*R**,5*S**,6*R**)-3,5-dimethyl-6-(methylethyl)-3,4,5,6-tetrahydropyran-2-one (Swedenborg et al., 1993). Lactone from both males and females elicited flight initiation, upwind anemotaxis, and casting by male wasps in a wind tunnel (Swedenborg and Jones, 1992a). In addition, when either source was paired with synthetic (*Z*)-4-tridecenal, a previously identified female-derived sex pheromone that elicits flight, landing, and courtship behavior in a wind tunnel, behavioral responses were observed to increase synergistically in laboratory and field studies (Swedenborg and Jones, 1992b). Subsequent to the isolation and identification of the lactone, the stereospecific synthesis of its 3*R*,5*S*,6*R* and 3*S*,5*R*,6*S* enantiomers (denoted below as *RSR* and *SRS*, respectively) was reported (Shin et al., 1993). This paper reports the response of male *M. grandii* to the synthetic lactone enantiomers, to natural lactone derived from males and females, and to (*Z*)-4-tridecenal in laboratory and field bioassays.

METHODS AND MATERIALS

Wasps. Laboratory studies were conducted with *M. grandii* reared from parasitized corn borer larvae (Ding et al., 1989; Guthrie et al., 1971). Field studies were conducted with feral populations of *M. grandii* at the Minnesota Agricultural Experiment Station, University of Minnesota, Rosemont, Minnesota. Voucher specimens have been placed in the University of Minnesota insect museum.

Chemicals for Testing. Lactone from male and female sources was extracted and purified as described by Swedenborg et al. (1993). The synthetic enantiomers were synthesized in the laboratory of Hung-wen Liu (Shin et al., 1993).

Purities of the synthetic enantiomers were: chemical, ≥99% *RSR* and ≥99% *SRS*; and optical, ≥99% *RSR* and ≥99% *SRS*. Optical purity was determined using gas chromatography. Samples (synthetic *RSR*, *SRS*, and male- and female-derived lactone) were injected onto a Chiraldex G-TA capillary column (30 m × 0.32 mm ID, Advanced Separation Technologies, Inc. Whippany, New Jersey). Using helium carrier gas (1.4 ml/min) and an isothermal program (130°C), baseline separation was achieved with a mixture of the synthetic enantiomers. Synthetic *RSR* eluted at 9.55 min and synthetic *SRS* eluted at 9.78 min. When injected separately, neither synthetic isomer showed evidence of the other enantiomer. Natural lactone from both males and females had a retention time identical to the *SRS* enantiomer. There was no evidence of the *RSR* enantiomer or other isomers in either natural source.

Laboratory Tests. Laboratory bioassays were conducted in a wind tunnel (31 × 42 × 92 cm; airspeed ca. 25 cm/sec) as described by Swedenborg and Jones (1992a). Chemical treatments were applied to 4-cm watch-glass plates with each plate attached to a Boston clip. The plates (treatment and control) were hung at the upwind end of the tunnel on prepositioned wire hooks 16 cm apart. About 150–200 males were used for each bioassay. Male behavior included upwind flight and casting in front of the source. Subsequent landing on the source was infrequent; therefore, the upwind flight response was quantified by recording the number of males flying upwind through a white Teflon O ring (8 cm ID; 8.5 cm OD). O rings were supported 7 cm directly downwind of each of the watch glass plates. Male- and female-derived lactones and the synthetic lactone enantiomers were diluted in pentane. Immediately after evaporation of the solvent, the treatments were bioassayed. All bioassays were run for 1 min. Treatments were randomized and paired with controls. Replications were performed over time (day). Chemical positions were exchanged for each replicate. Data were analyzed by a one-sided paired t test and analysis of variance (ANOVA) with mean separations by PLSD, $\alpha \leq 0.05$ (Steel and Torrie, 1980).

Field Tests. Field bioassays were conducted with feral populations of *M. grandii* from August 29 to September 13, 1992, in plots of hybrid field corn. Treatments were set up in a randomized complete block design with rows of corn as blocks. Blocks were a minimum of 20 rows apart and some blocks were in separate corn fields. No more than three blocks were run on the same day. Treatments were the synthetic *RSR* and *SRS* enantiomers, the female-derived lactone, and (*Z*)-4-tridecenal. The synthetic and female-derived lactones (all sources in pentane) were applied to 5 × 9-mm sleeve-type rubber stoppers (Thomas Scientific, catalog no. 1780J07). (*Z*)-4-Tridecenal was incorporated into a Hercon polymer-bonded slow-release wafer formulation (0.15 mg isomer/cm^2) and was used at a target release dosage of 50 ng/hr (release rate not verified). Traps were Pherocon AM (sticky panels folded back to back) and were hung over corn ears ca. 1 m above ground. Traps were 75 m apart within

a block and were used for only one experiment. Pheromone baits were pinned onto the traps in the center of one of the sticky panels. Control traps were identical to treatment traps but without bait. Traps and treatments were set out by 0900 hr and collected by 1300 hr the same day. Field data was transformed to log $(x + 1)$ to stabilize variance and tested for homogeneity with Levene's test. An ANOVA was applied with mean separations by PLSD, $\alpha \leq 0.05$.

RESULTS

Evaluation of male *M. grandii* response to the four lactone sources (concentrations 1–500 ng) in the wind tunnel indicated that the synthetic *SRS* enantiomer was as attractive as the male- or female-derived lactone sources as measured by flight through the O ring (Table 1). A concentration of 10–50 ng was necessary to initiate a behavioral flight response for each source. When tested at the maximum concentration (500 ng), there was literally a "cloud" of activity in front of the male- and female-derived lactones and the synthetic SRS enantiomer sources. At the same concentration there was virtually no activity in front of the synthetic *RSR* enantiomer source. Concentrations greater than 500 ng were not tested due to a limited amount of material. The synthetic *RSR* enantiomer was clearly not as attractive as the *SRS* enantiomer; however, there

TABLE 1. MEAN RESPONSE OF MALE[a] *Macrocentrus grandii* TO SYNTHETIC ENANTIOMERS OF (*SRS*)- AND (*RSR*)-3,5-DIMETHYL-6-(METHYLETHYL)-3,4,5,6-TETRAHYDROPYRAN-2-ONE AND MALE- AND FEMALE-DERIVED SOURCES OF LACTONE AT VARIOUS CONCENTRATIONS IN A WIND TUNNEL

	Mean[b] ($N = 6$)					
Treatment	1 ng	10 ng	50 ng	100 ng	300 ng	500 ng
Synthetic *RSR*	0.5a	0.7a	2.5a*	2.7a	3.2a*	6.0a**
Control	0.3	0.8	0.0	0.3	0.3	0.2
Synthetic *SRS*	1.0a	1.0a	14.7b**	16.8b**	34.5b**	43.3b**
Control	0.3	0.3	0.3	0.5	0.0	0.0
Female-derived lactone	0.8a	1.0a	8.2c**	18.0b**	27.2b**	42.8b**
Control	0.2	0.7	0.3	0.7	0.0	0.0
Male-derived lactone	0.8a	1.0a	13.2bc**	17.7b**	31.8b**	54.0b**
Control	0.7	0.8	0.3	0.2	0.2	0.0

[a] 150–200 males 1–5 days old were tested.
[b] Counts represent the mean number of wasps flying upwind through a Teflon O ring (8 cm ID) 7 cm directly downwind of the source during a 1-min test. Column means followed by different letters are significantly different (PLSD, $\alpha < 0.5$). * and ** imply significant differences from control at the $\alpha \leq 0.05$ and $\alpha \leq 0.01$ levels (one-sided paired t test).

was some flight response to the *RSR* enantiomer. Combining the responses to the four attractive concentrations (50–500 ng), the *RSR* enantiomer flight response averaged 14% of the *SRS* enantiomer.

Various ratios of the two synthetic enantiomers were bioassayed in the wind tunnel (Table 2). Replacement of the *SRS* enantiomer with the *RSR* enantiomer generally decreased the upwind response (Series 1). However, when the SRS enantiomer made up 50% or more of the treatment, there was no observable response decrease. Addition of the *RSR* enantiomer at various concentrations (0–200 ng) to 100 ng of the *SRS* enantiomer produced a response not unlike *SRS* alone (Series 2). However, with the highest concentrations of the *RSR* enantiomer (100, 150, and 200 ng), some additional flight activity appeared to occur in the wind tunnel, but this was not measurable by flight through the O ring.

Field testing of the synthetic and female-derived lactones was conducted separately and in combination with (*Z*)-4-tridecenal, the previously identified primary sex pheromone for *M. grandii* (Table 3). Blocking effectively reduced residual variability in the analysis of variance. This was important because some

TABLE 2. MEAN RESPONSE OF MALE[a] *Macrocentrus grandii* TO SYNTHETIC ENANTIOMERS OF (*SRS*)- AND (*RSR*)-3,5-DIMETHYL-6-(METHYLETHYL)-3,4,5,6-TETRAHYDROPYRAN-2-ONE AT VARIOUS RATIOS IN A WIND TUNNEL

Series 1		Series 2	
Ratio[b] *RSR*:*SRS*	Mean[c] (N = 8) Trt/control	Ratio *RSR*:*SRS*	Mean (N = 5) Trt/control
0:100	14.6a**/0.0	0:100	17.8a**/0.0
1:99	15.0a**/0.0	1:100	21.2a**/0.0
5:95	14.6a**/0.0	10:100	14.4a**/0.0
25:75	16.1a**/0.0	50:100	18.8a**/0.0
50:50	12.8a**/0.0	100:100	23.4a**/0.0
75:25	5.9b**/0.0	150:100	15.0a**/0.0
95:5	4.2b**/0.2	200:100	22.8a**/0.0
99:1	1.8b /0.5	100:0	2.4b* /0.0
100:0	1.2b* /0.0		

[a] 150–200 males 1–5 days old were tested.

[b] Enantiomers were solvated in pentane and mixed together on a 4-cm watch-glass plate. The total chemical applied (*RSR* plus *SRS*) for each treatment in series 1 was 100 ng, for series 2 each percent was equivalent to 1 ng (100 = 100 ng).

[c] Counts represent the mean number of wasps flying upwind through a Teflon O ring (8 cm ID) 7 cm downwind of the source during a 1-min test. Column means followed by different letters are significantly different (PLSD, $\alpha < 0.05$). * and ** imply significant differences from control at the $\alpha \leq 0.05$ and $\alpha \leq 0.01$ levels (one-sided paired t test).

TABLE 3. TRAP CATCHES OF FERAL MALE *Macrocentrus grandii* IN THE FIELD BY SYNTHETIC ENANTIOMERS OF (*RSR*)- AND (*SRS*)-3,5-DIMETHYL-6-(METHYLETHYL)-3,4,5,6-TETRAHYDROPYRAN-2-ONE, FEMALE-DERIVED LACTONE, AND (*Z*)-4-TRIDECENAL

Treatment[a]	Mean[b] ($N = 6$)
Z4-13:Al + female-derived lactone	143.0a
Z4-13:Al + synthetic *SRS*	136.2a
Z4-13:Al + synthetic *RSR*	66.3b
Z4-13:Al	27.6b
Female-derived lactone	27.2bc
Synthetic *SRS*	32.8bc
Synthetic *RSR*	13.3cd
Control (no bait)	3.5d

[a] All lactone sources were formulated at a dose of 10 μg/bait and were applied to sleeve-type rubber stoppers with equal volumes of pentane. (*Z*)-4-Tridecenal was released via a Hercon polymer-bonded slow-release wafer at a target dosage of 50 ng/hr. Traps were Pherocon AM.
[b] Column means followed by different letters are significantly different (PLSD, $\alpha < 0.05$).

blocks were run on different days and/or in different corn fields. Individually, the female-derived lactone, the *SRS* enantiomer, and (*Z*)-4-tridecenal were significantly attractive relative to the control trap. Trap catches by the *RSR* enantiomer alone were not significantly different ($\alpha \leq 0.05$) from the control trap. Pairing the female-derived or *SRS* enantiomer lactone sources with (*Z*)-4-tridecenal elicited a synergistic response. These two treatments were comparable, the most attractive of the treatments, and significantly more attractive than the *RSR* enantiomer plus (*Z*)-4-tridecenal, which was not significantly different from (*Z*)-4-tridecenal alone.

Increasing the concentration of the *SRS* enantiomer (10, 50, and 100 μg) increased the trap catches; however, trap catch increases were not proportional to the concentration increase (Table 4). This could simply be due to the number of feral males available to respond.

In addition to male wasp captures, eight of 96 field traps captured one or two female wasps. In seven of the eight cases, the trap catch of males was below the mean of the remaining five replicates. Therefore, capture of a female did not appear to affect male capture positively, yet the possibility can not be dismissed. The capture of a female(s) was observed in seven different treatments, including the control. We believe the yellow trap, not the bait, attracted female wasps since female wasps were found on control traps as well as baited traps in equivalent numbers. Laboratory-reared females were not found to be attracted to male- or female-derived extracts in wind-tunnel bioassays (Swedenborg and Jones, 1992a).

TABLE 4. TRAP CATCHES OF FERAL MALE *Macrocentrus grandii* IN THE FIELD BY SYNTHETIC ENANTIOMER (*SRS*)-3,5-DIMETHYL-6-(METHYLETHYL)-3,4,5,6-TETRAHYDROPYRAN-2-ONE AT VARIOUS CONCENTRATIONS WITH (*Z*)-4-TRIDECENAL

Treatment[a]	Mean[b] (N = 6)
Z4-13:Al + synthetic *SRS* (100 μg)	261.5a
Z4-13:Al + synthetic *SRS* (50 μg)	218.8a
Z4-13:Al + synthetic *SRS* (10 μg)	143.0ab
Z4-13:Al	83.3cd
Synthetic *SRS* (100 μg)	103.8cd
Synthetic *SRS* (50 μg)	84.7bc
Synthetic *SRS* (10 μg)	36.2d
Control (no bait)	3.7e

[a]The lactone was applied to a sleeve type rubber stopper. (*Z*)-4-Tridecenal was released via a Hercon polymer-bonded slow-release wafer at a target dosage of 50 ng/hr. Traps were Pherocon AM.
[b]Column means followed by different letters are significantly different (PLSD, $\alpha < 0.05$).

Because so many wasps were captured over a short period, it was easy to watch male behavior at the more attractive traps. In all cases, males flew into the treatment area and oriented to the trap during flight, flew to the trap, landed, and became stuck. In addition, many wasps also were observed to search the neighboring corn plants. This coincides with previous observations in which males were observed to respond to female cocoon masses at eclosion by searching the corn plants adjacent to the cocoon for female wasps (Swedenborg et al., 1993). Female wasps were never observed flying in the vicinity of the treatments.

DISCUSSION

Coupled with the identification information, this evidence verifies the *SRS* enantiomer as the natural pheromone responsible for male *M. grandii* attraction. These enantiomers fit the prevalent response scenario in insects to enantiomers in which one optical isomer is biologically active and the other is less active or inactive.

Observations on male flight activity around the treatments indicated the lactone baits lost much of their attractiveness by the end of the testing period (ca. 4 hr). Incorporating the lactone into a slow-release formulation analogous to the (*Z*)-4-tridecenal bait should be beneficial to increase trap catches. Additionally, since *RSR* does not appear to be inhibitory, using the racemic lactone

for field use, instead of pure *SRS*, would be less expensive yet effective. Further investigation is necessary to correlate European corn borer parasitism and economic damage by the European corn borer with adult male *M. grandii* captures. These baits should prove useful for field sampling of male *M. grandii*.

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